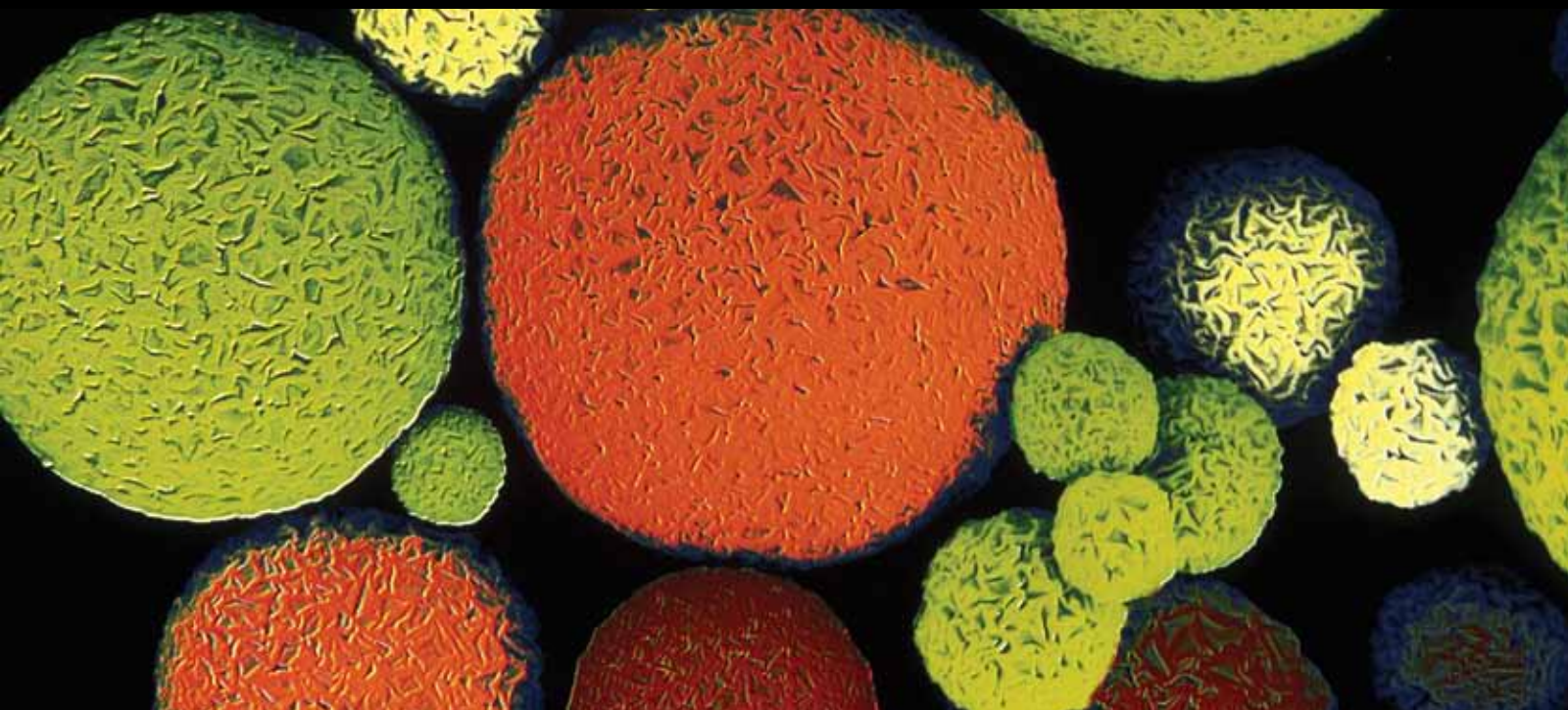# Analytical Method Development and Validation of Pharmaceutical Analysis Using Chromatographic Techniques

Guest Editors: Bengi Uslu, Henk Lingeman, Sibel A. Ozkan, Meehir Palit, and Burcu Dogan-Topal

Analytical Method Development and Validation of Pharmaceutical Analysis Using Chromatographic Techniques

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Editorial Board

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Editorial

Analytical Method Development and Validation of Pharmaceutical Analysis Using Chromatographic Techniques

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Analytical methods including chromatographic methods are commonly used for the quantitative and qualitative analysis of raw materials, drug substances, drug products, and compounds in biological samples in pharmaceutical industry. The components monitored include chiral or achiral drugs, process impurities, residual solvents, excipients such as preservatives, degradation products, extractable and leachable from container and closure or manufacturing process, pesticide in drug product from plant origin, and metabolites.

Validation of an analytical method which is used during drug development and drug manufacturing is required to demonstrate that the methods are fit for their intended purpose. Additionally, the pharmaceutical industry around the world is subject to extensive regulations due to the nature of its products.

Analytical chemists play important roles in monitoring the drugs in their dosage forms and biological samples. From the viewpoints mentioned above, the title of this special issue was chosen so as to ask chemists to appreciate their great roles in chemistry science. This special issue features 8 research articles. In this special issue, development of chromatographic methods such as high performance liquid chromatography, gas chromatography, micellar liquid chromatography, and their validations is presented.

The purpose of this special issue will be to serve as a guide to what chromatographic methods bring to analytical and medicinal chemistry and other pharmaceutical sciences as well as briefly review their role in drugs and the new developments and validation of assay methods of pharmaceutically active compounds. Also, recent developments of application, evaluation, and validation of chromatographic methods are focused on by key topics in drug developments and analysis by assessment of the distinguished authors of this special issue. We hope that the reader will find a number of topics of interest and that additional new ideas will emerge from this special issue.

Acknowledgments

Research Article

Analysis of Some Biogenic Amines by Micellar Liquid Chromatography

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Micellar liquid chromatography (MLC) with the use of high performance liquid chromatography (HPLC) was used to determine some physicochemical parameters of six biogenic amines: adrenaline, dopamine, octopamine, histamine, 2-phenylethylamine, and tyramine. In this paper, an influence of surfactant's concentration and pH of the micellar mobile phase on the retention of the tested substances was examined. To determine the influence of surfactant's concentration on the retention of the tested amines, buffered solutions (at pH 7.4) of ionic surfactant—sodium dodecyl sulfate SDS (at different concentrations) with acetonitrile as an organic modifier (0.8/0.2 v/v) were used as the micellar mobile phases. To determine the influence of pH of the micellar mobile phase on the retention, mobile phases contained buffered solutions (at different pH values) of sodium dodecyl sulfate SDS (at 0.1 M) with acetonitrile (0.8/0.2 v/v). The inverse of value of retention factor ($1/k$) versus concentration of micelles (C_M) relationships were examined. Other physicochemical parameters of solutes such as an association constant analyte—micelle (K_{ma})—and partition coefficient of analyte between stationary phase and water (hydrophobicity descriptor) ($P_{sw}\Phi$) were determined by the use of Foley's equation.

1. Introduction

Micellar liquid chromatography (MLC) is a mode of conventional reversed-phase liquid chromatography which uses a surfactant solution (anionic, cationic, or nonionic) above the critical micellar concentration (cmc) as a mobile phase [1, 2]. The retention of a compound in MLC depends on the type of interaction (electrostatic and/or hydrophobic) with the micelles and with a surfactant-modified stationary phase [2–5]. There is an assumption that there exist three different partition equilibria between: micellar mobile phase and water, micellar mobile phase and stationary phase, and stationary phase and aqueous mobile phase [1]. There are a lot of equations which describe interactions between these equilibria.

From the beginning of MLC in 1980, the technique has evolved up to become a real alternative in some instances to classical RPLC with hydro-organic mixtures, owing to its peculiar features and unique advantages [6]. MLC is an effective technique for analysis of the organic compounds with biological activity [7–9]. Amongst many advantages of MLC the following can be specified as the most important [1, 10–12]:

 (i) low price of a mobile phase,

 (ii) little toxicity in comparison with classical mobile phases in RP-HPLC,

 (iii) possibility of separation of ionic and nonionic substances in the same column packing and the same distributing cycle,

 (iv) possibility of control of system's selectivity through the modification of simple parameters of mobile phase such as surfactant's concentration, pH, or ionic strength,

 (v) possibility of fast modification of concentration gradient.

The biogenic amines are the group of compounds which are formed during many important intracellular metabolic processes [13]; these amines are the products of transformation of amino acids or their derivatives. In the living organism, the biogenic amines take physiological roles which are important for the correct course of many biological processes [14]. The biogenic amines are necessary for keeping an existence of cell. They are the part of coenzymes and might be hormones. In cells, among other things, amines have an effect on DNA replication and cell permeability and might control mammal's lactation. Some of these are carcinogenic.

In this paper, some biogenic amines (adrenaline, dopamine, octopamine, 2-phenylethylamine, tyramine, and histamine) were determined.

Biogenic amines are damaging to health because they are substrates in the carcinogenic compound synthesis—nitrosoamines. The presence of histamine in food (i.e., in cheese or fish) can result in strong alimentary intoxication [15, 16].

Adrenaline is a hormone and neurotransmitter which takes a role of stress mechanism that is lightning reactions of living organism to stress. Adrenaline can increase heart rate, constrict blood vessels, dilate air passages, and participate in the fight-or-flight response of the sympathetic nervous system. Adrenaline also increases cerebral blood flow and hence oxygen delivery to the brain [17, 18].

Dopamine is called the hormone of happiness. Dopamine is available as an intravenous medication acting on the sympathetic nervous system, producing effects such as increased heart rate and blood pressure. It is involved in the regulation of a variety of functions, including locomotor activity, emotion and affect, and neuroendocrine secretion [19].

Histamine is a powerful biologically active substance, and therefore it can exert many responses within the body. Histamine exerts its effects by binding to receptors on cellular membranes which are found in the cardiovascular system and in various secretory glands [20].

Octopamine is an endogenous biogenic amine that is closely related to noradrenaline. It has effects on the adrenergic and dopaminergic systems. Octopamine has been reported to influence animal behavior, such as avoidance conditioning or motor activity [21, 22].

Many derivates of 2-phenylethylamine are psychoactive and psychedelic. This amine as a hormone and neurotransmitter takes a fundamental role in nervous system. Moreover, it is present in food, mainly in chocolate, and also in some species of mushrooms [23, 24].

Tyramine acts mainly indirectly by releasing noradrenaline from the sympathetic nervous system which causes an increase of the blood pressure by peripheral vasconstriction and by increasing the cardiac output [15].

Due to biogenic amines are a large group of naturally occurring biologically active compounds, which are present in food products and can act as hormones and neurotransmitters, there are many different analytical techniques for their quantitative and qualitative determination. Amongst chromatographic techniques the most commonly used are RP-LC [25–28], MEKC [29, 30], and IEC [31–33]. MLC

technique for the determination of biogenic amines is rather rarely applied.

Paleologos and coworkers successfully separated nine biogenic amines (cadaverine, tyramine, putrescine, agamatine, spermidine, tryptamine, phenylalanine, spermine, and histamine) in the form of benzoyl derivatives by the use of MLC with gradient elution. Investigated biogenic amines were quantitatively and qualitatively determined in fish products [34].

Bose and coworkers used MLC technique for the determination of dopamine and serotonine and their metabolites (homovalinic acid (HVA) and hydroxyindoleacetic acid (HIAA)) and tyramine in the serum samples [35]. Other biogenic amines (such as tyramine and tryptamine and their precursors: thyrosine and tryptophan) were determined in MLC systems in vine samples [36].

2. Experimental

2.1. Apparatus. Chromatographic data were obtained using Shimadzu Vp liquid chromatographic system equipped with an LC 10AT pump, an SPD 10A UV-VIS detector, an SCL 10A system controller, a CTO-10 AS chromatographic oven, and a Rheodyne injector valve with 20 μL loop. This system utilizes a class-Vp computer program to control hardware, acquire and store data, and determine retention times.

A stainless-steel column (125 × 4 mm, I.D.) packed with 5 μm RP-8 endcapped Purospher (Merck) was used in each experiment.

The flow rate was 1.0 mL min^{-1}. All measurements were carried out at 20°C. The test compounds were detected at 230 nm.

2.2. Reagents and Materials. Stock standards of six biogenic amines (Table 1): adrenaline, dopamine, histamine, octopamine, 2-phenylethylamine, and tyramine were purchased from Sigma Aldrich (St. Louis, USA).
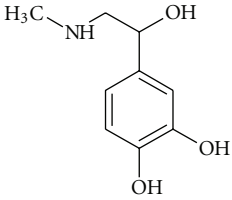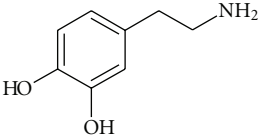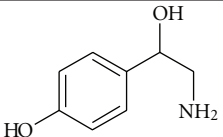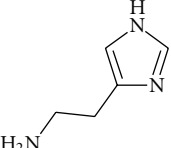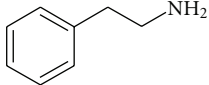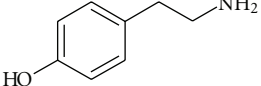
All stock solutions of the analytes were prepared with the concentration of about 0.1 mg mL^{-1} in methanol.

For the determination of the influence of surfactant's concentration on the retention, mobile-phases-buffered solutions at pH 7.4 of sodium dodecyl sulfate (SDS) at the concentrations of 0.02 M, 0.04 M, 0.06 M, 0.08 M, and 0.1 M + acetonitrile as an organic modifier (for HPLC) at the concentration of 20% v/v were used. After measuring of SDS concentration, the organic modifier was added, so the real concentrations of SDS were correspondingly 0.016 M, 0.032 M, 0.048 M, 0.064 M, and 0.08 M.

The buffer was prepared with Na$_2$HPO$_4$ (0.02 M) and citric acid (0.01 M) and was vacuum-filtered through 0.45 μm membrane filter before use. pH value of this buffer was measured before the preparation of the mobile phases. Distilled water was obtained from Direct-Q UV apparatus (Millipore). Acetonitrile (for HPLC) at the concentration of 20% v/v was utilized as an organic modifier. Micellar mobile phases were degassed in the ultrasonic bath for about two minutes before use.

For the determination of the influence of pH of the micellar mobile phase on the retention, mobile-phases-buffered

Table 1: Structures of tested substances.

Number	Name	Structures of tested substances
1	Adrenaline	
2	Dopamine	
3	Octopamine	
4	Histamine	
5	2-phenylethylamine	
6	Tyramine	

solutions at pH 3, 4, 5, 6, 7.4, 8, and 9 of sodium dodecyl sulfate (SDS) at the concentration of 0.1 M were used. The buffer in the same way as presented above was prepared. Acetonitrile (for HPLC) at the concentration of 20% v/v was utilized as an organic modifier.

3. Result and Discussion

Besides that MLC is an acknowledged analytical technique, it has high significance in elementary research.

There are many agents in MLC which have an effect on retention such as [5, 37]:

(i) type of organic modifier,

(ii) concentration of organic modifier,

(iii) concentration of surfactant (concentration of micelles),

(iv) pH of mobile phase.

In this paper, an influence of surfactant's concentration and pH of the micellar mobile phase on the retention of tested substances were examined.

3.1. An Influence of Surfactant's Concentration on the Retention of Tested Amines. For this purpose, the retention of

tested substances was defined in the following mobile phase: buffer (pH 7.4) + acetonitrile + SDS (of variable concentration).

The buffer solution at pH 7.4 was used for the reason that in biology and medicine, this value is often referred to as physiological (the pH of blood is usually slightly basic with a value of pH 7.365) [38] and tested substances are very closely connected with many processes in the living organism.

The research of variation of retention factor in relation to the concentration of surfactant can give much important information applying to interactions of tested substances with components of the micellar mobile phase.

The analysis of the relationship: inverse of value of retention factor ($1/k$) versus concentration of micelles (C_M) can give information about the type of interaction between tested solutes and micelles [39] and can determine such physicochemical parameters of solutes as an association constant analyte—micelle (K_{ma})—and can compare partition coefficient between micellar mobile phase and water ($P_{sw}\Phi$) [12, 40].

If substance does not work with micelle, the change of retention was not observed with the change of concentration of surfactant.

If substance acts with micelle, decrease of retention was observed with increase of surfactant's concentration (the so-called binding analyte) [39].

In case of the increase of retention, it was noticed that substance from micelle was eliminated with the increase of surfactant's concentration (the so-called antibinding analyte) [39].

Linear, ascending relationship between $1/k$ value and concentration of micelles with high coefficient of determination ($R^2 > 0.96$) was obtained for all investigated solutes (Figure 1). The character of relationships involves the fact that all tested biogenic amines act with micelles of the mobile phase (are so-called binding analytes).

3.2. Calculations of Chosen Physicochemical Parameters. Linear relationships $1/k = f(C_M)$ can be presented in the following way [12, 40] (Foley's equation):

$$\frac{1}{k} = \frac{K_{ma}C_M}{P_{sw}\Phi} + \frac{1}{P_{sw}\Phi}, \tag{1}$$

where C_M: concentration of micelles, K_{ma}: analyte-micelle association constant, P_{sw}: partition coefficient of analyte between stationary phase and water; hydrophobicity descriptor, and Φ—volume ratio of stationary phase to volume of mobile phase.

The slope of a straight line from (1) is formulated as $K_{ma}/(P_{sw}\Phi)$ and free term in an expression as $1/P_{sw}\Phi$.

The slope of a straight line informs about substance's sensitivity to the change of concentration of surfactant, therefore shows the possibility of modification retention through the change of surfactant's concentration. Moreover, the slope of a straight line $1/k = f(C_M)$ depends on the interaction of the substance with micelle (K_{ma}) and on partition coefficient between stationary phase and water (P_{sw}) (Table 2).

Adrenaline

$y = 1.8156x + 0.0471$

$R^2 = 0.9966$

(a)

Dopamine

$y = 1.2581x + 0.0222$

$R^2 = 0.9956$

(b)

Octopamine

$y = 1.7688x + 0.0309$

$R^2 = 0.9826$

(c)

Histamine

$y = 1.1038x + 0.0174$

$R^2 = 0.9765$

(d)

2-phenylethylamine

$y = 0.8631x + 0.0105$

$R^2 = 0.9692$

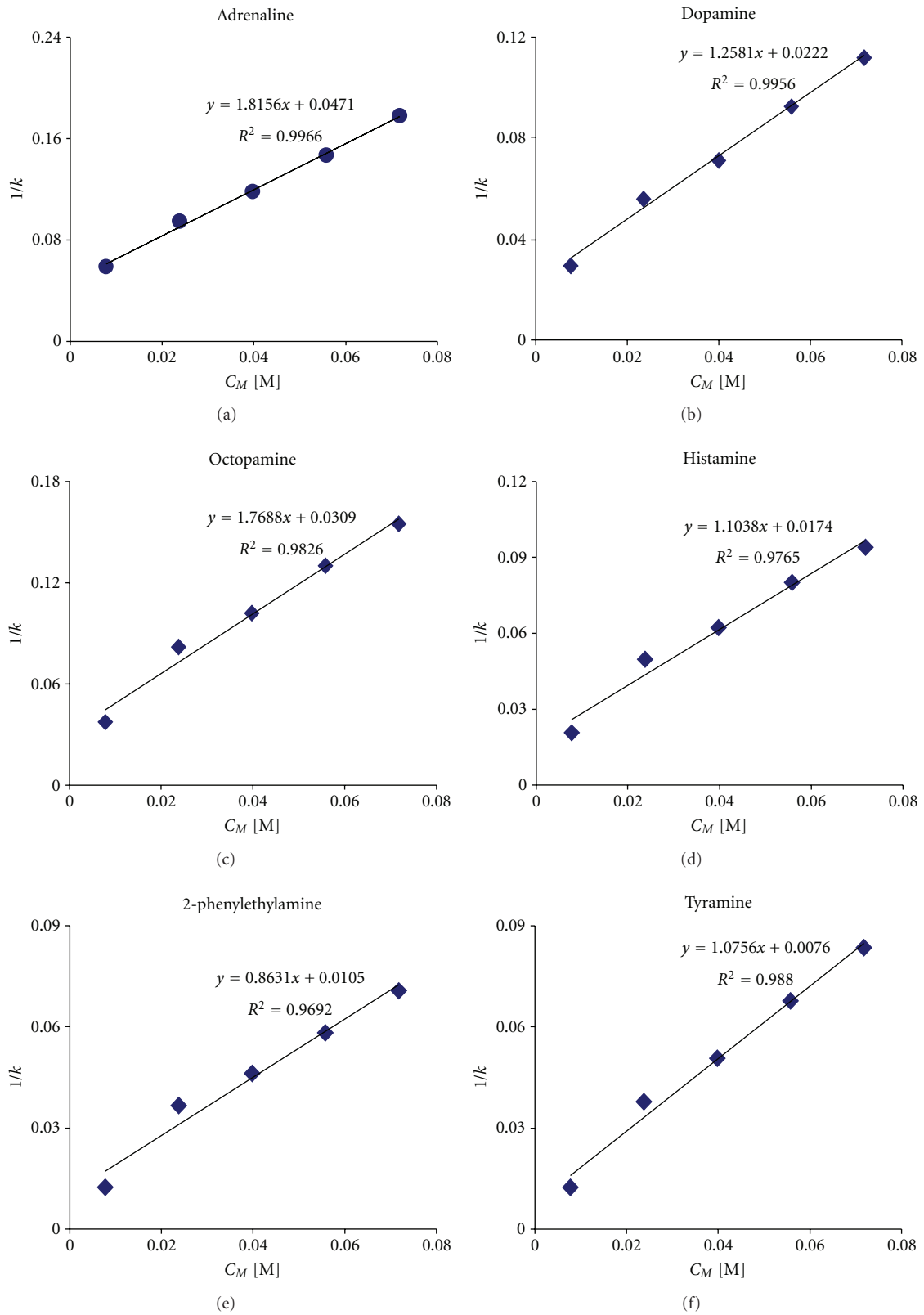(e)

Tyramine

$y = 1.0756x + 0.0076$

$R^2 = 0.988$

(f)

FIGURE 1: $1/k$ versus C_M relationships obtained for all tested biogenic amines.

TABLE 2: $K_{ma}/P_{sw}\Phi$, K_{ma} and $P_{sw}\Phi$ values of tested biogenic amines according to (1).

Solute number	Name	$K_{ma}/P_{sw}\Phi$	K_{ma}	$P_{sw}\Phi$
1	adrenaline	1.82	38.55	21.23
2	dopamine	1.26	56.67	45.05
3	octopamine	1.77	57.24	32.36
4	histamine	1.10	63.44	37.47
5	2-phenylethylamine	0.86	82.20	95.24
6	tyramine	1.08	141.53	131.58

Obtained values of K_{ma} (Table 2) demonstrate that tyramine acts the most strongly and adrenaline the most weakly with SDS micelle. Almost all of calculated values are larger than 1, which means that analyte-micelle interaction has higher influence than analyte-modified stationary phase on the retention of biogenic amines. Only 2-phenylethylamine demonstrates other type of interaction which suggests higher impact of analyte-modified stationary phase interaction on the retention of this amine.

Moreover, $1/b = P_{sw}\Phi$ value can inform also about hydrophobicity relations of tested substances.

Because of large difficulty to determine Φ value, it is given as $P_{sw}\Phi$ value, because at the given chromatographic system Φ value is constant. $P_{sw}\Phi$ value informs about relative hydrophobicity of tested substances. When analyte acts more strongly with micelle, it acts more weakly with water.

On the basis of the $P_{sw}\Phi$ values, it was demonstrated that tyramine is the most hydrophobic solute and adrenaline the least. The obtained K_{ma} values show that the increase of solutes hydrophobicity increases K_{ma} values.

In the micellar systems, the retention of the solutes depends on their hydrophobic properties.

Therefore, $\log k$ versus $\log P_{sw}\Phi$ relationships were determined for all chromatographic systems (Figure 2).

Very good $\log k$ versus $\log P_{sw}\Phi$ linear relationships ($R^2 > 0.98$) suggest that $P_{sw}\Phi$ parameter should be considered as good hydrophobicity descriptor for tested solutes despite using different concentrations of surfactant in the micellar mobile phases.

3.3. An Influence of pH of the Micellar Mobile Phase on the Retention of Tested Substances.

Due to possible existence of many different forms of biogenic amines [41], the pH of the micellar mobile phase is a very important agent which can influence the retention of tested substances [42].

For this purpose, the retention of tested substances was defined in the following mobile phases: acetonitrile (4 : 1 v/v) + SDS at the concentration of 0.1 M + citric buffer at different pH (3, 4, 5, 6, 7.4, 8, and 9). Due to stability of chromatographic column, the range of pH was as present above.

In Figure 3 there is a comparison of obtained relationships (k versus pH) with the literature data [41] related to the range of pH in which particular forms of tested substances exist for chosen biogenic amines.

As we can observe, depending on the pH value, the forms of tested substances are replaced. These changes influence the retention value.
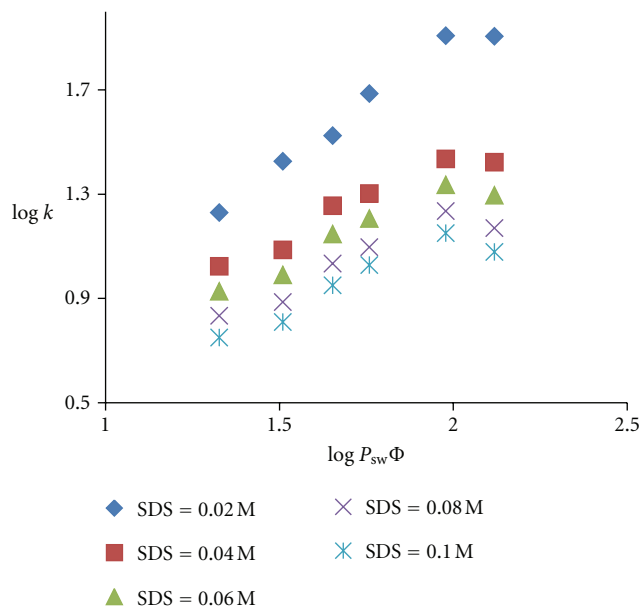
FIGURE 2: $\log k$ versus $\log P_{sw}\Phi$ relationships for all types of micellar mobile phases.

TABLE 3: pKa values of tested biogenic amines in the examined pH range.

Substance name	pKa value
adrenaline	8.91
dopamine	9.27
histamine	9.58
octopamine	8.98
2-phenylethylamine	9.79
tyramine	9.66

MLC technique can be used as an instrument to verify or even determine such physicochemical parameters as pKa in examined pH range [41] (Table 3). In this case, the limitation of pH range, caused by the stability of chromatographic column, makes the calculation of pKa impossible.

3.4. The Analytical Aspect of MLC.

Except for defined physicochemical parameters through the data obtained from the retention, the MLC performs a role as the analytical method. In this paper, it was also examined what was a separation possibility of analyzed biogenic amines in the tested systems. Because surfactant's concentration (concentration of micelles) effects on retention of tested substances, in the mobile phases at the different concentration of SDS (but at the same amount of other components), the longest time of retention was observed at the lowest concentration of surfactant.

For the micellar mobile phase: 0.02 M SDS + acetonitrile (4 : 1 v/v) at pH 7.4, the best separation was achieved for five tested amines out of six that were tested (except 2-phenylethylamine which retention time is almost the same as the retention time of tyramine). However, time of analysis

(a)

(b)

(c)

(d)
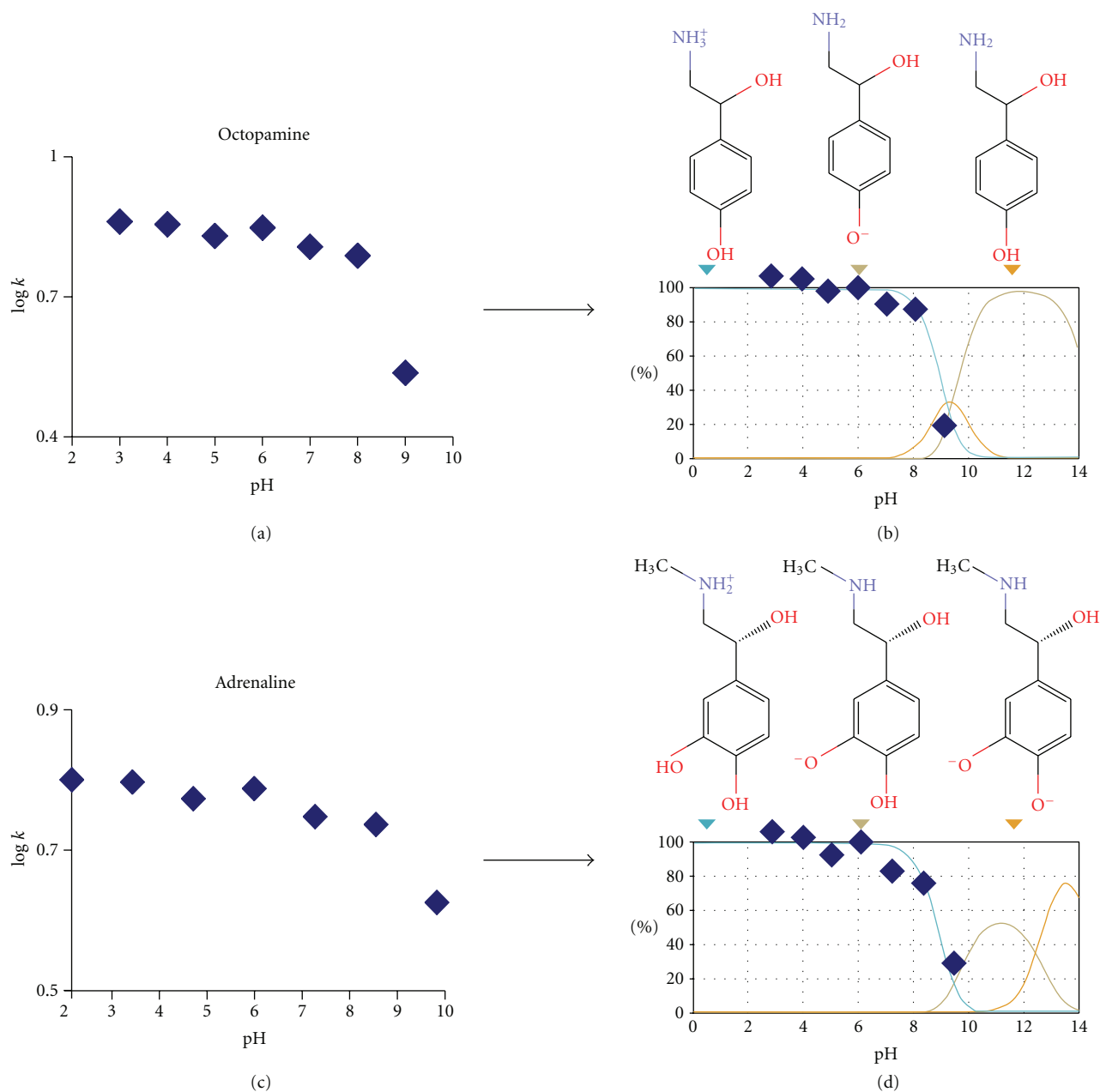
FIGURE 3: The comparison of obtained relationships $\log k$ versus pH of the micellar mobile phase with the pKa of tested amines.

using this mobile phase is about 50 minutes and peaks of the substances at the longest retention time (histamine and tyramine), are diffused. It is comprehensible, because it is a result of a diffusion. On the other hand, the separation of adrenaline (peak 1), octopamine (peak 2), and dopamine (peak 3) is satisfactory ($R_{s12} = 5.04$, $R_{s23} = 3,30$). Time of analysis for these three compounds is about 25 minutes. Therefore, for the determination of these amines mobile phase with 0.02 M SDS will be the most appropriate (Figure 4).

The retention time decreases with the surfactant's concentration. The increase of surfactant's concentration to 0.04 M induces the worse separation of adrenaline and

octopamine ($R_{s12} = 0.42$), but separation of octopamine and dopamine is acceptable ($R_{s23} = 1.29$) (to the zero line). The improvement of the shape of peaks 4 and 5 (histamine and tyramine) is noticeable in comparison with the mobile phase presented above, peaks 3 and 4 overlap.

The successive increase of SDS concentration to 0.06 M does not have an effect on the systems' resolution, but the time of analysis is observably shorter.

The next increase of SDS concentration results in no separation of adrenaline and octopamine, but dopamine, histamine, and tyramine to the zero line are separated and for such mixture, mobile phase with 0.08 M SDS would be better than mobile phase with 0.06 M SDS. In this mobile phase,
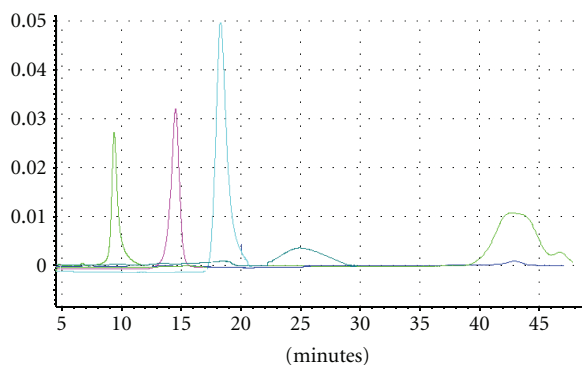
Figure 4: The chromatogram of the separation of tested amines: 0.02 M SDS (pH 7.4) + acetonitrile (20% v/v).

the separation and the identification of 4 out of 5 tested amines is practically possible.

At the mobile phase with the highest concentration of SDS (0.1 M) (retention times are the shortest), the overlapping of all peaks is observed, therefore this phase is not suitable for the analytical purpose.

4. Conclusions

For all tested biogenic amines, the decrease of retention (increase of $1/k$) with the increase of surfactant's concentration in the micellar mobile phase was observed. Very good $1/k$ value versus C_M linear relationships with high coefficient of determination confirm the above dependence. An impact of the surfactant's concentration in the micellar mobile phase on retention of tested amines was proved. The slope of the change of retention line $1/k = f(C_M)$ depends mainly on hydrophobicity of analytes. Linear, ascending relationship between $1/k$ value and concentration of micelles with high coefficient of determination was obtained for all investigated solutes (Figure 1). The character of relationships involves the fact that all tested biogenic amines act with micelles of the mobile phase (are binding analytes).

To determine this attribute P_{sw} and K_{ma} values were calculated. On the basis of the $P_{sw}\Phi$ values it was demonstrated that tyramine is the most hydrophobic solute and adrenaline the least. The obtained K_{ma} values show that the increase of solutes hydrophobicity, involves the increase of K_{ma} values. Moreover, between K_{ma} values significant differences for different tested amines were visible. The rise of $P_{sw}\Phi$ value responds to the increase of analytes retention. The above results prove that the concentration of surfactant in the micellar mobile phase is a very important agent that influences the retention of organic compounds. Therefore, the optimization of chromatographic systems in MLC through the choice of concentration of surfactant in the micellar mobile phase is necessary.

The investigated micellar chromatographic systems can be used for the separation and the determination of tested biogenic amines.

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Research Article

Stability-Indicating RP-TLC/Densitometry Determination of Raloxifene Hydrochloride in Bulk Material and in Tablets

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A stability-indicating RP-TLC/Densitometry method for analysis of Raloxifene hydrochloride both in bulk material and in tablets was developed and validated. Densitometric analysis of Raloxifene hydrochloride was carried out at 311 nm on TLC aluminium plates precoated with silica gel 60RP-18 F_{254}S as the stationary phase and methanol : water : ammonia (95 : 05 : 0.1 v/v) as mobile phase. Raloxifene hydrochloride was well resolved at R_f 0.55 ± 0.02. The linear regression analysis data for the calibration plots showed good linear relationship with $r^2 = 0.9969 ± 0.0015$ with respect to peak area in the concentration range 100–600 ng per band. The mean value ± SD of slope and intercept was found to be 15.05 ± 0.44 and 201.9 ± 29.58 with respect to peak area. The limits of detection and quantification were 9.27 ng and 27.10 ng, respectively. Raloxifene hydrochloride was subjected to acid and alkali hydrolysis, oxidation, dry heat, and photodegradation. The drug underwent degradation under basic and oxidation conditions. This indicates that the drug is susceptible to alkali hydrolysis and oxidation. The proposed developed RP-TLC/Densitometry method can be applied for identification and quantitative determination of Raloxifene hydrochloride in bulk material and tablets.

1. Introduction

Raloxifene hydrochloride (RLX), [6-Hydroxy-2-(4-hydroxy-phenyl) benzo [b] thien-3-yl] [4-[2-(1-piperidinyl)-ethoxy] phenyl]-methanone-, hydrochloride (Figure 1) is a selective estrogen receptor modulator (SERM) used in the treatment of osteoporosis in postmenopausal women [1]. Clinically, it is effective in the treatment of breast cancer [2, 3].

Literature survey revealed that RLX was analyzed by HPLC [4–9], Stability-indicating UPLC [10], and several UV-spectrophotometric [11–14] in pharmaceutical formulations. Few methods such as LC-MS-MS [15] and HPLC [16] have been reported for estimation of RLX in biological samples. Although the RP-HPLC and UPLC procedures are accurate and effective means of assaying RLX, they are time and solvent consuming, and therefore, disadvantageous for serial estimation for a large number of samples [17]. However, the prominent application of HPTLC is that many samples can be run simultaneously using a small quantity of mobile phase unlike HPLC, thus reducing the analysis time and cost per analysis. In-reverse phase chromatography, polar mobile phase is used and the stationary phase is nonpolar. It is increasingly being experienced that different components of formulation which could not be resolved using normal-phase TLC could easily be resolved by reverse-phase TLC. Further, in RP-TLC, the impurities either at starting line or near the solvent front can be detected [18].

In view of the above factors, an HPTLC method was well thought-out, to be cheaper, faster, and sometimes more efficient than RP-HPLC and UPLC. From the literature survey, it is revealed that no stability-indicating RP-TLC/Densitometry method has been reported in the literature for analysis of RLX as bulk material or in pharmaceutical formulations.

Hence, the objective of the present investigation was to develop a simple stability-indicating RP-TLC/Densitometry method offering lower analysis time and less cost per analysis for estimation of RLX in bulk material and in tablets and to validate the method according to the ICH guidelines [19, 20].
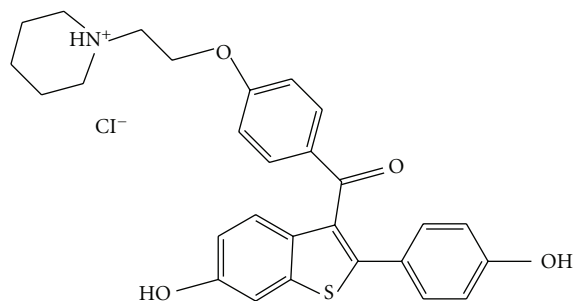
Figure 1: Chemical structure of Raloxifene hydrochloride (RLX).

2. Experimental

2.1. Chemicals and Reagents. Raloxifene hydrochloride (RLX) was procured from Cipla India Ltd, Mumbai, India. All chemicals and reagents used were of Analytical grade and were purchased from Merck Chemicals, India.

2.2. HPTLC Instrumentation. Chromatography was performed on aluminium plates precoated with Silica gel 60 RP-18 F_{254} S (20×10 cm, E. Merck, Germany). The plates were prewashed with methanol and activated at $100°C$ for 10 min prior to chromatography. The samples were spotted in the form of bands of 6 mm width with a Camag microlitre syringe using a Camag Linomat 5 applicator with a constant rate of application, 150 nL per second. Linear ascending development with methanol : water : ammonia ($95 : 05 : 0.1$ v/v) as mobile phase was performed in a 20×10 cm twin-trough glass chamber (Camag), with tightly fitting lid, previously saturated with mobile phase vapour for 25 min at room temperature ($25 \pm 2°C$). The development distance was 8 cm. After development, the plates were dried in current of air by an air dryer. Densitometric scanning was then performed at 311 nm with a Camag TLC Scanner 3 in absorbance mode operated by winCATS software. The source of radiation was a deuterium lamp. Slit dimensions were 5 mm $\times$ 0.45 mm and the scanning speed 20 mm per second.

2.3. Preparation of Stock Standard Solution and Linearity Study. Stock standard solution was prepared by dissolving 10 mg of RLX in 10 mL methanol. From it, appropriate volume 0.2–1.2 mL was transferred into six separate 10 mL volumetric flask and volume was made up to the mark with methanol. With the help of linomat 5 applicator, 5 μL of each solution was applied on RP-TLC plates to obtain concentration in the range of 100 to 600 ng per band, developed and scanned as described above.

2.4. Preparation of Sample Solution. Twenty tablets (RALISTA, label claim: 60 mg of RLX per tablet) were weighed and crushed into fine powder. The quantity of powdered drug equivalent to 50 mg of RLX was weighed and transferred in 100 mL volumetric flask containing 50 mL methanol, sonicated for 10 min, volume was adjusted to mark and filtered using 0.45 μm filter (Millifilter, Milford, MA). From the filtrate 1.0 mL was further diluted to 10 mL with methanol. Appropriate volume 8 μL was spotted for assay of RLX. The RP-TLC plates were developed and scanned as described above.

2.5. Method Validation

2.5.1. Precision. Repeatability of sample application and measurement of peak area were performed using six replicates of the same spot (400 ng per band of RLX). The intra, and interday variation for the estimation of RLX was carried out at three different concentration levels of 200, 300, and 500 ng per band.

2.5.2. Limit of Detection (LOD) and Limit of Quantification (LOQ). In order to determine detection and quantification limit, RLX concentrations in the lower part of the linear range of the calibration curve were used. From the stock standard solution RLX 100, 120, 140, 160, 180, and 200 ng per band was applied in triplicate on RP-TLC plate. The LOD and LOQ were calculated using equation LOD = $3.3 \times$ N/B and LOQ = $10 \times$ N/B, where "N" is standard deviation of the peak areas of the drugs ($n = 3$), taken as a measure of noise, and "B" is the slope of the corresponding calibration curve.

2.5.3. Specificity. The specificity of the method was checked by analyzing drug standard and sample. The band for RLX in sample was confirmed by comparing the R_f values and spectra of the band with that of standard. The peak purity of RLX was assessed by comparing the spectra at three different levels, that is, peak-start (S), peak-apex (M), and peak-end (E) positions of the band.

2.5.4. Ruggedness. Ruggedness of the method was performed by spotting 400 ng of RLX by two different analysts keeping same experimental and environmental conditions.

2.5.5. Accuracy. The preanalysed samples (200 ng per band) were spiked with extra 80, 100, and 120% of the standard RLX, and the mixture was then reanalysed by the proposed method. At each level of the amount, three determinations were performed. This was done to check the recovery of the drug at different levels in the formulations.

2.5.6. Robustness. By introducing small deliberate changes in the mobile-phase composition, the effects on the results were examined. Mobile phases having different composition of methanol : water : ammonia ($96 : 4 : 0.1$ v/v) and ($94 : 6 : 0.1$ v/v) were tried and chromatograms were run. The amount of mobile phase was varied in the range of ± 2 mL. The plates were prewashed by methanol and activated at 100 $\pm 5°C$ for 5 and 15 min prior to chromatography. Time from spotting to chromatography and from chromatography to scanning was varied from 0, 20,40 min.

2.5.7. Stability of Sample Solution in Methanol and Mobile Phase. To assess the stability of RLX in methanol and mobile

TABLE 1: Repeatability and Intraday, Interday precision.

Parameters	Concentration ng per band	% Amount found	% RSD
Repeatability ($n = 6$)	400	100.94	1.67
	200	99.98	1.27
Intraday ($n = 3$)	300	101.44	0.94
	400	99.70	0.41
	200	99.50	1.37
Interday ($n = 3$)	300	99.68	1.33
	400	101.97	1.35

n: number of determinations.

TABLE 2: Recovery studies.

Drug	Initial amount (ng per band)	Amount of drug standard added (%)	% Drug recovered	% RSD ($n = 3$)
	200	0	100.20	1.2
	200	80	99.78	1.2
RLX	200	100	99.74	1.4
	200	120	100.57	1.5

n: number of determinations.

phase; the sample solutions were separately prepared in methanol and mobile phase and stored at room temperature for 24 (h). The sample solutions were assayed at an interval of 6 (h) for 24 (h).

2.5.8. Stability of Sample Solution on RP-TLC Plate. The sample solution was applied on RP-TLC plate, kept for 72 (h), and scanned at an interval of 12 (h) as described above.

2.6. Forced Degradation of RLX

2.6.1. Acid, Base and Oxidation Degradation. Accurately weighed quantity 10 mg of RLX was separately dissolved in 10 mL methanolic solution of 0.5 M HCl and 0.5 M NaOH and 3% (v/v) hydrogen peroxide, respectively; solutions were kept for period of 12 (h) at room temperature in dark to avoid likely degradative effect of light. An appropriate volume 1.0 mL of above solution was taken, neutralized, and diluted up to 10 mL with methanol. The resultant solution was applied on RP-TLC plates in triplicates (5 μL each, i.e, 500 ng per band). The chromatogram was developed and scanned as described above.

2.6.2. Dry Heat Degradation. Accurately weighed quantity 10 mg of RLX stored at 80°C for 24 (h) in an oven. It was transferred to 10 mL volumetric flask containing methanol and volume was made up to the mark. The 1.0 mL of above solution was taken and diluted up to 10 mL with methanol. The resultant solution was applied on RP-TLC plate in triplicate (5 μL each, i.e, 500 ng per band). The chromatogram was developed and scanned as described above.

2.6.3. Photodegradation. Accurately weighed quantity 10 mg of RLX was dissolved in 10 mL methanol and solutions was kept for period of 24 (h) in light. An appropriate volume 1.0 mL of above solution was taken and diluted up to 10 mL
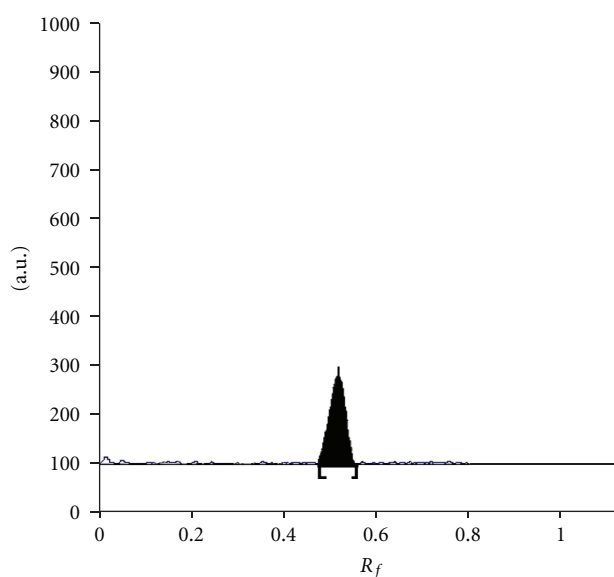
FIGURE 2: Chromatogram of RLX standard (R_f: 0.55 ± 0.02) at 311 nm, in mobile phase methanol : water : ammonia (95 : 05 : 0.1 v/v).

with methanol. The resultant solution was applied on RP-TLC plate in triplicate (5 μL each, i.e, 500 ng per band). The chromatogram was developed and scanned as described above.

3. Results and Discussion

3.1. Development of Optimum Mobile Phase. For the selection of appropriate mobile phase for RLX, several runs were exercised using mobile phases containing solvents of varying polarity, at different concentration levels. Among the different mobile-phase combinations employed, the mobile phase

TABLE 3: Robustness of the method.

Parameter	±SD of peak area	% RSD ($n = 6$)
Mobile-phase composition: methanol : water : ammonia (96 : 04 : 0.1 v/v)	50.89	1.5
Mobile-phase composition: methanol : water : ammonia (94 : 06 : 0.1 v/v)	52.74	1.6
Mobile-phase volume (±2 mL)	47.91	1.4
Development distance (±0.5 cm)	44.03	1.3
Activation of TLC plate (±5 min)	48.84	0.9
Duration of saturation (±5min)	44.97	1.4
Time from spotting to chromatography (±10 min)	40.16	0.9
Time from chromatography to scanning (±10 min)	49.34	1.2
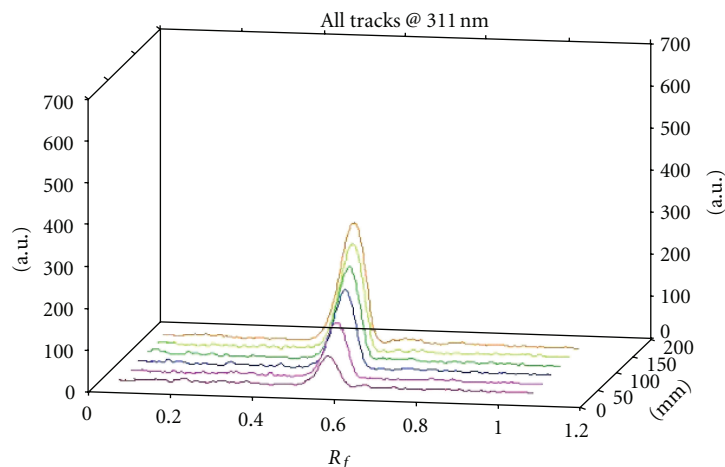
FIGURE 3: Three dimensional chromatograms of RLX sample (100–600 ng per band).

TABLE 4: Summery of validation parameter.

Parameter data	RLX
Linearity range (ng per band)	100–600
Correlation coefficient (r^2)	0.9969
Limit of detection (ng)	9.27
Limit of quantification (ng)	27.10
Recovery ($n = 3$)	99.74–100.57
Ruggedness (% RSD)	
Analyst-I ($n = 6$)	1.53
Analyst-II ($n = 6$)	1.33
Precision (% RSD)	
Repeatability of application ($n = 6$)	0.96–1.49
Interday ($n = 3$)	1.33–1.37
Intraday ($n = 3$)	0.41–1.27
Robustness	Robust
Specificity	Specific

$$y = 15.108x + 178.36$$
$$R^2 = 0.998$$

FIGURE 4: Calibration curve containing LOQ value as the lowest point.

3.2. Calibration Curve. The linear regression data for the calibration curves showed good linear relationship over the concentration range 100–600 ng per band. Linear regression equation was found to be $Y = 15.05X + 201.9$, $r^2 = 0.9969$ (Figure 3).

3.3. Validation of Method

3.3.1. Precision. The precision of the developed method was represented in terms of % relative standard deviation

consisting of methanol : water : ammonia (95 : 05 : 0.1 v/v) gave a sharp and well-defined peak at R_f value of 0.55 ± 0.02 (Figure 2). Well distinct bands were found when the chamber was saturated with the mobile phase for 25 min at room temperature.

TABLE 5: Summary of forced degradation studies.

Stress conditions	Time (h)	Recovery (%)	R_f of degradants
0.5 M HCl	12	99.5	No degradants formed
0.5 M NaOH	12	80.2	0.32,0.44,0.70
3% (v/v) H_2O_2	12	91.45	0.64
Day light (8 (h)/day)	24	99.9	No degradants formed
Heat (80°C)	24	99.6	No degradants formed
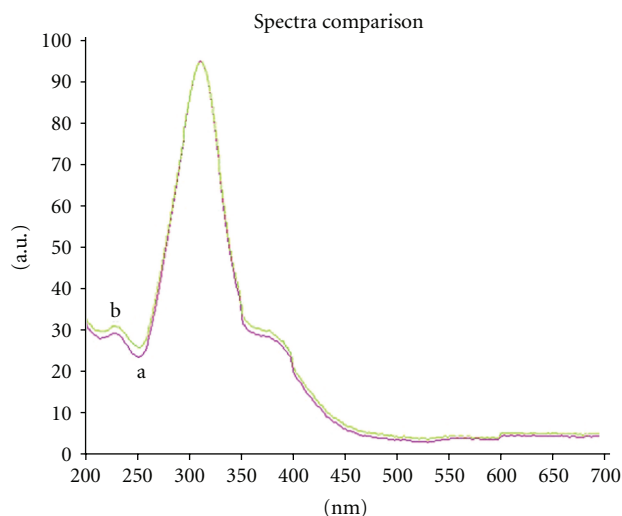
FIGURE 5: Peak purity spectra of RLX standard (a) and RLX extracted from tablets (b) scanned at peak-start, peak-apex and peak-end position.

(% RSD) of the peak area. The results depicted indicated high precision of the method are presented in Table 1.

3.3.2. LOD and LOQ. The LOD and LOQ were determined from the slope of the lowest part of the calibration plot. The LOD and LOQ were found to be 9.27 ng and 27.10 ng, respectively, which indicates the sensitivity of the method is adequate (Figure 4).

3.3.3. Recovery Studies. The recovery studies were executed out at 80%, 100%, and 120% of the test concentration as per ICH guidelines. The % recovery of RLX at all the three levels was found to be satisfactory. The amounts of drug added and determined and the % recovery are listed in Table 2.

3.3.4. Specificity. The peak purity of RLX was assessed by comparing the spectra at peak-start, peak-apex, and peak-end positions of the band, that is, $r^2(S, M) = 0.996$ and $r^2(M, E) = 0.9988$. Good correlation ($r^2 = 0.9989$) was also obtained between drug standard and drug extracted from tablet formulation (Figure 5).

3.3.5. Robustness of the Method. The standard deviation of peak areas was calculated for each parameter and % R.S.D. was found to be less than 2%. The low values of % RSD

values indicate robustness of the method; results are shown in Table 3.

3.3.6. Solution Stability and Mobile Phase Stability. The stability study of RLX in methanol demonstrates no significant change in the chromatogram obtained.

Similarly, the stability study of RLX in mobile phase also does not show any noticeable change in the chromatogram.

3.3.7. Stability on Plate. No major changes were observed in chromagram when plate's were scanned at 0, 12, 24, 36, 48, 72 (h) interval.

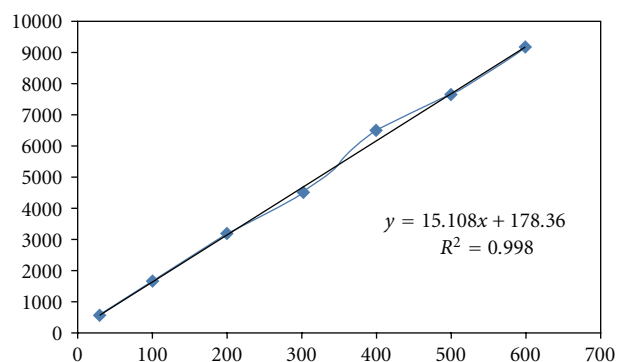The validation of the method is summarized in Table 4.

3.4. Analysis of the Marketed Formulation. A single spot at R_f 0.55 ± 0.02 was observed in the chromatogram of the drug samples extracted from tablets. There was no interference from the excipients which routinely occur in the tablets. The mean % drug content was found to be 100.28% of the label claim.

3.5. Stability-Indicating Property. The results of the forced degradation study of RLX are summarized in Table 5. RLX showed degradation in alkali and oxidative conditions. In the base-induced degradation (0.5 M NaOH) study, RLX showed additional peaks at R_f values 0.32, 0.44, 0.70, and in oxidative degradation (3% (v/v) H_2O_2) RLX showed only one additional peak at R_f 0.64 (Figure 6). The spots of the degraded products were well separated from the drug spots. The peak purity spectra of RLX recovered after degradation in 0.5 M NaOH, 3% (v/v) $H_2O_2$and RLX standard scanned at peak-start, peak-apex, and peak-end positions of the spot are shown in (Figure 7).

No additional peaks were found in acid, dry heat, and photodegradation. Therefore, RLX is stable in acidic, dry heat, and photoconditions.

4. Conclusion

The developed method was found to be simple, rapid, selective, sensitive, and suitable for determination of Raloxifene hydrochloride in bulk material and pharmaceutical dosage forms without any interference from excipients. As the method is stability-indicating one, it can be used to determine the purity of the drug available from various sources by detecting the related impurities. Furthermore, it can be concluded that the impurities present in the drug
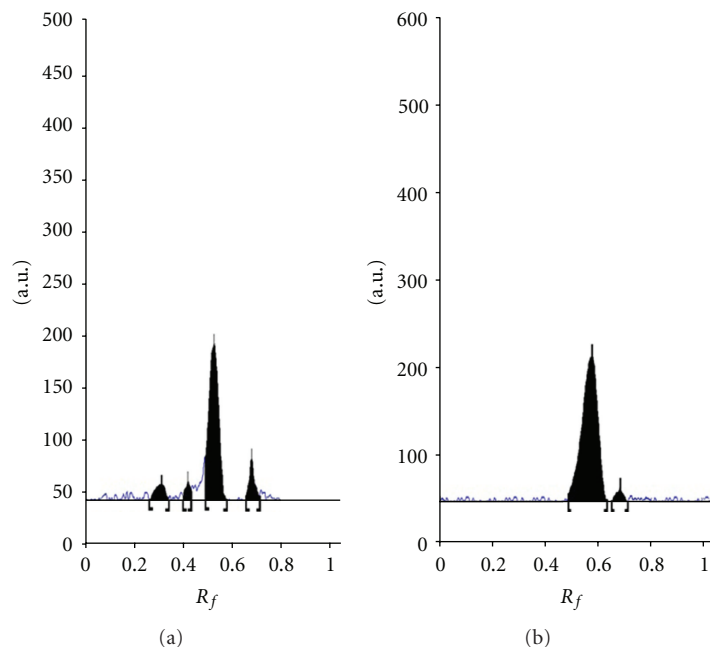
FIGURE 6: RP-TLC chromatogram obtained from forced degradation studies. (a) Base degradation (0.5 M NaOH, 12 (h), RT) showing three degradants at R_f values 0.32, 0.44, and 0.70 for RLX. (b) Oxidative degradation (3% (v/v) H_2O_2, 12 (h), RT) showing one degradant at R_f values 0.64 for RLX.
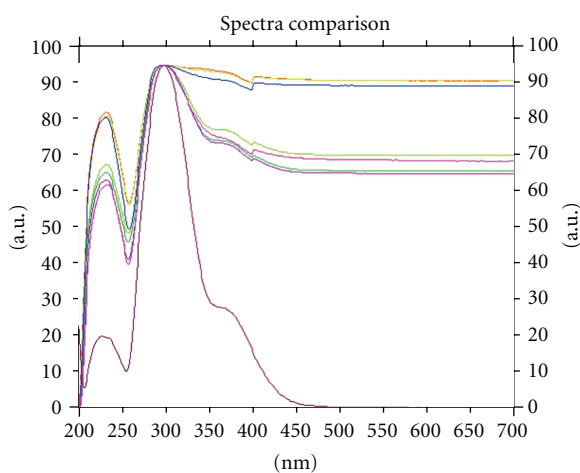
FIGURE 7: Peak purity spectra of RLX recovered after degradation in 0.5 M NaOH, 3% (v/v) H_2O_2, degradants, and RLX standard scanned at peak-start, peak-apex, and peak-end positions.

could be due to hydrolysis or oxidation during processing and storage of the drug.

The proposed procedure fits precision and accuracy usually requested by official methods and can be used as a convenient alternative to HPLC analysis for quantitation of Raloxifene hydrochloride in both bulk and tablet dosage forms. Therefore, the proposed RP-TLC/Densitometry method can be used as an alternative tool in the drug quality control laboratories for quantitative determination of Raloxifene hydrochloride.

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Research Article

Development and Validation of Stability-Indicating GC-FID Method for the Quantitation of Memantine Hydrochloride and Its Nonchromophoric Impurities in Bulk and Pharmaceutical Dosages

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A stability-indicating method has been developed and validated for the quantitative determination of memantine hydrochloride and its nonchromophoric impurities in drug substance and drug product using gas chromatography coupled with flame ionization detector (GC-FID). The stability-indicating nature of the method has been proved by establishing peak purity and confirming the mass balance of all samples by subjecting them to stress conditions like hydrolysis, oxidation, photolysis, and thermal degradation studies. The chromatographic separation was performed on a fused silica capillary (HP-5, 30 meter, 0.32 mm and 0.25 μm film thickness) column. The method validation results indicate that the method has acceptable specificity, accuracy, linearity, precision, robustness, and high sensitivity with detection limits and quantitation limits ranging from 0.001% to 0.01% and 0.004% to 0.03%, respectively. The effectiveness of the technique was demonstrated by analysis of different bulk sample of Memantine hydrochloride. The proposed GC-FID method was also found to be specific and selective for the analysis of commercial formulation samples.

1. Introduction

Memantine hydrochloride is a 1-amino-3,5-dimethyladamantane derivative developed by Merz co. for the treatment of Alzheimer's disease by decreasing abnormal activity in the brain. This medication was approved by US-FDA during 2007 and marketed under the brand name Namenda. Memantine helps people with dementia to think more clearly and perform daily activities more easily [1–3]. Memantine and its process-related impurities, namely, Imp-A, Imp-B, and Imp-C (Figure 1) are small molecules insensitive to UV due to lack of ultraviolet chromophores, and hence the determination of memantine and its related substances in drug substances and drug product was a critical activity during drug testing. Several analytical methods have been reported for the determination of memantine on high-performance liquid chromatography (HPLC) in combination with mass spectrometry and ultraviolet detection or fluorescence detection usually after derivatization with a suitable chromophores or fluorophore [4–11]. Most of these reported liquid chromatography-mass spectrometry (LC-MS) methods require tedious extraction procedures, which are time-consuming, complex, and expensive [4, 5]. However, some of HPLC methods are intended for the determination of memantine in biological matrices requiring multiple sample preparation, derivatization and thus are not suitable for routine testing in quality control laboratory [6–9]. The one more drawback of fluorescent derivatization method is that the fluorescence detectors are not as common as UV-visible detector in the analytical laboratory
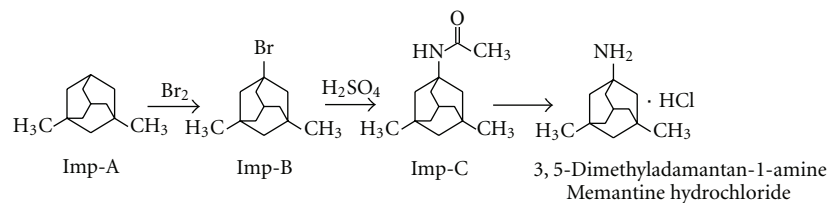
FIGURE 1: Synthetic scheme of memantine hydrochloride.

of a pharmaceutical company. A gas chromatography-mass spectrometry (GC-MS) method has also been reported for determination of memantine in plasma and which is also not convenient for routine analytical testing [10, 11]. In addition, Michail et al. recently report the photometric methods for determination of memantine by derivatization with NBD-Cl or OPA/NAC reagents in alkaline media [12]. Thus, we felt a need of a simple, suitable, accurate, and stability-indicating method for the quantification of memantine and its impurities in bulk and formulation samples. Literature survey indicates that there is no stability-indicating GC-FID method available for the determination of memantine and its nonchromophoric impurities, and thus we aimed to develop the same in our analytical laboratory.

Development of "analytical method" for a pharmaceutical product during process research and development in pharmaceutical companies is a critical activity for analytical chemists especially engaged in the generic product due to varied impurities in the API as scheme selected are very specific for their preparation during its life cycle. Understanding of the impurity profile, its structure, and its physical and chemical properties and the study of degradation pathway of API are very important to establish the suitable analytical method. An ideal stability-indicating method is one that quantifies the standard drug alone and also resolves its degradation products. With the advent of International Conference on Harmonization (ICH) guidelines, the requirement of establishment of stability-indicating method has become more clearly mandated [13, 14]. The guidelines explicitly require the conduction of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, and so forth, and separation of the drug from degradation products. The establishment of purity of chromatographic peak in stressed samples is essential for the validation of the chromatographic method. This is particularly important when developing a stability-indicating method for determination of impurities. Currently various methods are employed for measuring peak purity using a photodiode array detector, which essentially compares the entire UV spectra recorded at various points across the liquid chromatography (LC) peak with the spectrum collected at the apex of the peak. Sometimes this concept is not useful as there are often no spectral data available for the impurity. Gas chromatography (GC) is a very well-established technique for determination of residual solvents, nonchromophoric impurities in drug substance, drug product, and environmental contaminant identification. GC is very rarely used for quantitative determination of impurities in pharmaceutical

preparation. Developing a stability-indicating method on GC for impurity profiling in active pharmaceutical ingredients or in pharmaceutical formulation is a challenging task. Complexities involved in development of method are extraction of impurities in presence of salts, selection of suitable diluents for gas chromatographic analysis, and proving stability-indicating nature of the method. The analytes are typically detected using a flame ionization detector (FID) technique. These FID detectors can be used for almost all pharmaceutical products, as they are able to detect any analyte which contains a C–C or C–H bond. However, in order to analyze compounds using gas chromatography, the compounds must have a high enough vapor pressure to allow them to be volatilized prior to interaction with the chromatographic column. Gas chromatography is a highly efficient, sensitive method used to analyze complex mixtures of substances and nonchromophoric impurities. In our study, a simple stability-indicating GC-FID method was developed and validated for determination of related substance of memantine hydrochloride in drug substances and drug product. The peak purity of stressed samples has been established by comparing mass ion fragmentation pattern with the memantine reference standard using GC-MS. All three non-chromophoric impurities (IMP-A, IMP-B, and IMP-C) are well separated from each other. The method was validated as per ICH guideline [15] and successfully applied for separation of all compound of interest in the pharmaceutical formulation. The method reported here may find utility both in the pharmaceutical and chemical industries, and it may potentially be applied to the purity/impurity analysis of other pharmaceutical product analysis.

2. Experimental

2.1. Materials and Reagents. The investigated sample of memantine hydrochloride and its potential process-related impurities (Figure 1) were received from synthetic laboratory of Megafine Pharma (P) Ltd, Nashik, India. In addition, analytical reagent grade chloroform and sodium hydroxide were purchased from Merck, (Mumbai, India). Highly pure water obtained from Millipore system (Millipore Inc., USA) was used throughout the analysis.

2.2. GC (Analytical) Instrumentation and Operating Conditions. The GC system used for method development and method validation was Agilent 1200 series (manufactured by Agilent technologies, Waldbronn, Germany) with a liquid

autosampler. The detection was performed by means of flame ionization detector (FID). The output signal was monitored and processed using Ezchrome Elite software version 3.2.1. The HP-5 (30 m length × 0.32 mm ID, 0.25 μm film thickness) column has been procured from Agilent technologies made in USA and used for the method development and method validation studies. The column oven was programmed as follows: initial column oven temperature, 90°C; hold for 5 min, and increased to 250°C at the rate of 10°C/min; hold for 10 min. The run time of analysis was 31 min. The injector and detector temperature was kept at 250°C and 280°C, respectively. Nitrogen was used as a carrier gas with a constant pressure of 7.0 psi. The split ratio was set at 20 : 1 and a 4 mm i.d., deactivated open-glass tube liner packed with fused silica wool was employed. Sample was injected by the instrument's autosampler with injection volume of 1.0 μL and chloroform as the syringe cleaning solvent between injections.

2.3. Preparation of Standard Solutions and Sample Solutions.

All the samples used for method development and validation were prepared volumetrically. A solution of memantine hydrochloride was prepared at a concentration of 50 mg/mL and 2 mg/mL in the chloroform for related substances determination and assay determination, respectively. The individual stock solutions of each impurity at concentration of about 375 μg/mL (impurities Imp-A, B, and C) were prepared in chloroform and diluted further adequately to study the validation attributes. The resultant final diluted solutions were separately transferred into different 125 mL separating funnel. Extracted as follows, 25 mL of water and 1.0 mL aqueous sodium hydroxide solution (40% w/v) were added to each separating funnel, shaked well, and allowed the layers to separate. Collected the lower chloroform layer into another different 125 mL separating funnel and washed the organic layer by 20 mL water washing to remove the inorganic salts and allowed the layers to separate. Collected the washed lower chloroform layer in 25 mL volumetric flask containing about 2.0 gm of anhydrous sodium sulphate, shaked well, and the supernatant liquid was taken for injection. The specification limits used for validation studies were 0.15% for the known impurities (Imp-A, Imp-B, and Imp-C) and 0.10% for the any individual unknown impurity. Hence, the system suitability solution of all impurities and memantine was prepared at specification level by diluting above stock solutions.

The test solution was prepared by taking powdered tablets, and a known amount of memantine hydrochloride equivalent to 500 mg was transferred to a 50 mL volumetric flask along with 10 mL chloroform and 25 mL water. The powdered material was dispersed by mixing in an ultrasonic bath for 20 min and diluted to 50 mL with diluent. Above solution was centrifuged at 4000 rpm for 15 min in order to eliminate insoluble excipients and transferred into 125 mL separating funnel, treated, and extracted as per the above extraction procedure of drug substances. Blank chloroform was prepared by the extraction treatment given to the chloroform.

2.4. Analytical Procedure.

1.0 μL of blank chloroform, six replicate injections of system suitability solution, and test sample solution were separately chromatographed. A resolution of not less than 3.0 between any two peaks was set as a system suitability requirement in system suitability solution. To verify the system precision, six replicate injections of system suitability solution were injected and the relative standard deviation (RSD) for Imp-A, Imp-B, Imp-C, and memantine peak areas obtained from six injections were calculated. The RSD of not more than 5.0% was set as system precision acceptance criteria for Imp-A, Imp-B, Imp-C, and memantine peak areas. All the known related substances Imp-A, Imp-B, and Imp-C in test sample were determined against mean area of respective impurities obtained from replicate injections of system suitability solution. Also, the unknown impurities in test sample were calculated against mean area of memantine obtained from replicate injections of system suitability solution.

2.5. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis.

The GC-MS analyses was carried out on Shimadzu GC-MS-QP 2010 gas chromatograph fitted with a HP-5 (methylphenylsiloxane, 30 m × 0.32 mm i.d.) capillary column and was used under the following conditions: carrier gas, nitrogen with a flow rate of 1.0 mL/min; column temperature, 5 min hold for 90°C, 90 to 250°C at 10°C min^{-1}, 10 min hold at 250°C; injector temperature, 250°C; volume injected, 1 μL of impurity solution; split ratio, 50 : 1. The MS operating parameters were as follows: ionization potential, 70 eV; ion source temperature, 290°C; quadrupole 100°C, scan time 3.1 to 31 min, scan speed 2000 amu/s, and scan range 45–450 amu, ion mode EI^{+ve}.

2.6. Specificity/Application of Stress (Forced Degradation Study).

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. The specificity of the developed GC method for memantine hydrochloride was demonstrated in the presence of its impurities (namely Imp-A, Imp-B, and Imp-C) and degradation products. Forced degradation study was conducted on bulk drug substance in order to prove the stability-indicating property and selectivity of the established method [16]. Forced degradation of memantine was carried out under acid/base hydrolytic, oxidative, thermolytic, and photolytic stress conditions. The drug substances were exposed with concentrated hydrochloric acid (refluxed for 36 h), aqueous 5 M sodium hydroxide (refluxed for 36 h), and aqueous 30% hydrogen peroxide (kept for 24 h at RT). After the degradation, these solutions were extracted with chloroform to a known concentration and analyzed by the proposed method. For thermal stress, sample of drug substance was placed in oven with controlled temperature of 60°C for 8 days. For photolytic stress, the sample was exposed to photolytic conditions for 9 days as per ICH guideline. After the exposure to the above stress conditions, solutions of these samples were prepared by dissolving respective samples of known concentration in chloroform and extracted as per extraction procedure and further subjected to analysis

TABLE 1: System suitability test results.

Compound	Retention time (min)	RRT	Theoretical plate	USP tailing factor (T)	Resolution (R_s)
Imp-A	8.04	0.74	212697	0.99	—
Memantine	10.93	1.00	410875	1.40	41.92
Imp-B	13.40	1.23	728847	1.04	37.66
Imp-C	17.36	1.59	1354612	1.00	64.75

RRT: relative retention time.

TABLE 2: Forced degradation results.

Stress condition	% Assay of memantine	% of degradant	Observation and mass balance	Peak purity by GC-MS
Undegraded	98.8	—		
Acid hydrolysis (Conc. HCl, 36 h refluxed)	100.6	Nil	No any known and unknown degradation product formed (Mass balance: 100.6%)	Mass spectrum similar to the mass spectrum of reference standard
Base hydrolysis (5 M NaOH, 36 h refluxed)	101.1	Nil	No any known and unknown degradation product formed (Mass balance: 101.1%)	Mass spectrum similar to the mass spectrum of reference standard
Oxidation (30% H_2O_2, 24 h at RT)	76.6	24.0	24.0% of unknown minor degradation products formed (Mass balance: 100.6%)	Mass spectrum similar to the mass spectrum of reference standard
Thermal (60°C, 8 days)	99.2	Nil	No any known and unknown degradation product formed (Mass balance: 99.2%)	Mass spectrum similar to the mass spectrum of reference standard
Photolytic as per ICH	99.2	Nil	No any known and unknown degradation product formed (Mass balance: 99.2%)	Mass spectrum similar to the mass spectrum of reference standard

Mass balance = % assay + % sum of all impurities + % sum of all degradants.

using the proposed method. All the stressed samples were quantified for memantine and its impurities. The mass balance (% assay + % of impurities + % of degradation products) was calculated for all of the samples. GC-MS analysis was employed to check and ensure the homogeneity and purity of memantine peak in all the stressed sample solutions.

2.7. Synthesis of Memantine Hydrochloride. The reaction scheme for the synthesis of memantine hydrochloride is shown in Figure 1 [17]. The chemical name of possible three process related impurities is *1,3-dimethyladamantane* (Imp-A), *1-bromo-3,5-dimethyladamantane* (Imp-B), and *N-(3,5-dimethyl-1-adamantyl)acetamide* (Imp-C), whose area percentage in the crude samples is ranged from 0.05 to 0.2% consistently.

3. Results and Discussion

3.1. Detection of Impurities. Laboratory batches of crude memantine hydrochloride were analyzed for their related substances identification using the developed GC-FID method. These samples were subjected to GC-MS analysis. Imp-B and Imp-C were detected in the crude memantine hydrochloride batch sample. The masses of the detected peaks observed on GC-MS were noted. On the basis of m/z value and NIST library search (Shimadzu, Kyoto, Japan),

the possible structures for potential impurities suggested are shown in Figure 1. Evaluation of synthetic scheme adopted for memantine hydrochloride and basic raw materials used for the construction of intermediates were considered for understanding the potential process related impurities. These impurities (Imp-A, Imp-B, and Imp-C) were received from R&D laboratory of Megafine Pharma (P) Ltd and coinjected with memantine hydrochloride to confirm the retention times.

3.2. Development of Chromatographic Conditions. The core-objective of the present study is to develop a simple, rapid, and stability-indicating GC-FID method capable of eluting and resolving memantine, its process-related and degradation-related impurities within the short run time that complies with the general requirement of system suitability. During the method development, the optimized conditions using the HP-5 column gave excellent separation of the impurities in memantine hydrochloride bulk drug substance but poor peak shape of memantine was noticed. The memantine hydrochloride assay concentration was injected with different chromatographic conditions, but the peak shape was not improved satisfactorily (Figure 2(a)) mainly could be due to hydrochloride salt. The poor peak shape of memantine designated that there was need to optimize the suitable extraction procedure and then need to optimize the chromatographic conditions.
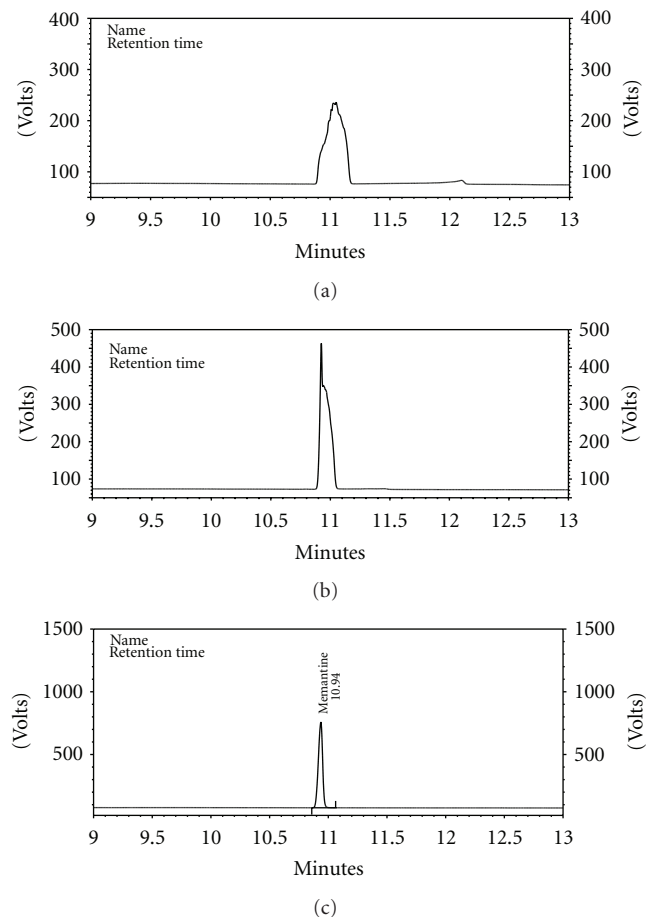
(a)

(b)

(c)

FIGURE 2: GC-FID method development chromatogram during extraction procedure optimization; (a) test preparation injection before extraction, (b) test preparation injection during optimization of extraction procedure, and (c) test preparation injection after extraction.

3.2.1. Optimization of Extraction Conditions.

In order to obtain best extraction efficiency, we used the peak area of memantine standard as the GC response and peak shape to evaluate the extraction efficiency under different conditions. To optimize the method, all extractions were initially carried out on standard stock solution. Suitable organic solvent for GC analysis was identified based on the extractability of memantine hydrochloride from aqueous solution. Solvents such as n-heptane, methylene dichloride, n-hexane, and chloroform were screened for extraction of memantine hydrochloride from aqueous samples. Memantine hydrochloride being highly soluble in chloroform as compared with other solvents experiments with chloroform showed best extraction efficiency of memantine and its three impurities. Efficient extraction in chloroform also played an important role in improving the peak shape of memantine substantially as shown in Figure 2.

3.2.2. Optimization of Chromatographic Conditions.

During the method development, different chromatographic parameters were optimized to obtain an acceptable peak shape and resolution between the two peaks with acceptable recoveries to satisfy the GC system suitability. These parameters include flow rate (5–10 psi, at constant pressure), initial column temperature (80–150°C), and injector temperature (±10°C). Also various capillary GC columns (HP-5, DB-1701, and SPB-1 with different film thickness) were screened during the method optimization. Among those tested, the SPB-1 (30 m length × 0.32 mm ID, 1 μm film thickness) and DB-1701 (30 m length × 0.32 mm ID, 1 μm film thickness) columns gave reasonable retention times at lower temperatures, but these stationary phases shown slightly more bleed and had a baseline shift over the temperature range used. Satisfactory selectivity, sensitivity, resolution, and speed of chromatographic separation with stable baseline were only achieved on HP-5 column (30 m length × 0.32 mm ID, 0.25 μm film thickness) with nitrogen as a carrier gas under the chromatographic conditions as described in Section 2.2. The run time of analysis was 31 min. In the optimized conditions, the memantine and all the three nonchromophoric impurities were well separated with a resolution greater than ten. The typical retention times of Imp-A, Memantine, Imp-B, and Imp-C were about 8.1, 10.9, 13.4, and 17.3 min, respectively (Figure 3). The developed GC-FID method was found to be specific for determination related substances and assay in drug substance and drug product. For peak identification, a solution containing Imp-A, Imp-B, and Imp-C spiked with authentic samples of memantine hydrochloride at levels of 0.15% (with respect to target analytical concentration) was injected, and the peak identities were confirmed by GC-MS.

3.3. System Suitability Criteria.

A system suitability test was defined based on the results obtained in several representative chromatograms. The column efficiency determined from the analyte peak >100000, resolution between two analyte peaks >10.0, and RSD for impurity areas in six replicate injection of system suitability solution was <5.0%. All the system suitability criteria during validation of the study and batch analysis study were within the acceptance limit. The results of system suitability are depicted in Table 1.

3.4. Validation

3.4.1. Specificity (Selectivity).

The stability-indicating property of analytical method for memantine hydrochloride was established by targeting 10–30% degradation of memantine. The degradation was not observed in memantine hydrochloride sample when sample exposed to stress conditions like photolytic, thermal, acid, and base hydrolysis. Considerable degradation of memantine hydrochloride was observed under oxidative hydrolysis only. The GC-MS data (mass-ion fragmentation) of memantine indicated that the memantine peak is homogenous and spectrally pure in all the stress conditions tested. The typical chromatogram of memantine test sample after degradation under oxidative conditions is shown in Figure 4 and GC-MS spectra for memantine in unspike test preparation, spiked test preparation and peroxide treated test preparation are shown in Figure 5. The mass balance is a process of adding together the assay value

TABLE 3: Linearity, limit of detection (LOD), and limit of quantitations (LOQ) data for memantine hydrochloride and related substances.

Component	Linearity results (LOQ to 250% of specification level[a])		LOQ LOD results	
	Regression equation	CC[b] (r)	LOQ μg/mL, (% w.r.t.)[c]	LOD μg/mL, (% w.r.t.)[c]
Imp-A	$y = 1236.60x - 346.02$	1.00000	2.941 (0.006)	0.971 (0.002)
Imp-B	$y = 817.18x - 5032.36$	0.99951	5.421 (0.011)	1.789 (0.004)
Imp-C	$y = 992.21x - 602.22$	0.99999	3.491 (0.007)	1.152 (0.002)
Memantine	$y = 921.02x - 2420.30$	0.99991	5.985 (0.012)	1.975 (0.004)

[a] 0.15% of all related substances and 0.10% of memantine hydrochloride.
[b] CC: correlation coefficient.
[c] LOD LOQ values are in % with respect to test concentration of 50 mg/mL.
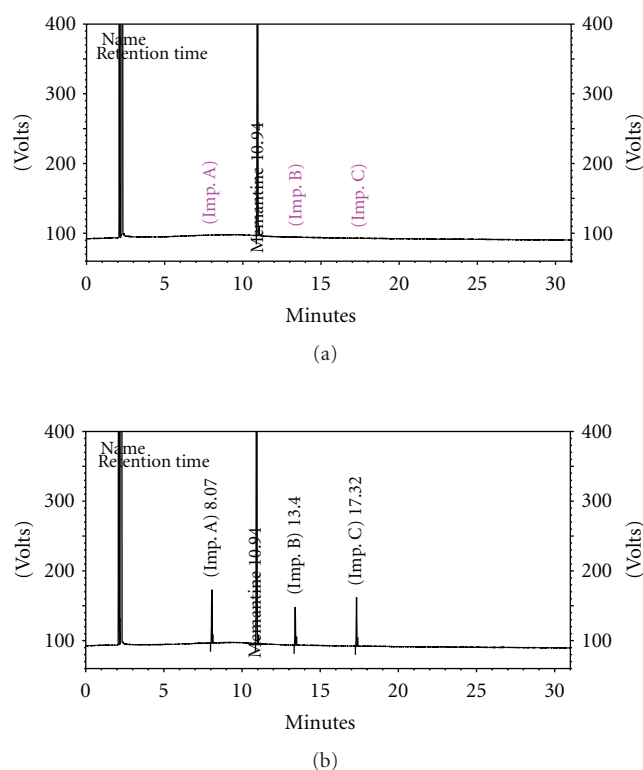
FIGURE 3: Typical GC-FID chromatograms of (a) memantine hydrochloride unspiked test preparation, (b) memantine hydrochloride spiked test preparation with known impurities (Imp-A, Imp-B, and Imp-C).

FIGURE 4: Typical GC-FID-forced degradation chromatogram of peroxide treated test preparation.

peak, suggesting the method enabled specific analysis of memantine in the presence of its degradation products.

3.4.2. Linearity. The linearity of method was established at two different levels. The assay linearity was studied by preparing five different solid weighings of memantine hydrochloride from 50 to 150% w/w (50, 75, 100, 125, and 150% w/w) with respect to target analytical concentration (2000 μg/mL) and injected. Linearity test solutions for related substance were prepared by diluting the impurity stock solution (as described in the Section 2.3) to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 250% (LOQ, 50, 100, 150, 200, and 250%) with respect to the specification level of impurities. The data were subjected to statistical analysis using a linear-regression model; the regression equations and correlation coefficients are given in Table 3. The results showed an excellent correlation between the peak areas and concentrations of Imp-A, Imp-B, Imp-C, and memantine.

3.4.3. Limits of Detection and Quantitation (LOD and LOQ). According to ICH Q2R1 recommendations, the limits of detection (LOD) and quantitation (LOQ) for memantine and its key impurities (Imp-A, Imp-B, and Imp-C) were estimated by calibration curve method (residual standard deviation (σ) and the slope (S) of regression line), by injecting series of dilute solutions of known concentration.

and the levels of degradation products to see how closely these add up to 100% of initial value with due consideration of the margin of analytical error [18].

The resultant mass balance of stressed samples was about 99.0%. The data on forced degradation studies are summarized in Table 2. The results on degradation studies revealed that the degradation products were well separated from the memantine. No peaks coeluted with the memantine

Rt-9.04 (base peak *m/z* 108)

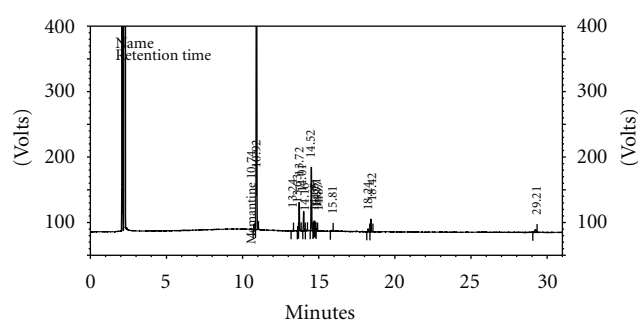(a)

Rt-9.04 (base peak *m/z* 108)

(b)

FIGURE 5: Continued.

Rt-9.05 (base peak *m/z* 108)

(c)

FIGURE 5: Typical GC-MS spectra of memantine in (a) unspiked test preparation, (b) spiked test preparation, and (c) peroxide treated test preparation.

TABLE 4: Validation data results of related substances and assay of memantine hydrochloride.

| Compound | Related substances results | | | Assay results |
	Imp-A	Imp-B	Imp-C	Memantine
Precision (mean results ±%RSD)				
Method precision ($n = 6$)	0.155 ± 1.29	0.167 ± 1.20	0.153 ± 1.31	99.9 ± 0.44
Intermediate precision ($n = 6$)	0.165 ± 1.21	0.165 ± 1.82	0.168 ± 1.19	100.4 ± 0.73
Overall results ($n = 12$)	0.160 ± 3.13	0.166 ± 1.81	0.161 ± 4.97	100.1 ± 0.62

The LOQ and LOD of memantine hydrochloride were 5.985 μg/mL and 1.975 μg/mL, respectively, whereas the LOD and LOQ for the related substances were in the range of 0.971–1.789 μg/mL and 2.941–3.491 μg/mL, respectively. The calculated LOQ concentrations of all the components were further verified for precision by injecting six individual preparations of Imp-A, Imp-B, Imp-C, and memantine hydrochloride. The RSD of LOQ precision was in the range of 0.83–7.19%. These limits of quantification levels of the impurities were helpful for the process research work to control the impurities at the accepted level during the optimization of the process. The results are depicted in Table 3.

3.4.4. Precision. System precision for assay method was verified by injecting the six replicate injections of standard concentration (5 mg/mL), and RSD of memantine peak area was evaluated and found to be 0.72%. System precision for related substances determination was verified by system suitability solution, which was analyzed for six times and RSD of memantine and all impurities peak areas were evaluated and found to be 0.58%.

Precision of the method was studied for method precision and intermediate precision. The assay method precision was examined by analysing six determinations of the sample solution at working concentration versus a standard concentration, and RSD of obtained results was evaluated and found to be 0.44%. Related substances method precision was demonstrated by analyzing six separate memantine hydrochloride sample solutions that were prepared by spiking the related substances, namely, Imp-A, Imp-B, and Imp-C at specification level. The RSD (1.20–1.31%, $n = 6$) for each related substance was evaluated.

TABLE 5: Accuracy data of related substances.

| Component | | Recovery results (mean % recovery (%RSD))[a] | | |
	LOQ level; amount (% w/w)	50% of specification level[b]; amount (% w/w)	100% of specification level[b]; amount (% w/w)	150% of specification level[b]; amount (% w/w)
Imp-A	116.67 (0.00)	106.67 (3.00)	104.00 (2.00)	104.15 (3.00)
Imp-B	116.67 (0.00)	114.86 (3.00)	111.94 (2.00)	113.36 (3.00)
Imp-C	112.50 (0.00)	102.67 (3.00)	100.22 (2.00)	100.30 (3.00)

[a] Average % recovery and % RSD of results of three determinations.
[b] 0.15% of all related substances.

TABLE 6: Results (in %) of formulation tablet and bulk drug batches sample analysis.

| Sample source | Related substances results | | | | Assay results |
	Imp-A	Imp-B	Imp-C	SUMI	
Formulation product analysis results					
Formulation-1	ND	ND	ND	ND	99.4
Formulation-2	ND	ND	ND	ND	99.9
Batch analysis results (%)					
B.NO. MEM/A163/IV/08	ND	ND	ND	0.01	99.7
B.NO. MEM/A163/IV/09	ND	ND	ND	0.01	99.2
B.NO. MEM/A163/IV/10	ND	ND	ND	0.01	99.3

ND: not detected
SMUI: single maximum unknown impurity.

In the intermediate precision study, the similar procedure of method precision was carried out by a different analyst, diluent preparations, and instrument on a different day with different lot of same brand column. The percentage relative standard deviation of the results for assay method and related substances method was evaluated and found to be 0.73% and 1.19–1.82%, respectively. The percentage overall RSD for results of intermediate precision and results of method precision was calculated. The overall RSD ($n = 12$) of assay and related substances results was found within 0.62% and 4.97%, respectively. The results were reported in Table 4.

3.4.5. Accuracy (Recovery). Accuracy of the method for all the related substances was determined by analyzing memantine hydrochloride sample solutions spiked with all the related substances at four different concentration levels namely, LOQ, 50, 100, and 150% of specification level. Each level has been analyzed in triplicate. Table 5 provides validation data results of related substances of memantine hydrochloride. The recovery of all these related substances was found to be in between the predefined acceptance criteria of 80.0–120.0% [19].

3.4.6. Robustness. To assess robustness of the method, the experimental conditions were deliberately altered and system suitability parameter was evaluated. Nitrogen was used as a carrier gas with a constant pressure of 7.0 psi. To study the effect of flow rate on the resolution, the same was altered by 0.7 units that is from 6.3 to 7.7 psi. The effect of column temperature was studied at 93 and 87°C instead of 90°C. The effect of changing the injector temperature by ±10°C (240°C and 260°C instead of 250°C) on resolution was also studied.

All the other chromatographic conditions were held constant as described above.

In all the deliberate varied chromatographic conditions (flow rate, column temperature, and injector temperature), the all system suitability criteria were within the limits (theoretical plate > 100000, resolution between two analyte peak > 10.0, and RSD < 5.0%), illustrating the robustness of the method.

3.4.7. Stability of Analytical Solution. Sample solutions of memantine hydrochloride spiked with related substances at specified level were prepared and analyzed immediately at different time intervals up to 48 hrs to determine the stability of sample solution. The cumulative % RSD of memantine and all impurities peak areas was evaluated at each time interval and found to be less than 5.0%. The results from these studies indicated that the sample solution was stable for 48 hrs at room temperature.

3.5. Application of the Method. The analysis of commercial formulation sample and bulk drug sample indicated that the method is specific and selective for determination of related substances in the formulation and bulk drug samples (Table 6). The developed method is capable for quantitative analysis of memantine hydrochloride in the bulk drug and in a pharmaceutical dosage form. The values of impurities detected indicate that the method is capable of detecting known and unknown impurities in routine analysis of bulk drug and formulation sample.

4. Conclusion

This is the first method reported in the literature for the separation and quantitation of memantine and its impurities/degradants. The GC-FID method is specific, linear, sensitive, accurate, precise, and robust. This approach may potentially be applied to the purity/impurity analysis of other acid chlorides or acid halides. The degradation pathway of memantine hydrochloride is established as per ICH recommendations. The stability-indicating GC-FID method developed and used for stress studies is also fit for quantitative, related substance, and assay determination of memantine hydrochloride. The method is validated as per ICH requirements. The developed method is stability indicating which can be used for the impurity testing and assay determination in routine analysis of production samples and also to analyze stability samples.

Acknowledgments

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Research Article

A Validated LC Method for Separation and Determination of Tetralone-4-O-β-D-Glucopyranoside and 4-Hydroxy-α-Tetralone in *Ammannia multiflora*

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A rapid, sensitive, and reproducible RP-HPLC method was developed for the determination of two constituents in *Ammannia multiflora*, namely, tetralone-4-O-β-D-glucopyranoside (**1**) and 4-hydroxy-α-tetralone (**2**). The samples were separated on a Spherisorb ODS2 column (250×4.6 mm, i.d., $10\,\mu$m) and binary elution of water and methanol ($2:3$) with flow rate of 0.8 mL/min at λ 254 nm. The LOD and LOQ were found 0.05 and 0.18 μg/mL for compound **1** and 0.06 and 0.18 μg/mL for compound **2**, respectively. All calibration curves showed good linearity ($r^2 > 0.999$) within test ranges for both the analytes. The RSD values for intra-and inter-day precisions were less than 1.1%. The successful application of the developed method on five different samples revealed an average 0.0206% and 0.7636% (w/w) of compounds **1** and **2**, respectively in *A. multiflora* indicating that the developed LC assay method may be readily utilized as a quality control method for the plant.

1. Introduction

The genus Ammannia (Family Lythraceae) is commonly called as "red stems," and species of this genus are nearly cosmopolitan in distribution including India. One species of this genus, *Ammannia baccifera* is frequently used in traditional Chinese medicine to cure human female infertility, gastroenteropathy, as antipyretic, antidiuretic, antimicrobial, antirheumatic, anticancer, rubefacient, and as an external remedy for ringworm and skin diseases [1]. *Ammannia multiflora* grows in marshy places along the banks of rivers and rivulets and generally found in cultivated paddy fields. In Tanzania, the juice of fresh leaves of *A. multiflora* is used to treat sight problems in human adults, caused by filaria [2]. The preliminary phytochemical analysis of *A. multiflora* showed presence of glycosides, flavonoids, carbohydrates, steroids, phenols, and absence of alkaloids [3]. The GC-MS analysis of hexane and chloroform fraction led to identification of fourteen constituents, many of them are well-known bioactive phytomolecules [3]. Recently,

we isolated and characterized compounds **1** and **2** from *A. multiflora* and reported bioenhancing activity of **2** and its various semisynthetic acyl and aryl derivatives [1]. Compound **2** has shown potent antituberculosis [4], antidiabetic [5], and antileishmanial [6] activities in earlier studies. Until now there has been no quantitative method for the analysis of *A. multiflora*, hence the current study was aimed at developing and validating a simple, rapid, and sensitive reverse phase HPLC method for the quantification of these two constituents in order to have quality control in *A. multiflora*. The present work is part of a series of efforts towards developing analytical methods for plant drug analysis.

2. Experimental

2.1. Plant Material and Sample Preparation. The samples of *A. multiflora* were collected from the Lucknow, Faizabad, Gorakhpur, Barabanki, and Sitapur districts of Uttar

TABLE 1: Solvent system for the separation of analytes **1** and **2** from the MeOH extract of *A. multiflora*.

Time (min)	Water conc. (%) (pump A)	Methanol conc. (%) (pump B)
0.00	40	60
15.00	70	30
20.00	40	60

Pradesh, India in the month of November 2009 and identified by Dr. D. C. Saini, Scientist, Birbal Sahni Institute of Palaeobotany (BSIP), Lucknow and Dr. S. C. Singh, Scientist, Botany Division, Central Institute of Medicinal & Aromatic Plants (CIMAP), Lucknow, India. A voucher specimen (no. −9455) has been deposited at the CIMAP herbarium.

The dried and finally milled plant material (particle size about 60 mesh, 0.1 g) of *A. multiflora* (whole plant) was extracted by sonication for 30 min using methanol as solvent. The solvent was removed under vacuum at 40°C, and the extract was redissolved in 1 mL of methanol and centrifuged at 10000 rpm for 10 min, filtered through a 0.45 μm Millipore membrane (Millipore, Billerica, MA, USA).

2.2. Chemicals and Standards. The solvents used for extraction were of analytical grade, while the methanol and water used for chromatographic separation were of HPLC grade (all purchased from E. Merck Ltd., Mumbai, India). The reference marker compounds **1** and **2** were isolated in high purity (98.7%) in our laboratory following previously described procedures [1] (see details in supporting information). The purity of isolated compounds was determined by HPLC using area normalization method. Before use in HPLC, the solvents were filtered through a 0.45 μm Millipore membrane. The samples and mobile phases were degassed by sonicator.

2.3. Apparatus and Chromatographic Conditions. HPLC analysis was performed on a Shimadzu LC-10AD Liquid chromatograph equipped with a SPD-M10A VP Diode array detector, a SIL-10ADVP autoinjector and CBM-10 interface module. Data were collected and analyzed using a class LC-10 Work Station. A prepacked Waters Spherisorb ODS2 (250 × 4.6 mm, i.d., 10 μm) column was selected for HPLC analysis. The separation was achieved with an isocratic program for pump A (water) and pump B (methanol) (Table 1). The injection volume was 20 μL, and flow rate was 0.8 mL/min throughout the run. Column temperature was maintained at 25 ± 1°C. The data acquisition was performed in the range of 200–400 nm, and quantification was performed at 254 nm. Sonicator (Microclean 109, Oscar Ultrasonic, Mumbai, India) was used for sample preparation. The 300 MHz NMR (Avance, Bruker, Switzerland) was used to record ^{1}H and ^{13}C NMR with tetramethylsilane (TMS) as internal standard. Hyphenated LC-PDA-MS (Prominence LC and mass MS-2010EV, Shimadzu, Japan) was used for mass spectra.

2.4. Preparation of Standard Solutions. Standard stock solutions of compounds **1** and **2** were prepared as 1.0 mg/mL in methanol. A serial dilution was made for each stock solution at concentrations of 2–10 μg/mL by adding methanol, and 20 μL of each was used for plotting the standard curve for **1** and **2**, respectively.

3. Results and Discussion

3.1. Extraction Method. In order to obtain satisfactory extraction efficiency, extraction method and extraction time were investigated. Initially, two samples of 0.1 g each were separately extracted with 10 mL of methanol at 50°C for 30 min; one by refluxing on water bath while the other by ultrasonication. The w/w% yield of compounds **1** and **2** was 0.014 and 0.702 in refluxing and 0.020 and 0.751 in ultrasonication. As ultrasonication gave better yields, extracting time optimization was studied for 15, 30, 45, and 60 min, respectively. The results showed that the targeted compounds were completely extracted in 30 min. Hence, 30 min ultrasonic extraction with methanol at 50°C was considered as optimal extracting condition.

3.2. Optimization of Chromatographic Conditions. Since HPLC with UV detection is the most popular method for analysis of bioactive compounds, method development in the present study was based on HPLC coupled with photodiode array detector (PDA) [7]. Different types of columns and mobile phase compositions were carefully tested in order to determine the optimal chromatographic conditions. It was found that better separation and peak shapes were achieved with Waters Spherisorb ODS2 column (250 × 4.6 mm, i.d., 10 μm). For the baseline separation first we tried acetonitrile and water in different proportions, but the results were not satisfactory. Hence we switched to water and methanol, which gave us better separations. The optimized programme for pump A (water) and pump B (methanol) was carried out, and the results were good and reproducible in water-methanol (2 : 3). The retention times were 4.1 ± 0.02 and 5.2 ± 0.04 min for **1** and **2**, respectively. The absorption maxima for both analytes were found at 254 nm. Peaks were identified by comparing their retention times and UV absorption spectra with those acquired for standards analyzed under the same chromatographic conditions. Column performance report for *A. multiflora* plant extract is presented in Table 2. As a measure of column performance, the number of theoretical plate counts (N) for compounds **1** and **2** was 2053 and 3214, respectively. The representative chromatograms of standards and *A. multiflora* extract are shown in Figure 1. LOD, LOQ, accuracy, and precision were evaluated for quantitative purposes. The samples were analyzed within same day and for consecutive days to assess intraday and interday precision and accuracies. The low values of % RSD (<1.1%) reflect the high precision of the method. Hence, the proposed RP-HPLC method is found to be precise and accurate. Further, the method is sensitive enough for the analysis of compounds **1** and **2** in *A. multiflora*.

TABLE 2: Column performance report for the determination of analytes **1** and **2** ($n = 5$).

Analyte	Retention time (min)	Retention factor	Tailing factor	Theoretical plates
1				
Mean	4.1 ± 0.02	3.13	1.58 ± 0.0152	2053 ± 0.08
% RSD	0.48	0.31	0.96	0.003
2				
Mean	5.2 ± 0.04	4.25	1.28 ± 0.0142	3214 ± 0.07
% RSD	0.76	0.35	0.95	0.002

TABLE 3: Linearity and sensitivity parameters for determination of analytes **1** and **2**.

Parameter	1	2
Retention time (min $\pm$ SD)	4.1 ± 0.02	5.2 ± 0.04
Linear regression parameters		
Slope	1612.4	1605.6
Y-intercept	300.0	325.6
r^2	0.999	0.999
LOD (μg/mL)	0.05	0.06
LOQ (μg/mL)	0.18	0.18
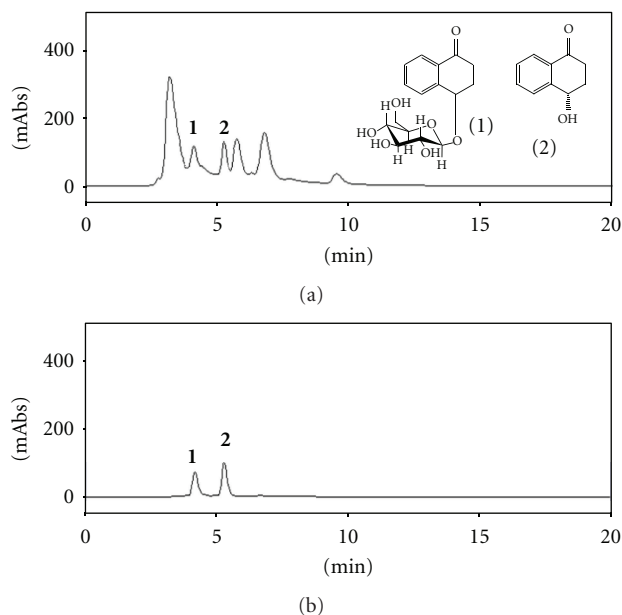
FIGURE 1: HPLC chromatogram. (a) methanolic extract of *A. multiflora*. (b) artificial mixture of **1** and **2**.

3.3. Method Validation. Method validation was performed on the parameters such as linearity, LOD and LOQ, specificity, precision, accuracy, and recovery as per ICH [8] guidelines. All data were evaluated using standard statistical packages for Windows and Graph Pad Prism 4.0 (Graph Pad Software, Inc., USA).

3.3.1. System Suitability Test. System suitability was assessed by five replicate analyses of the analytes at a concentration of 10 μg/mL. The acceptance criterion was ± 2% for the percentage relative standard deviation (% RSD) of peak area and retention time. The retention and tailing factors were also determined and the results are given in Table 2.

3.3.2. Linearity. The calibration curves for **1** and **2** were plotted with five different concentrations at 2–10 μg/mL. The detector response was linear, and the linearity was assessed by calculating the slope, Y-intercepts and coefficient of correlation (r^2) using least squares regression equation. The detail descriptions of regression curves are depicted

in Table 3. Good linearity ($r^2 = 0.999$ for both analytes) was observed within the examined concentration range. A statistical residual plot analysis also demonstrated that residuals were randomly distributed around the zero value. This confirms that the model choice is linear.

3.3.3. LOD and LOQ. The limit of detection (LOD) and limit of quantification (LOQ) of this method were determined as S/N ratio 3 for LOD and 10 fold for LOQ. The low values of LOD and LOQ, that is, 0.05 and 0.18 μg/mL for **1** and 0.06 and 0.18 μg/mL for **2**, respectively suggest the high detection capability of the method.

3.3.4. Precision and Accuracy. The accuracy of the assay was measured in terms of recovery of analytes **1** and **2**. The three different concentrations diluted from the stock solution were added to an extract with a known content of compounds **1** and **2**, and the recovery of respective constituents was calculated. The recovery (R) was calculated as $R = (C_{\text{found}} - C_{\text{sample}})/C_{\text{added}}$, where C_{found} is the concentration in spiked sample and C_{sample} is the concentration in the sample prior to spiking, and C_{added} is the concentration of added standard. The results of recovery of the tests were acceptable as the percentage recoveries of compounds **1** and **2** for intraday accuracies were 98.40–99.60 and 99.40–100.40%, respectively, and those for interday accuracies were 98.2–101.6 and 98.40–100.2%, respectively, (Table 4). All percentage recoveries were within 98.2–101.6% indicating the good accuracy of the method.

The intraday and interday precision of the method were assessed by measurement of relative standard deviation (% RSD) of the results of the recovery for compounds **1** and **2** (Table 4). The % RSD values for intraday precision were 0.214–0.812 and 0.406–1.015 for **1** and **2**, respectively, and those for interday precision were 0.484–0.590 and 0.548–0.853, respectively. The low values of % RSD (<1.1%) reflect the high precision of the method.

3.3.5. Assay of Compounds 1 and 2 in A. multiflora. The developed and validated method was applied for the quantification of two compounds (**1** and **2**) in five different samples of *A. multiflora* collected from Lucknow, Faizabad, Gorakhpur, Barabanki, and Sitapur districts of Uttar Pradesh, India. The sample collected from Lucknow was found to contain

TABLE 4: Intra-and inter-day precision and accuracy parameters of analytes **1** and **2** ($n = 6$).

Analyte	Amount added (μg/mL)	Amount found (μg/mL)	% RSD	% Recovery
		Intraday		
1	5	4.92 ± 0.040	0.812	98.40
	10	9.96 ± 0.026	0.260	99.60
	15	14.94 ± 0.032	0.214	99.60
2	5	5.02 ± 0.051	1.015	100.40
	10	9.94 ± 0.094	0.945	99.40
	15	15.02 ± 0.061	0.406	100.13
		Interday		
1	5	5.08 ± 0.030	0.590	101.6
	10	9.82 ± 0.048	0.488	98.2
	15	14.85 ± 0.072	0.484	99.0
2	5	4.92 ± 0.042	0.853	98.40
	10	10.02 ± 0.064	0.638	100.2
	15	14.96 ± 0.082	0.548	99.7

TABLE 5: Assay of analytes **1** and **2** in methanolic extracts of *A. multiflora*.

Sample name	Amount (% w/w) in methanolic extracts	
	1	**2**
Lucknow	0.024 ± 0.003	0.823 ± 0.002
Faizabad	0.019 ± 0.002	0.648 ± 0.006
Gorakhpur	0.021 ± 0.003	0.813 ± 0.003
Barabanki	0.023 ± 0.006	0.716 ± 0.007
Sitapur	0.016 ± 0.002	0.818 ± 0.004

maximum amount of marker compounds **1** and **2**. The assay results of different samples have been summarized in Table 5.

4. Conclusions

A new RP-HPLC method has been developed for the simultaneous determination of two compounds: tetralone-4-O-β-D-glucopyranoside (**1**) and 4-hydroxy-α-tetralone (**2**) in *A. multiflora*. The method is simple, precise, selective, and sensitive having acceptable precision, accuracy, and linearity ($r^2 > 0.999$) within the test ranges for both the analytes. The quantification of compounds **1** and **2** in *A. multiflora* is being reported here for the first time. Due to simple extraction procedure, high precision, accuracy, and short run time of 6 min, the method may be of immense application in the screening of raw materials as well as in the quality control of finished herbal products of *A. multiflora*. The extraction and isolation procedures of marker compounds (**1** and **2**) including their ^{1}H, ^{13}C NMR, and mass spectroscopic data are accessible as Supplementary Material available online at doi:10.1155/2012/162302.

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Research Article

Development and Validation of Dissolution Test for Fluconazole Capsules by HPLC and Derivative UV Spectrophotometry

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The purpose of this study is to develop and validate a dissolution test for fluconazole, an antifungal used for the treatment of superficial, cutaneous, and cutaneomucous infections caused by *Candida* species, in capsules dosage form. Techniques by HPLC and UV first derivative spectrophotometry (UV-FDS) were selected for quantitative evaluation. In the development of release profile, several conditions were evaluated. Dissolution test parameters were considered appropriate when a most discriminative release profile for fluconazole capsules was yielded. Dissolution test conditions for fluconazole capsules were 900 mL of HCl 0.1 M, $37 \pm 0.5\,^{\circ}$C using baskets with 50 rpm for 30 min of test. The developed HPLC and UV-FDS methods for the antifungal evaluation were selective and met requirements for an appropriate and validated method, according to ICH and USP requirements. Both methods can be useful in the registration process of new drugs or their renewal. For routine analysis application cost, simplicity, equipment, solvents, speed, and application to large or small workloads should be observed.

1. Introduction

The dissolution can be defined in a narrow sense as the process by which a solid substance is incorporated into the solvent to form a solution. However, in a broad sense, it is more than a simple measurement of solubility rate and can be better described as physical test to predict the drug release from a dosage form, for a given area for some precise time. Fundamentally, this process is controlled by the affinity between the solvent and the solid substance and the way by which the pharmaceutical system releases the drug [1, 2]. According to Mehta and coworkers [3] dissolution test provides an indication of bioavailability of a drug and, thus, pharmaceutical equivalence from batch to batch. The dissolution test is an important tool in quality control of drugs and it becomes more important for drugs with relatively low water solubility, including broad spectrum antifungal fluconazole. Some characteristics of this

drug are not well defined, for instance, its classification as high or low soluble in water or its classification in the biopharmaceutical classification system [4–6]. Well-defined drug features, together with the dissolution studies, can be an important tool to justify the use of in vitro methods instead of in vivo methods when they are requested. The aim of this dissolution study is to contribute to define fluconazole dissolution conditions, what can be the focus for further studies.

A dissolution method should be discriminatory, and it should allow evaluating the performance of the product and possible changes it may suffer from during the stability study. According to Marcolongo [7], many variables can influence the results of a dissolution test. Among these variables we can find solubility, chemical nature of the drug, the dosage form, excipients and manufacturing technology employed, the apparatus used, the stirring speed, the use of devices for floating dosage forms (sinkers), the volume of media

used, pH and temperature of the media, the filtration, and analytical method employed.

To develop a dissolution method the characteristics of the drug and its behavior in the chosen test media should be taken in account. Moreover, the dissolution conditions must follow the sink conditions (final concentration equivalent to 10% of saturation concentration) [7], and the quantitation method should be sensitive, selective, accurate, and precise.

Although there are many published analytical methods for fluconazole, there are few dissolution studies for fluconazole, as it has been well compiled in a review by Corrêa and Salgado [8]. Fluconazole dissolution has not been recommended in any pharmacopeia until December 2010 when it has been incorporated in the capsule monograph in the Brazilian Pharmacopeia, 5th edition [9]. However, FDA [10] has recommended dissolution methods since 2004 for fluconazole suspension and since 2006 for tablets; the FDA-recommended method for tablets was tested in this study without good results. In the Brazilian Pharmacopeia [9], the dissolution method recommended for fluconazole capsule uses 900 mL of 0.1 M HCl at 37°C and baskets with 100 rpm for 30 min with quantitation by spectrophotometry at $\lambda = 261$ nm.

The aim of this work is to develop and validate a discriminative dissolution method for fluconazole capsules employing two analytical methods to determine fluconazole by high-performance liquid chromatography (HPLC) and by first-order derivative UV spectrophotometry (FDS). Problems encountered by the UV spectrophotometric method, recommended by the Brazilian Pharmacopeia 5th edition [9] are discussed.

2. Experimental

2.1. Material and Equipment. Fluconazole chemical reference (assigned purity 100%) was purchased from Sigma Aldrich. Bulk drug was kindly donated (EMS, Hortolândia, SP, Brazil) and was standardized against fluconazole chemical reference. Capsules were purchased from local market with 150 mg drug label claim. A Hanson SR 8 *Plus* dissolution system containing six vessels was used for dissolution tests.

LC grade methanol was purchased from Tedia (Fairfield, USA). Purified water was prepared in-house by using Direct-Q water system (Millipore, Billerica, MA, USA). Prior to use, mobile phase solvents were degassed in an ultrasonic bath for 30 min. Purified water (>18 MOhm cm) was used to prepare the mobile phase. Solvents were filtered through a 0.45 μm membrane filter.

An HP 8453 UV-Visible spectrophotometer (Agilent Technologies, Inc., Santa Clara, CA, USA) with photodiode array (PDA) and HP ChemStation software with automatic differentiation was used. A liquid chromatograph (Waters Corporation, Milford, MA, USA) equipped with a Waters 1525 binary pump, a Rheodyne Breeze 7725i manual injector, and a Waters 2487 UV-VIS wavelength detector was used. HPLC analysis was conducted in an RP C18 column (Symmetry, 5 μm, 4.6 mm × 250 mm, Waters, Milford, MA, USA).

2.2. Preparation of Standard and Sample Solutions

2.2.1. Dissolution Performance. Fluconazole 150 mg capsules were dissolved in 900 mL of HCl 0.1 M at 37°C for 30 min, using baskets. The collected samples were filtered through quantitative filter paper, when evaluated by FDS, or through 0.45 μm regenerated cellulose membranes, when evaluated by HPLC.

2.2.2. LC and UV Determination Method. A stock solution was prepared by dissolving 50.0 mg of fluconazole in a 100 mL volumetric flask using HCl 0.1 M with 30 min sonication. Five standard solutions were prepared from stock solution in different concentrations (135, 150, 165, 180, and 195 μg/mL) by dilution with the same solvent. The range of concentrations chosen has the used concentration (150 mg of fluconazole in 900 mL of dissolution media) as the central concentration. The samples were filtered using quantitative filter paper, when they were evaluated by FDS, and using membranes of regenerated cellulose, 0.45 μm, when they were evaluated by HPLC. Sample, standard, and placebo-enriched solutions were prepared in the selected central concentration in the same way.

3. Results and Discussion

3.1. Analytical Development

3.1.1. UV Determination Method. The selectivity of the Brazilian-Pharmacopoeia-recommended method was evaluated using the compounding fluconazole and its excipients (capsules and placebo). The calculations of interference were performed in accordance with the recommendations of USP 32 [11], since the Brazilian Pharmacopoeia does not cite this calculation, using the following equation. The interference must not exceed 2%:

$$100C \times \left(\frac{\text{Ap}}{\text{As}}\right) \times \left(\frac{V}{L}\right), \tag{1}$$

where C is the concentration (mg/mL) of standard solution, Ap and As are the absorbances of placebo and standard solutions, respectively, V is the volume of medium (mL), and L is the dosage of the product (mg).

Spectrum results showed a strong interference, caused by both capsule shells and placebo in the analytical result. Using the analytical method recommended by the Brazilian Pharmacopoeia the mean percentage of dissolution for six fluconazole capsules was 115.21% and the mean percentage of response for placebo and capsule was 3.94% and 10.70%, respectively, a total of 14.64%. These results are in accordance with those obtained by Oliveira and coworkers [12], who reported fluconazole capsules dissolution. They have shown a huge interference of the excipients in fluconazole determination by UV.

In order to eliminate the interference of the excipients (placebo and capsule) UV-FDS was tested. In general, the spectral derivation provides simultaneous drugs determinations in association, as well as, increased selectivity. In addition, there are often an increased sensitivity and
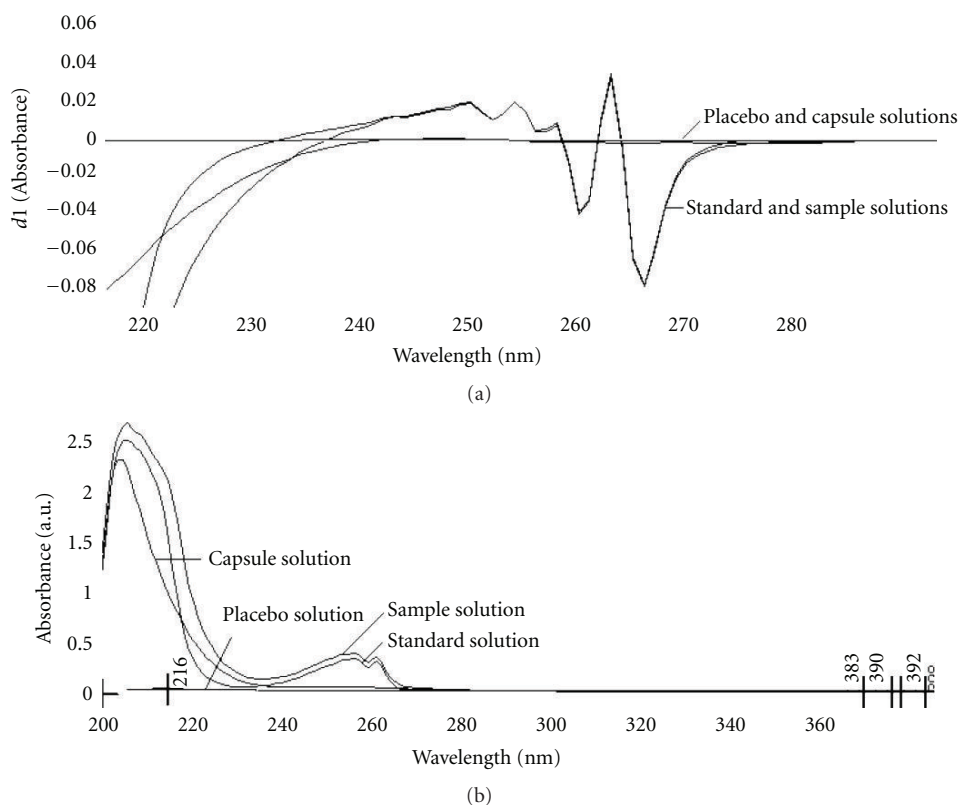
(a)

(b)

Figure 1: (a) First-order derivative spectra overlay of fluconazole standard, dissolution sample, and placebo and capsule shells solutions. (b) Zero-order derivative spectra overlay of standard solution, dissolution of fluconazole sample, and capsule shells and placebo solutions.

improved detection limits. The increased sensitivity observed in the derivative spectroscopy is based on the observation that the amplitude of the absorbance derivative relative to the wavelength is inversely proportional to the bandwidth of the ordinary spectrum. The order of the derivative must be carefully selected since there is usually an increase in noise level with increasing order of differentiation [13, 14].

Figure 1 shows the first-order derivative of absorbance of fluconazole that has an intense and well-defined valley at 268 nm. It was the wavelength chosen. Fluconazole capsule samples were tested according to the method recommended by the Brazilian Pharmacopeia [9]. The same samples were employed to evaluate the derivative spectrophotometric method. The average interference from placebo and capsules was again calculated in the same way but using the derivative spectrophotometric method. The interference was equal to 0.1% due to placebo and 1.98% due to capsules shells. These values have low analytical significance and are satisfactorily acceptable. Thus, the interference of excipients (placebo and capsule) was considerably reduced by using the differentiation.

3.1.2. HPLC Determination. HPLC is a widely used method of separation with high precision and accuracy. It allows the separation between the drug and excipients, as well as degradation products, which is useful as an indicative stability method.

The analytical development must be developed to obtain a simple and optimal method. Good results were obtained using reversed phase C18 (250×4.6 mm, 5 mm) Water Symmetry endcapped column, 1.0 mL/min, water, and methanol (60 : 40, v/v), injection of $20\,\mu$L monitored at $\lambda = 261$ nm.

The samples used to test the method recommended by the Brazilian Pharmacopeia [9] were now employed to evaluate the HPLC method. The results showed that the HPLC method is selective, and it was able to separate the drug from the placebo and capsule (Figure 2).

Figure 2 shows that capsule shells and placebo absorb energy in UV region (peaks at 5.15 min); however, they could be well separated from fluconazole (peak at 3.65 min). The capsule peak was observed in capsule shells and sample solutions. The peak at 2.45 min, present in all samples, refers to the dissolution media (HCl 0.1 M), including the blank solvent.

3.1.3. Dissolution Performance. Fluconazole three different pK_a [15] values, 11.01 ± 0.29, 2.94 ± 0.10, and 2.56 ± 0.12, correspond to the groups alcohol (proton donor) and two nitrogens (proton acceptors), respectively. Thus, the aqueous solubility of fluconazole is greater in solutions with extreme pH values (above 11.01 and below 2.94), situations in which the drug would be fully ionized.

However, dissolution of fluconazole capsules was evaluated in deionized water, as recommended by FDA [10], and
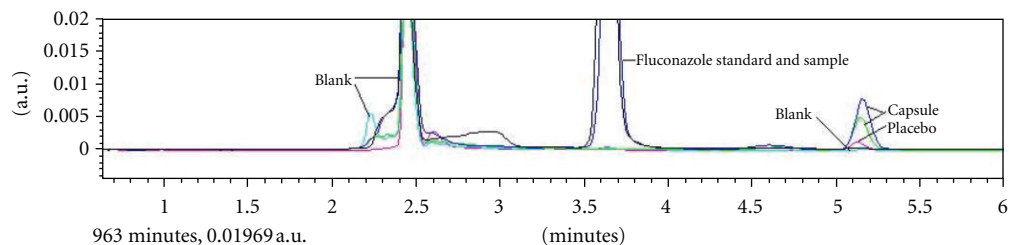
FIGURE 2: Chromatograms overlay of fluconazole dissolution samples, placebo and capsule shells, at $\lambda = 261$ nm.

in HCl 0.1 M at 37°C. Both baskets and paddles (with and without sinkers) apparatuses were used in a total medium volume of 900 mL in all tests to evaluate dissolution profile. The dissolution media were degassed by sonication for 30 min at 37°C before initiating the dissolution test. The final concentration of fluconazole in 900 mL was nearly 165 mg/mL as capsule products contained 150 mg of the drug. This is in agreement with required sink conditions, desirable to prevent saturation. Thus, the concentration 165 mg/mL was included to be the central point of the range used to validate the method.

In the dissolution profile, samples were collected after 5, 10, 15, 20, 30, 45, 60, and 65 min, filtered through quantitative filter paper, and fluconazole was quantified by UV-FDS. The dissolution medium was used as blank. The replacement of media was performed after each collection using the dissolution media at 37°C. Both mass withdrawn and the dilution made for each replacement of media were taken into account in the calculations for the construction of the dissolution profiles.

Rotation speeds 75 and 100 rpm were used in each test with baskets and 50 and 75 rpm with paddles. During the last 5 min, the speed of rotation was changed to 150 rpm in all tests. The speed can be increased at this point to verify if the drug contained in the capsule was entirely released during the test. The results of dissolution profiles using the described conditions are shown in Table 1 and Figure 3.

Water was used as dissolution medium in Tests 1 and 2, as recommended by FDA [10]. It shows not to be an appropriate medium for fluconazole capsules even after 65 min test at the highest speed because the drug percentage release was below 57%. The HCl 0.1 M dissolution media tested showed better results.

The paddle apparatus was tested at 50 and 75 rpm, with and without sinker. The use of sinker made the dissolution slower, and it is shown comparing Tests 4 and 6 to Tests 3 and 5. Tests 3 and 4, when 75 rpm was employed, showed fast dissolution in the beginning and low discriminatory power. Tests 5 and 6 showed slow dissolution in the beginning; however, at the end the drug was not released from the dosage form to the dissolution media. It could be realized after employing 150 rpm for 5 minutes in the end of test that the concentration of drug increased rapidly.

Baskets were employed in Tests 7 and 8 using HCl 0.1 M as dissolution media and 100 and 75 rpm, respectively. Test
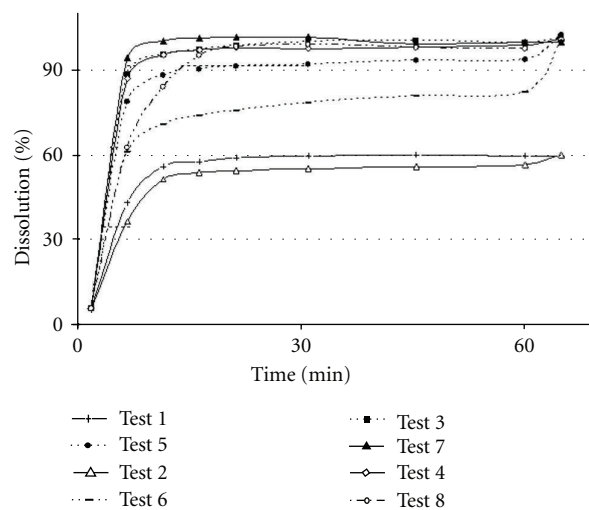
FIGURE 3: Dissolution profile obtained for testing fluconazole 150 mg capsules by UV-FDS. Conditions: as in tests 1–8 specified in Table 1.
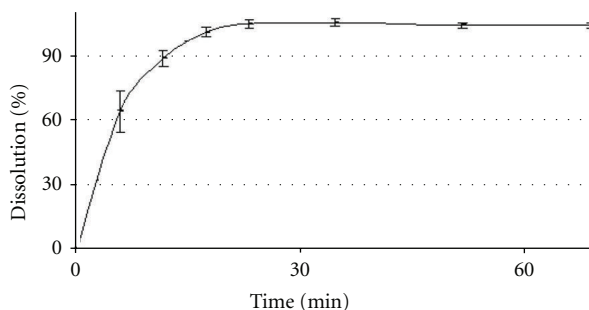
FIGURE 4: Dissolution profile for fluconazole capsules. Samples analyzed using 0.1 M HCl at 37°C as dissolution media, basket, and 75 rpm. UV method was employed.

7 has shown fast initial dissolution with more than 90% of drug release after 5 min and therefore a low discriminatory power. In Test 8, there were small release increases in fluconazole amount along the test, which has shown a discriminative profile (Figure 4).

Therefore, basket apparatus, 75 rpm, 900 mL of 0.1 M HCl at 37°C as dissolution media, and 30 min of testing

TABLE 1: Dissolution profiles for fluconazole determination: tested parameters and results.

Test	Time (min)	Medium	Medium volume (mL)	Apparatus	Rotation (rpm)	Average % dissolution	% R.S.D.
1	5	water	900	Basket	100	39.05	10.58
	10					52.79	3.26
	15					55.73	3.14
	20					56.73	1.16
	30					56.65	0.38
	45					56.82	0.31
	60					56.74	0.60
	65				150	56.69	1.11
2	5	water	900	Paddle	75	32.29	0.07
	10					48.09	4.78
	15					50.31	3.21
	20					51.01	2.02
	30					51.77	1.57
	45					52.48	1.25
	60					53.52	1.26
	65				150	56.99	0.89
3	5	0.1 M HCl	900	Paddle	75	86.79	6.23
	10					94.14	3.47
	15					95.89	2.72
	20					97.32	3.43
	30					98.64	2.40
	45					99.19	1.58
	60					98.24	1.68
	65				150	100.24	2.37
4	5	0.1 M HCl	900	Paddle + sinker	75	85.45	9.69
	10					93.92	1.13
	15					95.70	0.26
	20					96.63	0.73
	30					96.34	1.31
	45					97.06	1.71
	60					97.57	1.37
	65				150	100.00	1.43
5	5	0.1 M HCl	900	Paddle	50	76.67	5.84
	10					86.41	1.35
	15					88.70	2.58
	20					89.78	3.47
	30					90.44	3.65
	45					91.79	2.94
	60					92.36	3.81
	65				150	101.24	2.77
6	5	0.1 M HCl	900	Paddle + sinker	50	58.10	4.62
	10					68.09	6.98
	15					71.54	7.02
	20					73.34	5.75
	30					76.28	4.53
	45					78.76	4.70
	60					80.19	4.78
	65				150	99.05	4.55

TABLE 1: Continued.

Test	Time (min)	Medium	Medium volume (mL)	Apparatus	Rotation (rpm)	Average % dissolution	% R.S.D.
	5					92.96	6.34
	10					98.96	0.32
	15					100.07	0.20
7	20	0.1 M HCl	900	Basket	100	100.56	0.39
	30					100.47	0.38
	45					98.09	0.15
	60					98.66	0.23
	65				150	98.92	0.87
	5					59.50	15.07
	10					82.07	4.45
	15					93.61	2.19
8	20	0.1 M HCl	900	Basket	75	97.00	1.93
	30					97.64	1.36
	45					96.43	1.35
	60					96.38	1.23

were the conditions chosen for the dissolution of fluconazole capsules. This method was validated by FDS and HPLC.

3.2. Method Validation. The quantitative methods were validated according to the ICH [16] and USP [11] guidelines for development and validation of dissolution methods. Because nonuniform drug distribution may affect the dissolution test in the single-dose units, fluconazole capsule samples were evaluated regarding uniformity content (UC). All fluconazol capsules UC results obtained by HPLC (between 91.42–100.2% of labeled value) were within accepted specifications.

The calibration curves were obtained at five fluconazole concentration levels from 135 to 195 μg/mL for HPLC ($\lambda =$ 261 nm) and UV-FDS ($\lambda = 268$ nm). Lambert-Beer law was observed at this concentration range. Linearity was evaluated by the least square method with determinations in triplicate at each concentration level. Both methods were linear with this model. Regression equations were $y = 2.5 \times 106X - 1.1 \times$ 104 ($r^2 = 0.996$) for HPLC and $y = 0.51439X - 2.9 \times 10^{-3}$, ($r^2 = 0.998$) for UV-FDS. The standard deviations of the regression were 0.83% for HPLC and 0.62% for UV-FDS. The validity of the assay was verified by means of ANOVA. According to ANOVA there is a statistically significant linear regression ($F_{calculated} > F_{critical}$; $P = 0.05$) and there is no deviation from linearity ($F_{calculated} < F_{critical}$; $P = 0.05$) for both methods.

The precision of the methods was determined by repeatability (intraday) and intermediate precision (interday). For repeatability test three curves were constructed with the established five concentration levels using standard solutions in the same day; for intermediate precision three curves were constructed with three concentration levels (high, intermediate, and low) using standard solutions in a different day. An interval of two days between repeatability and intermediate precision was observed. The results were expressed as percentage of relative standard deviation (R.S.D.). The

intraday precision tests showed R.S.D. of 0.81%, HPLC and 0.75%, FDS and interday precision tests showed R.S.D. of 2.29%, HPLC and 2.55%, FDS. These results indicate good precision.

The accuracy was performed in triplicate using the standard addition method (enriched placebo). Known amounts of standard of fluconazole were added to placebo in order to reach five established concentration levels. The mean percentage recovery of fluconazole standard found was 98.56 ± 0.82% for HPLC and 98.35 ± 0.88% for UV-FDS (Table 2). These results indicate an agreement between the true values and found values.

This paper compares the methods to determine fluconazole after dissolution test regarding their precision accuracy, and repeatability (Table 3). Both methods showed to be specific, precise, accurate, and linear in the range of concentration tested.

3.2.1. Dissolution Performance Validation. After several conditions tested for the dissolution test development, appropriate parameters were considered optimized whether provided most discriminatory dissolution profile for fluconazole capsules. That means test conditions must be able to show differences in drug release from batch to batch products, as well as to distinguish possible changes that may occur during stability studies or shelf-life of the product. The optimal parameters for fluconazole capsules dissolution are 900 mL of HCl 0.1 M, 37 ± 0.5°C using baskets with 50 rpm during 30 min.

Validation of dissolution performance was carried out by the two methods of quantification, HPLC and FDS. The concentration of 150 mg of fluconazole in 900 mL of dissolution media is nearly equal to the central concentration (165 mg/mL) at the range established.

The precision was determined by repeatability (intraday) and intermediate precision (interday). The repeatability

Table 2: Recovery data for fluconazole standard solutions added to the placebo by using the proposed HPLC and UV-FDS.

Method	Added amount (μg/mL)	Found[a] amount (μg/mL)	Bias (%)	Recovery[a] (%) ± R.S.D.
HPLC	135	133.92	0.80	99.20 ± 0.77
	150	148.00	1.33	98.67 ± 1.36
	165	161.28	2.25	97.75 ± 0.30
	180	177.30	1.50	98.50 ± 0.20
	195	192.41	1.33	98.67 ± 0.65
FDS	135	134.54	0.34	99.66 ± 1.02
	150	147.51	1.66	98.34 ± 0.17
	165	161.93	1.86	98.14 ± 0.08
	180	175.68	2.40	97.60 ± 0.22
	195	190.96	2.07	97.93 ± 0.15

[a] Average of three replicates.

Table 3: Validation parameters for different analytical methods, UV-FDS and HPLC, to determine fluconazole in capsules.

Parameters	FDS	HPLC
Analytical curve	$0.51439X - 2.9 \times 10^{-3}$	$2.5 \times 10^{6}X - 1.1 \times 10^{4}$
Intercept values	-2.9×10^{-3}	-1.1×10^{4}
Standard error of slope	8.8346×10^{-7}	19.63
Correlation coefficient (r^2)	0.998	0.996
R.S.D. of repeatability (%)	0.75	0.81
R.S.D. intermediate (%)	2.55	2.29
Accuracy (%)	98.35	98.56
R.S.D. of accuracy (%)	0.88	0.82
LOQ	4.9×10^{-3}	1.28×10^{-8}
LOD	1.4×10^{-3}	3.84×10^{-9}

Table 4: Recovery data of dissolution performance obtained by HPLC and UV-FDS methods.

Method	Added amount (μg/mL)	Found[a] amount (μg/mL)	Bias (%)	Recovery[a] (%) ± R.S.D.
HPLC	135	133.55	1.07	98.93 ± 0.23
	165	160.33	2.83	97.17 ± 0.55
	195	191.14	1.98	98.02 ± 0.63
FDS	135	135.11	0.08	100.08 ± 0.42
	165	163.40	0.97	99.03 ± 0.32
	195	190.05	2.54	97.46 ± 0.34

[a] Average of three replicates.

4. Conclusions

A discriminative dissolution test for fluconazole capsules determination was presented in this study. Selective, sensitive, precise, and accurate analytical methods were used for quantitation. The results showed that the determination of fluconazole capsules using direct UV spectrophotometry, recommended in the Brazilian Pharmacopeia, is not enough selective; however, the developed first-order UV derivative spectrophotometry and the HPLC showed to be selective and meet requirements for an appropriate validated method. Both methods are useful for the registration of new drugs or their renewal. The application of each method, as a routine analysis, should be observed considering cost, simplicity, equipment, solvents, speed, and application to large or small workloads.

Acknowledgments

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was tested by dissolution of five fluconazole capsules containing 150 mg of drug in duplicate, in the same day; and intermediate test was evaluated by the same way in a different day. An interval of two days between repeatability and intermediate test was observed. The results were expressed as percentage of dissolution and the R.S.D. The repeatability results were 97.96% ± 2.01% for FDS and 97.10% ± 2.44% for HPLC, and the interday results were 96.94% ± 2.48% for FDS and 95.02% ± 2.80% for HPLC. These results indicate good precision.

The accuracy of the dissolution performance was determined using enriched placebos. Known amounts of fluconazole standard were added to placebos in order to obtain three concentrations (high, intermediate, and low) of the range established. The accuracy test was performed in triplicate. The mean recovery was found to be 98.04% ± 0.89% for HPLC and 98.86% ± 1.20% for FDS (Table 4) indicating an agreement between the true values and the values found.

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Research Article

Pharmacokinetics of Single-Dose and Multi-Dose of Lovastatin/Niacin ER Tablet in Healthy Volunteers

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An extended-release (ER) niacin and lovastatin fixed-dose combination has been developed for the treatment of primary hypercholesterolemia and mixed dyslipidemia. The purpose of the present study was to examine the drug interaction between niacin and lovastatin after multi-dose oral administration of lovastatin/niacin ER combination in healthy Chinese volunteers. A single-center, randomized, open-label, 5-period crossover study was conducted in thirty healthy volunteers aged 18 to 45 years with a washout period of 8 days. Subjects were randomized to receive multiple doses of treatment A (1 500 mg niacin ER tablet), B (1 20 mg lovastatin tablet), C (1 20 mg lovastatin and 500 mg niacin-ER tablet), D (2 10 mg lovastatin and 350 mg niacin-ER tablets) or E (2 10 mg lovastatin and 500 mg niacin-ER tablets) in 1 of 5 sequences (ABCDE, BCDEA, CDEAB, DEABC, EABCD) per period. Lovastatin, niacin and its metabolites (nicotinuric acid and nicotinamide) were determined in plasma by LC/MS method. Pharmacokinetic parameters were calculated, and least square mean ratios and 90% confidence intervals for C_{max} and $AUC_{(0-24)}$ were determined for lovastatin/niacin ER versus niacin ER or lovastatin. It revealed that the formulation had no potential drug interaction in healthy Chinese volunteers when the dosage was increased from 500 mg to 1000 mg.

1. Introduction

Niacin (nicotinic acid, 3-pyridine-carboxylic acid, NA), which belongs to the hydrophilic vitamin B complex, is widely used to treat a diverse range of lipid disorders and prevent clinical CVD [1]. It is well known for its effects in reducing total cholesterol, triglycerides (TGs), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and lipoprotein (a) (L (pa)), and increasing high-density lipoprotein (HDL) level. NA is metabolized in two pathways: the first is the metabolic route to nicotinuric acid (NUA) through nicotinyl CoA by glycine conjugation, and the second is that to nicotinamide (NAM), which is utilized in NAD synthesis [2]. Lovastatin, as a fungal antibiotic, is a member of the drug class of statins and a specific and nonreversible competitive inhibitor of HMG-CoA reductase, used for lowering cholesterol (hypolipidemic agent) in the patients with hypercholesterolemia and so preventing cardiovascular disease [3]. Many clinical studies have shown that the combination tablet of extended-release (ER) niacin and lovastatin decreases LDL-C and increases HDL-C greater than either treatment alone in patients with dyslipidemia [4–6].

Moreover, lovastatin was primarily metabolized by the cytochrome P450 isoenzyme, especially CYP3A4, with less than 10% being excreted renally [7]. And an in vitro study indicated that NA and NUA inhibited CYP2D6 and NA inhibited CYP3A4, which was responsible for lovastatin metabolism [8]. Genetic variation in those isoenzymes has been surveyed in an ethnically diverse population [9, 10]. Previous work has observed the lack of a pharmacokinetic interaction between niacin and lovastatin after single-dose administration in healthy Hispanic volunteers [11].

The most common adverse events of niacin and lovastatin were flushing, itch of skin, headache, abdominal pain, malaise, dyspepsia, nausea, and hepatic toxicity [3, 4]. And several clinical trials showed that the rates of adverse event with the ER niacin/lovastatin tablet were similar to those with the ER niacin or the lovastatin in Caucasian patients [12–14].

However, it is still unknown whether potential increased risks of adverse events or a pharmacokinetic interaction of lovastatin and niacin exist in Chinese people.

2. Experimental

2.1. Chemicals and Reagents. Lovastatin/niacin ER tablets were supplied by Kangde Pharmaceutical Group Co. Ltd., (Zhejiang, China). Niacin extended-release tablet was purchased from Bejing Second Pharmaceutical Co. Ltd., (China). Lovastatin tablet was obtained from Hisun Pharmaceutical Co. Ltd. (Zhejiang, China). Chemical reference substances of NA, NAM, lovastatin, and simvastatin were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). NUA was purchased from Sigma (St. Louis, MO), and 6-methyl nicotinic acid as an internal standard was supplied by Aldrich. HPLC grade methanol was obtained from Merck (Darmstadt, Germany), and other chemical reagents were of analytical grade, obtained from Nanjing Chemical Reagent Co., Ltd.(Nanjing, China). Water was deionized and purified by using a Milli-Q system (Millipore, Milford, MA, USA) and was used to prepare all aqueous solutions.

2.2. LC-MS Instrumentation and Analytical Conditions. An agilent (Agilent Technologies, USA) 1100 series LC system equipped with degasser and agilent 1100 MS was used to detect NA and lovastatin during the study. For detecting NA, NAM, and NUA [15], seperation was carried out using a Dikma-C_{18} column (dp $3\,\mu m$, 2.1 $\times$ 150 mm ID, Dikma Technologies Inc.), with an isocratic elution system consisting of methanol (containing 0.1% acetic acid) and water (containing 0.3% isopropanol) (2/98, v/v) at a flow rate of 0.2 mL/min. Ion scan mode was with the following settings: the capillary voltage, 100 V; temperature, 350°C; drying gas, 600 L/h; nebulizer pressure, 40 psig. Quantitations of NA, NAM, NUR, and 6-methyl nicotinic acid were achieved by monitoring the ions at $[M + H]^+$, *m/z* 124.1, 123.1, 181.1, and 138.1, respectively (Figure 1). For detecting lovastatin [16], seperation was carried out using a Lichrospher C_{18} (dp $5\,\mu m$), 200 mm $\times$ 4.6 mm ID with a gradient elution system consisting of methanol and water (containing $50\,\mu mol/L$ sodium acetate) (see Table 1) at a flow rate of 1 mL/min. The column temperature was kept at 25°C. Detection was performed by mass spectrometer (MS) in positive ion mode. Ion scan mode was with the following settings: the capillary voltage, 140 V; temperature, 350°C; drying gas, 600 L/h; nebulizer pressure, 40 psig. Lovastatin and simvastatin, ions at $[M + Na]^+$ *m/z* 427.2 and 441.3 (Figure 2), were monitored, respectively. All data were collected and analyzed using Agilent Chemstation software.

2.3. Study Design. The pharmacokinetics of Lovastatin/Niacin ER tablet was studied in healthy Chinese subjects in accordance with the Declaration of Helsinki for biomedical research involving human subjects and Good Clinical Practice. The protocol and associated informed consent statements were reviewed and approved by the Committee on Human Rights Related to Human Experimentation, Xijing

TABLE 1: Mobile phrase program for lovastatin.

Time (min)	Water phase (B%)	Flow rate (mL/min)
0	12	1
6.5	12	1
6.6	0	1
10.6	0	1
10.7	12	1
16.0	12	1

Hospital, and the informed consent statements were signed by the volunteers. It was a single-center, randomized, open-label, crossover study with five treatment cycles separated by an eight-day washout cycle. Thirty healthy volunteers who aged from 18 to 45, body mass index (BMI) ranged 19 and 24 Kg/m^2, were enrolled in this study. All volunteers have passed an obtaining of complete medical history and physical examination before participate in the study. All subjects were fasted for at least 8 hours at last night before our study and were confirmed abstinence from other medications, alcohol, tobacco, and caffeinated products.

The subjects were randomly allocated into five groups (each group have 3 male and 3 female). Each group was randomized to receive multi-dose of treatment A (500 mg niacin ER, one tablet), B (20 mg lovastatin, one tablet), C (one lovastatin/niacin ER tablet (500/20)), D (two lovastatin/niacin ER tablet (350/10)), or E (two lovastatin/niacin ER tablet (500/10)) in 1 of 5 sequences (ABCDE, BCDEA, CDEAB, DEABC, EABCD) per period. Blood samples were collected in heparinized tubes before dosing at days 1, 4, 5, 6, and 7, and on the 1st and 7th day, and blood samples were also collected at 30, 60, and 90 minutes 2, 3, 4, 6, 5, 8, 10, 12, 15, and 24 hours after dosing. All samples were separated immediately by centrifugation at 3500 rpm for 10 min at 4°C and stored at −80°C until analysis.

2.4. Analytical Procedures

2.4.1. Preparation of Stock Solutions and Standard. Stock solutions of NA, NAM, and NUA were prepared by dissolving the drugs in methanol at the concentrations of 0.492, 0.508, 0.0993 mg/mL, respectively. Serial (working) dilutions of NA NAM, and NUA were prepared from the stock solutions by appropriate dilution with methanol at the concentrations of 98.4, 9.84, 0.984, 0.0984 $\mu g/mL$ for NA; 102, 10.2, 1.02, 0.102 $\mu g/mL$ for NAM; 9.93, 0.993, 0.0993 $\mu g/mL$ for NUA, respectively. Stock solution of lovastatin was prepared by dissolving 10.34 mg drug in methanol at a concentration of 1.034 mg/mL. Serial (working) dilutions of lovastatin were prepared with methanol at the concentrations of 103.4, 10.34, 1.034, 0.01034, 0.001034 $\mu g/mL$, respectively. Stock solutions of internal standards (IS) were prepared by dissolving the drug in methanol at concentrations of 0.500 mg/mL for 6-methyl nicotinic acid and 1.052 mg/mL for simvastatin. Working solutions of IS were prepared with methanol at concentrations of 2.0 $\mu g/mL$ for 6-methyl nicotinic acid and 0.2104 $\mu g/mL$ for simvastatin. All the stock
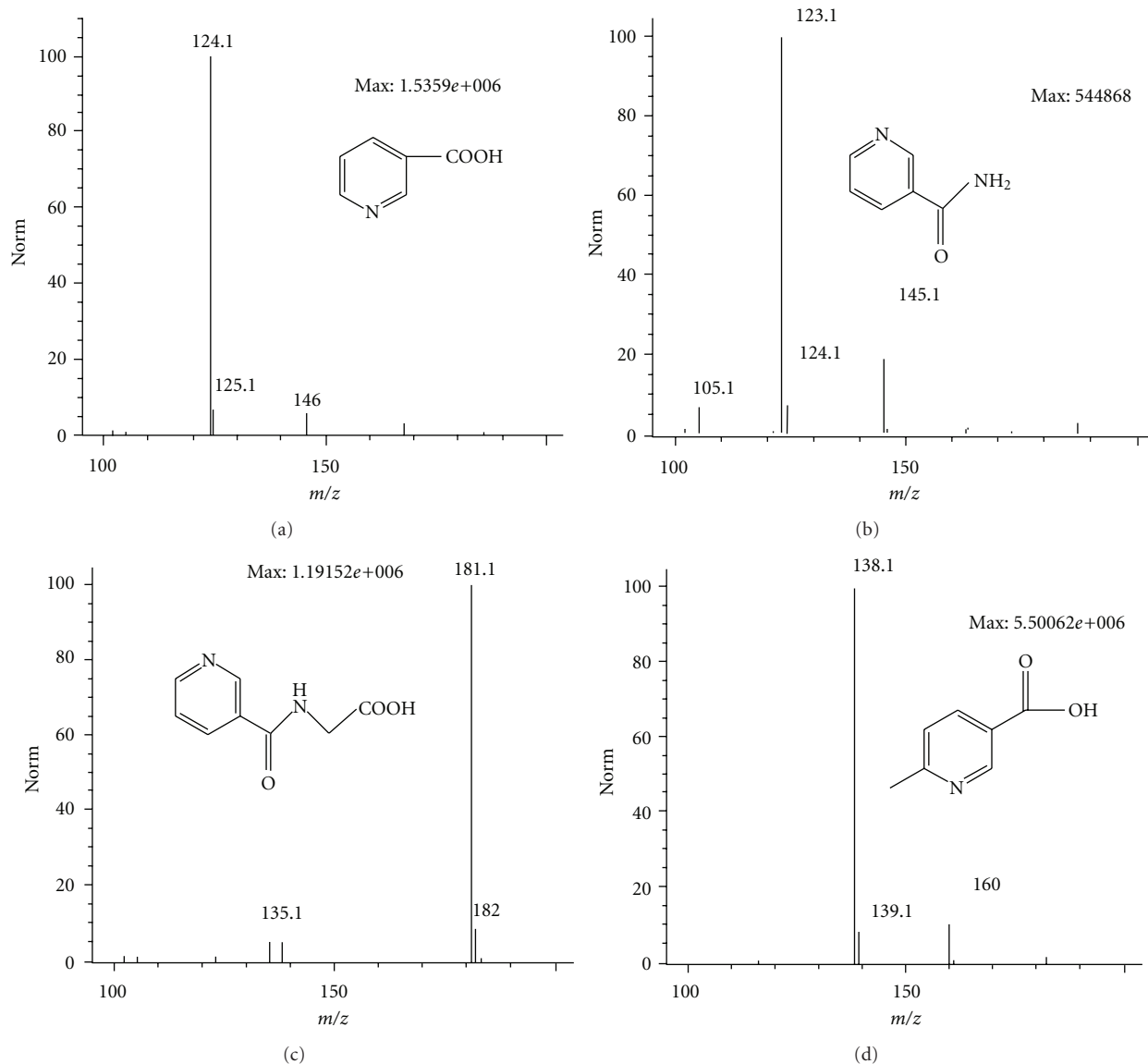
FIGURE 1: Electrospray product ion mass spectrum of the precursor ion of NA (a), NAM (b), NUA (c), and internal standard 6-methyl nicotinic acid (d).

and working solutions were stored at $-20°$C and prepared for calibration curve and quality controls.

2.4.2. Sample Preparation. To determine the niacin and its metabolites, $50\,\mu$L 6-methyl nicotinic acid ($2.00\,\mu$g/mL, IS) solution was added into $100\,\mu$L plasma sample and vortex-mixed for 30 s and then 0.6 mL methanol was added and vortex-mixed for 3 min. After centrifugation (16000 r/min, 6 min), the upper organic layer was separated and evaporated to dryness using a gentle stream of nitrogen. The residuum was reconstituted using the mobile phase and centrifugated 6 min at 16000 r/min. A $5\,\mu$L supernatant was autoinjected into the LC/MS system for analysis.

For lovastatin, 1 mL plasmas sample and $75\,\mu$L simvastatin solution ($0.2104\,\mu$g/mL, IS) were accurately added into 10 mL centrifuge tube, and vortex-mixed adequately, then

5 mL redistillate acetidin was added, and, after centrifugation (4000 r/min, 10 min), the upper organic layer was evaporated to dryness using nitrogen in a water bath at $30°$C. The residuum was dissolved with $200\,\mu$L mobile phase solution, and, after centrifugation (16000 r/min, 6 min), a $20\,\mu$L supernatant was transferred into the LC/MS system for analysis.

2.4.3. Calibration Curve. Calibration curves were prepared at the concentration levels of 0.00492, 0.0148, 0.0295, 0.0984, 0.295, 0.984, 1.97, 4.92, and $9.84\,\mu$g/mL for NA; 0.00508, 0.0152, 0.0305, 0.102, 0.305, 1.02, 2.03, 5.08, and $10.2\,\mu$g/mL for NAM; 0.00497, 0.0149, 0.0298, 0.0993, 0.298, 0.993, 1.99, 4.96, and $9.93\,\mu$g/mL for NUA; 0.0517, 0.1551, 0.3102, 1.034, 3.102, 10.34, 20.68, and 41.36 ng/mL for lovastatin by spiking an appropriate amount of the standard solutions
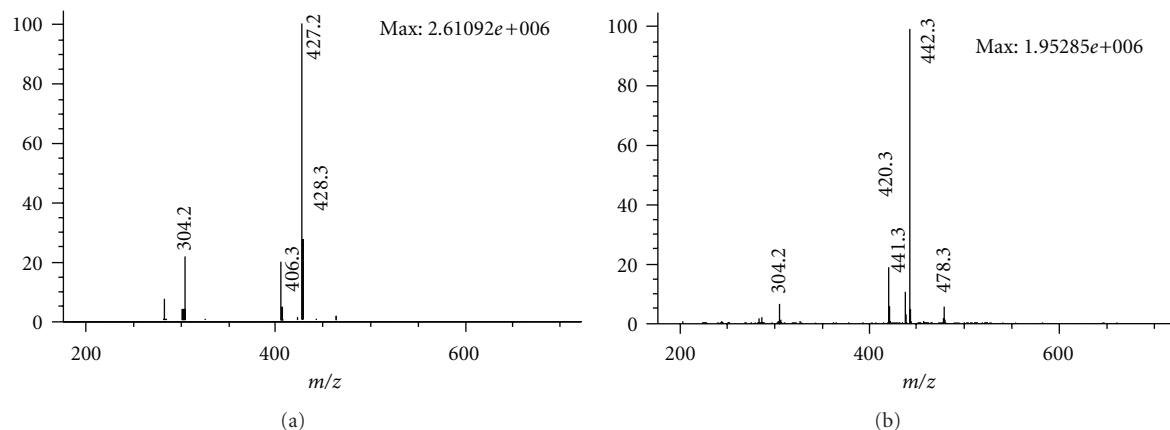
FIGURE 2: Electrospray product ion mass spectrum of the precursor ion of Lovastatin (a), and internal standard simvastatin (b).

in 1 mL blank plasma. The calibration curve was prepared and assayed along with quality control (QC) samples. QC samples were prepared in 1 mL blank plasma at three levels of 0.00984, 0.246, and 8.86 μg/mL for NA; 0.0102, 0.254, and 9.14 μg/mL for NAM; 0.00993, 0.248, and 8.94 μg/mL for NUA; 0.1034, 2.585, 36.19 ng/mL for lovastatin, respectively. The plasma samples were stored at −20°C.

2.4.4. Specificity. The specificity of the method was tested by screening six different batches of blank human plasma. Each blank sample was tested for interferences in the MS channels using the proposed extraction procedure and chromatographic/MS conditions, and the results were compared with those obtained for water solution of the analytes at a concentration near to the lower limit of quantification (LLOQ).

2.4.5. Precision and Accuracy. The intrarun precisions and accuracies were estimated by analyzing five replicates containing NA, NAM, NUA, and lovastatin at three different QC levels. The interrun precisions were determined by analyzing QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and a precision of within ±15% relative standard deviation.

2.4.6. Extraction Recovery. The recoveries of NA, NAM, NUA, and lovastatin were determined by comparing the peak area obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked after extraction to the same nominal concentrations (0.00984, 0.246, and 8.86 μg/mL for NA; 0.0102, 0.254, and 9.14 μg/mL for NAM; 0.00993, 0.248, and 8.94 μg/mL for NUA; 0.1034, 2.585, 36.19 ng/mL for lovastatin).

2.4.7. Stability. The stability of NA, NAM, NUA, and lovastatin in plasma under different temperature and timing conditions was evaluated. Plasma samples were subjected to short-term conditions, to long-term storage conditions (−20°C), and to three freeze-thaw stability studies. The autosampler stability was conducted by reanalyzing extracted

samples kept under the autosampler conditions for 0 and 48 h. All the stability studies were conducted at two concentration levels with three determinations for each.

2.5. Pharmacokinetic Analysis. The noncompartmental model analysis was used in the data processing of NA, NUA, NAM, and lovastatin. The maximum and minimum observed serum concentrations at steady state ($C_{\text{max-ss}}$, $C_{\text{min-ss}}$) and time to $C_{\text{max-ss}}$ ($T_{\text{max-ss}}$) were taken from raw data. Ke was determined by linear regression of the terminal linear portion of the concentration-time curve, and $T_{1/2}$ was calculated as $\ln(2)/Ke$. AUC_{ss} (steady-state area under the curve during T (dosing interval) and AUC_{inf} (steady-state area under the curve from 0 to infinity) were calculated by the linear trapezoidal rule. C_{av} (mean concentration between 2 administrations) was calculated as AUC_{ss}/T. The degree of fluctuation (DF) value was calculated as $(C_{\text{max}} - C_{\text{min}})/C_{\text{av}} \times 100\%$ and actual accumulation factor $R = \text{AUC}_{\text{ss}}/\text{AUC}_{\text{inf}}$. Clearance (CL/F) was calculated as dose/AUC_{inf}.

2.6. Safety Evaluation. Safety assessments included the recording of all adverse events, vital signs (blood pressure and heart rate), 12-lead electrocardiograms (ECG), laboratory investigations (including biochemistry, haematology, coagulation, and urinalysis), and full physical examinations.

3. Results and Discussion

3.1. Method Validation

3.1.1. Specificity and Selectivity. Good selectivity was observed, and there was no significant interference or ion suppression from endogenous substances observed at the retention time of the analytes. The retention time of NA, NAM, NUA, and 6-methyl nicotinic acid was 4.2, 7, 12, and 4.8 min, respectively. The retention time of lovastatin and simvastatin were 5.6 and 6.6 min, respectively.

3.1.2. Calibration Curve. NA and NAM can be both detected in the blank plasma samples therefore, the background level

TABLE 2: Intra- and interrun precision and accuracy of determination of NA, NAM, NUA, and lovastatin in human plasma.

	Concentration		RSD (%)	
	Nominal	Found C	Intrarun ($n = 5$)	Interrun ($n = 15$)
NA (μg/mL)	0.00984	0.0101	6.0	12.7
	0.246	0.237	4.2	8.3
	8.86	8.71	3.7	8.5
NAM (μg/mL)	0.0102	0.0103	5.5	12.0
	0.254	0.271	3.5	9.3
	9.14	9.58	3.2	13.5
NUA (μg/mL)	0.00993	0.0103	4.8	2.0
	0.248	0.268	2.3	11.3
	8.94	9.52	2.3	13.0
Lovastatin (ng/mL)	0.1034	0.1051	8.2	14.9
	2.585	2.688	5.0	8.6
	36.19	37.09	6.6	9.7

of peak area of NA and NAM will be deducted during the analysis process. Calibration curves of NA, NAM, and NUA in plasma were validated over the concentration ranges of 0.00492–9.84 μg/mL, 0.00508–10.2 μg/mL, and 0.00497–9.93 μg/mL, respectively. The r^2 values for the calibration curves were >0.99. Typical equations of calibration curves were as follows: $y = 0.363 \times C\text{-}0.000002$ ($r^2 = 0.9995$, $n = 5$) for NA, $y = 1.17 \times C\text{-}0.00107$ ($r^2 = 0.9998$, $n = 5$) for NAM, and $y = 1.07 \times C\text{-}0.000235$ ($r^2 = 0.9997$, $n = 5$) for NUA, respectively. Calibration curves of lovastatin in plasma were validated over the concentration ranges of 0.0517–41.36 ng/mL. The limit of quantification (LLOQ), defined as the lowest concentration on the standard curve that can be measured with acceptable accuracy and precision (<20%), was established at 0.00492 μg/mL for NA, 0.00508 μg/mL for NAM, 0.00497 μg/mL for NUA, and 0.0517 ng/mL for lovastatin, respectively (single-to-noise, S/N $\geq$ 10).

3.1.3. Accuracy and Precision. The intra- and interrun precision and accuracy of the assay were assessed by running a single batch of samples containing a calibration curve and five replicates at each QC level. The precision was calculated by using one-way ANOVA. The results, which were summarized in Table 2, demonstrated that the precision and accuracy values were within the acceptable range and the method was accurate and precise.

3.1.4. Extraction Recovery and Matrix Effects. The extraction recoveries of the four analytes were NA 95.2 $\pm$ 3.2%, 93.8 $\pm$ 2.3%, and 96.7 $\pm$ 2.9% at the concentrations of 0.00984, 0.246, and 8.86 μg/mL, respectively; NAM 105.6 $\pm$ 4.2%, 94.1 $\pm$ 4.5%, and 98.5 $\pm$ 4.3% at the concentrations of 0.0102, 0.254, and 9.14 μg/mL, respectively; NUA 92.1 $\pm$ 7.6%, 95.7 $\pm$ 3.3% and 101.7 $\pm$ 5.2% at the concentrations of 0.00993, 0.248 and 8.94 μg/mL respectively; NUA 92.1 $\pm$ 7.6%, 95.7 $\pm$ 3.3%, and 101.7 $\pm$ 5.2% at the concentrations of 0.00993, 0.248, and 8.94 μg/mL respectively; lovastatin 95.5 $\pm$ 3.31%, 87.8 $\pm$ 1.25%, and 82.8 $\pm$ 1.81% at the concentrations of 0.1034, 2.585, 36.19 ng/mL, respectively.

The matrix effect was defined as the direct or indirect alteration or interference in respond due to the presence of unintended or other interfering substances in the samples. It was evaluated by comparing the peak area of the analytes (background subtraction for NA and NAM) dissolved in the blank plasma sample's reconstituted solution (the final solution of the blank plasma after extraction and reconstitution) with that dissolved in mobile phase. Three different concentration levels of analytes were evaluated by analyzing five samples at each level, and the blank plasma used in this study was from five different batches of blank plasma. If the peak area ratio is less than 85% or more than 115%, a matrix effect will be implied. In this study, the peak area ratios of the analytes were NA 87.2 $\pm$ 1.6%, 107.8 $\pm$ 4.6%, and 89.3 $\pm$ 4.7%, NAM 99.1 $\pm$ 10.8%, 96.8 $\pm$ 3.1% and 104.6 $\pm$ 2.9%, NUA 104.9 $\pm$ 4.0%, 97.1 $\pm$ 3.4%, and 98.2 $\pm$ 7.7%, respectively, at concentrations of 0.01, 0.25, and 9 μg/mL; 6-methyl nicotinic acid 95.5 $\pm$ 10.5% at the concentration of 2.0 μg/mL; lovastatin 94.5 $\pm$ 3.21%, 89.8 $\pm$ 1.35%, and 92.8 $\pm$ 2.87% at the concentrations of 0.10, 2.6, 36.2 ng/mL, respectively; simvastatin 94.5 $\pm$ 7.8% at the concentration of 0.2 μg/mL. The results showed that there was no matrix effect of the analytes and IS from the matrix of plasma in this study.

3.1.5. Stability. The stability of NA, NAM, NUA, and lovastatin in plasma was determined by assessing low- and high-QC samples ($n = 3$ for each concentration). The results are summarized in Table 3. All analytes were found to be stable in plasma samples for at least 12 h at room temperature, for 2 months at $-20°$C freezing condition, and following three freeze-thaw cycles.

3.2. Pharmacokinetics

3.2.1. NA, NAM, and NUA Plasma Analysis. Mean pharmacokinetic parameters of the treatment A and C were provided in Table 4 for NA, NAM, and NUA. Mean plasma concentration versus time profiles for NA, NAM, and NUA were presented in Figure 3.

TABLE 3: Stability of NA, NAM, NUA, and lovastatin under different storage conditions ($n = 3$).

Storage conditions	Drug	Concentration	
		Nominal	Mean found C
Stability at room temperature for 8 h	NA	0.00984	0.00904 ± 0.00059
		8.86	8.51 ± 0.10
	NAM	0.0102	0.00948 ± 0.00007
		9.14	9.11 ± 0.07
	NUA	0.00993	0.0105 ± 0.0007
		8.94	9.81 ± 0.13
	Lovastatin	0.1034	0.09808 ± 0.0117
		36.19	37.15 ± 3.08
Stability at −80°C for 2 months	NA	0.00984	0.00904 ± 0.00031
		8.86	0.00953 ± 0.00052
	NAM	0.0102	0.00925 ± 0.00072
		9.14	8.49 ± 0.18
	NUA	0.00993	8.53 ± 0.17
		8.94	8.79 ± 0.12
	Lovastatin	0.1034	0.1014 ± 0.0009
		36.19	34.10 ± 1.81
Freeze-thaw stability	NA	0.00984	0.00872 ± 0.00036
		8.86	7.98 ± 0.13
	NAM	0.0102	0.0109 ± 0.0002
		9.14	8.76 ± 0.22
	NUA	0.00993	0.0108 ± 0.0003
		8.94	8.46 ± 0.24
	Lovastatin	0.1034	0.09911 ± 0.0051
		36.19	34.71 ± 1.39
Autosampler stability at 4°C for 24 h	NA	0.00984	0.00955 ± 0.00096
		8.86	8.86 ± 0.16
	NAM	0.0102	0.0100 ± 0.0001
		9.14	9.58 ± 0.30
	NUA	0.00993	0.0100 ± 0.0010
		8.94	9.77 ± 0.139
	Lovastatin	0.1034	0.09931 ± 0.00437
		36.19	40.19 ± 0.31

For the NA study, we found there was significant difference between C_{max}, AUC_{0-24}, and T_{max} for NA on the multiple dose of the treatment A or C, compared with single-dose of treatment A (500 mg niacin ER tablet) or the treatment C (one lovastatin/niacin ER tablet (500/20)). And a higher C_{max} and AUC_{0-24} and longer T_{max} of NA were obtained for the multi-dose treatment A or C. And, for the NAM pharmacokinetic study, the mean NAM C_{max} and AUC_{0-24} values were about 3 times higher for multi-dose administration of 500 mg niacin ER tablet (treatment A or C), comparing with single-dose of 500 mg niacin ER tablet (treatment A or C). It was indicated the metabolism of NA and NAM may exit the accumulation phenomenon in human body. However, there was no significant statistical difference ($P > 0.05$) in the main pharmacokinetic parameters of NA, NAM, NUA

(C_{max-SS}, C_{av}, AUC_{0-24}, T_{max}) between the two treatments (Figure 3). It was suggested that niacin had similar drug delayed release behavior in two treatments, and lovastatin had no effect on pharmacokinetic character of NA. The results initially indicated that no drug interaction existed between NA and lovastatin after multiple oral administration of lovastatin/niacin ER tablet in healthy Chinese volunteers.

For the multi-dose NA pharmacokinetic study, the mean pharmacokinetic parameters of NA, NAM, and NUA after multi-dose three different formulations (treatment C, D, E) were present in Table 5. The NA C_{max} and AUC_{0-24} were appropriately 30 times higher, when the dose was changed from 500 mg to 750 mg, but no big difference when the dose was changed from 750 mg to 1000 mg. It was indicated that there was a liver enzyme saturation phenomenon in NA
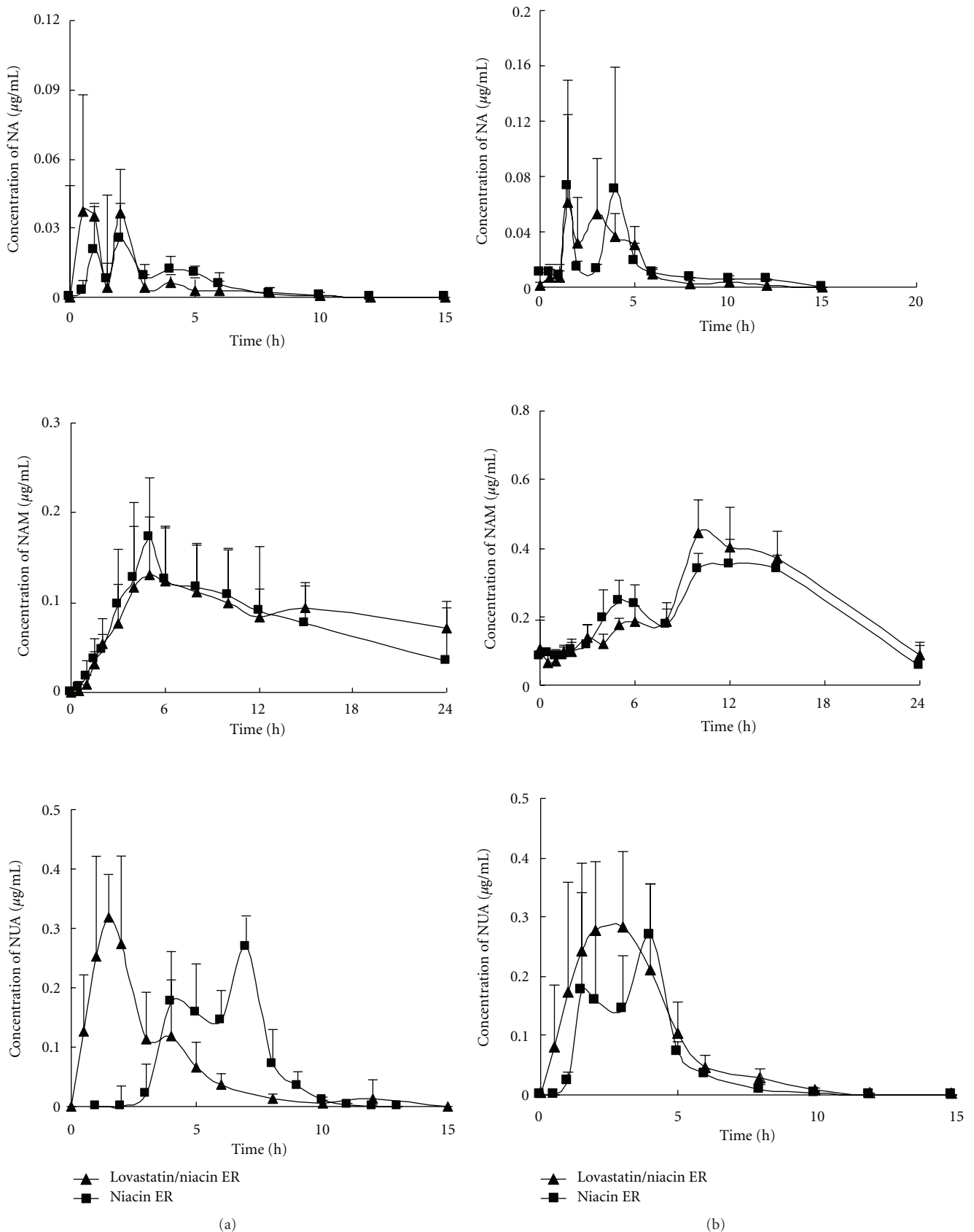
FIGURE 3: Plasma concentration-time curve of NA and NAM and NUA in 10 healthy volunteers after dose administration of lovastatin/niacin ER (20 mg/500 mg) and niacin ER (500 mg) ((a) single-dose; (b) multi-dose).

TABLE 4: The pharmacokinetic variables of NA, NAM, and NUA in volunteers after single- and multi-dose administration of the treatment A (niacin ER tablet, 500 mg) or the treatment C (lovastatin/niacin ER tablet, 20 mg/500 mg) (mean ± SD).

	Treatment		NA			NAM			NUA			
			T_{max} (h)	C_{max} (μg/mL)	AUC$_{0-24}$ (μg·h/mL)	T_{max} (h)	C_{max} (μg/mL)	AUC$_{0-24}$ (μg·h/mL)	T_{max} (h)	C_{max} (μg/mL)	AUC$_{0-24}$ (μg·h/mL)	$t_{1/2}$ (h)
C	Single	Mean	1.1	0.079	0.104	7.2	0.148	2.09	1.5	0.404	0.94	1.9
		±s	0.8	0.061	0.067	4.3	0.061	0.79	0.4	0.114	0.32	0.6
	Multi	Mean	2.6	0.097	0.221	10.2	0.448	5.81	2.4	0.375	1.19	1.4
		±s	1.1	0.043	0.055	0.6	0.104	1.29	1.1	0.127	0.38	0.2
A	Single	Mean	2.1	0.0392	0.0761	5.1	0.176	1.82	2.8	0.232	0.743	1.8
		±s	1.6	0.0253	0.0385	0.6	0.068	1.10	1.4	0.062	0.217	0.4
	Multi	Mean	3.3	0.155	0.221	11.6	0.375	5.31	3.15	0.340	0.781	1.3
		±s	1.3	0.121	0.157	3.1	0.050	1.01	1.1	1.18	0.205	0.07
	Single	C/A		1.06	1.14		0.96	1.08		1.12	1.09	
		90%		0.94, 1.21	0.98, 1.29		0.80, 1.25	0.92, 1.24		0.96, 1.30	0.94, 1.24	
	Multi	C/A		1.03	1.06		1.09	1.10		1.11	1.06	
		90%		0.88, 1.22	0.96, 1.16		0.94, 1.26	0.99, 1.20		0.81, 1.21	0.93, 1.19	

TABLE 5: The pharmacokinetic variables of NA, NAM, and NUA in volunteers of group I after multi-dose administration of three different dosage of lovastatin/niacin ER.

		NA			NAM			NUA		
Lovastatin/niacin ER	Variable	AUC$_{0-24}$ (μg·h·L^{-1})	C_{max} (μg/mL)	C_{av} (μg/mL)	AUC$_{0-24}$ (μg·h·L^{-1})	C_{max} (μg/mL)	C_{av} (μg/mL)	AUC$_{0-24}$ (μg·h/L)	C_{max} (μg/mL)	C_{av} (μg/mL)
20 mg/500 mg	Mean	0.221	0.097	0.00919	5.81	0.448	0.242	1.19	0.375	0.0496
	±s	0.055	0.043	0.00227	1.29	0.104	0.054	0.38	0.127	0.0159
20 mg/750 mg	Mean	7.15	3.54	0.298	7.16	0.804	0.298	4.31	1.38	0.179
	±s	3.76	1.90	0.157	1.26	0.168	0.052	0.96	0.35	0.037
20 mg/1000 mg	Mean	5.36	2.73	0.223	7.13	0.730	0.297	4.44	1.13	0.185
	±s	2.96	1.45	0.123	1.73	0.202	0.072	1.21	0.21	0.050

metabolism [11, 12] in Chinese healthy volunteers at the range of 500–1000 mg NA.

3.2.2. Lovastatin Plasma Analysis. For lovastatin study, mean pharmacokinetic parameters of lovastatin (treatment B and C) were provided in Table 6. Mean plasma concentrations versus time profiles for lovastatin were presented in Figure 4. Lovastatin was eliminated with a half-life of approximately 5 h, and peak plasma concentrations of lovastatin were reached within 0.7–1.8 h. These findings are in agreement with the previously reported pharmacokinetic parameters of lovastatin [16–20]. For both treatment B and C comparisons, the ratios ranged from 101% to 124% for C_{max}, 104% to 126% for AUC$_{(0-24)}$. The 90% CI for the ratios of both comparisons was within the 80% to 126% interval. It was indicated that there was no drug interaction between monotherapy and coadministration.

For multi-dose pharmacokinetics study of lovastatin, mean pharmacokinetic parameters of lovastatin (treatment C, D, and E) were provided in Table 7. No significant statistical difference was observed in the pharmacokinetic parameters of lovastatin among the three treatments. The results suggested that different doses of NA have no effect on the pharmacokinetic character of lovastatin and indicated that no drug interaction existed between NA and lovastatin after multiple oral administration of lovastatin/niacin ER tablet in healthy Chinese volunteers.

3.3. Adverse Events. Some subjects in the treatment A (1 subject), C (1 subjects), D (3 subjects), E (4 subjects) reported the adverse events of erubescence, slight fever, pruritus on the skin or mild stomach discomfort. Overall, all adverse events were mild and the volunteers recovered without treatment.

More detail information needs to be collected and analyzed from the phase-two clinical trials of the lovastatin/niacin ER tablet in Chinese patients.

4. Discussion and Conclusion

The study was completed with a sufficient number of subjects to meet the PK objectives. Although there are not observed mean PK differences between monotherapy and coadministration, the overall variability of the study was relatively high,

TABLE 6: The pharmacokinetic variables of lovastatin in volunteers after single-dose administration of lovastatin/niacin ER (20 mg/500 mg) and lovastatin (20 mg) (mean ± SD).

Variable	Lovastatin/niacin ER (treatment C)		lovastatin (treatment B)	
	Single-dose	Multi-dose	Single-dose	Multi-dose
T_{max} (h)	1.3 ± 0.5	1.3 ± 0.6	1.4 ± 0.2	1.5 ± 0.3
C_{max} (ng/mL)	10.94 ± 3.51	10.25 ± 3.12	9.54 ± 4.47	8.45 ± 2.77
AUC_{0-24} (ng·h/mL)	26.97 ± 6.2	29.85 ± 10.25	27.78 ± 10.33	26.86 ± 8.53
$t_{1/2}$ (h)	3.9 ± 0.8	4.6 ± 1.4	3.8 ± 1.5	5.1 ± 0.5
	Single (C/B, 90%)		Multi (C/B, 90%)	
C_{max}	1.12, (1.00, 1.24)		1.14, (1.04, 1.24)	
AUC_{0-24}	1.13, (1.01, −1.24)		1.15, (1.04, 1.25)	

TABLE 7: The pharmacokinetic variables of lovastatin in volunteers after multi-dose administration of three different formulations of lovastatin/niacin ER (mean ± SD).

Variable	One Lovastatin/niacin ER tablet (500/20)	Two Lovastatin/niacin ER tablets (375/10)	Two Lovastatin/niacin ER tablets (500/10)
T_{max} (h)	1.3 ± 0.6	0.9 ± 0.3	1.3 ± 0.4
C_{max} (ng/mL)	10.25 ± 3.12	10.45 ± 3.85	12.67 ± 6.09
C_{min} (ng/mL)	0.02719 ± 0.02879	0.03676 ± 0.02553	0.05462 ± 0.02128
C_{av} (ng/mL)	1.244 ± 0.427	1.389 ± 0.411	1.552 ± 0.567
DF	8.42 ± 1.48	7.51 ± 1.37	7.98 ± 1.39
AUC_{0-24} (ng·h/mL)	29.85 ± 10.25	33.33± 9.86	37.25 ± 13.61
$t_{1/2}$ (h)	4.6 ± 1.4	4.7 ± 1.1	5.8 ± 1.5
K (h^{-1})	0.167 ± 0.065	0.155 ± 0.043	0.127 ± 0.035
CL/F (L/h)	742.8 ± 247.2	667.3 ± 269.4	616.8 ± 263.6
V_d/F (L)	5001.4 ± 2196.6	4556.9 ± 1911.9	5122.4 ± 2488.6

(a)

(b)
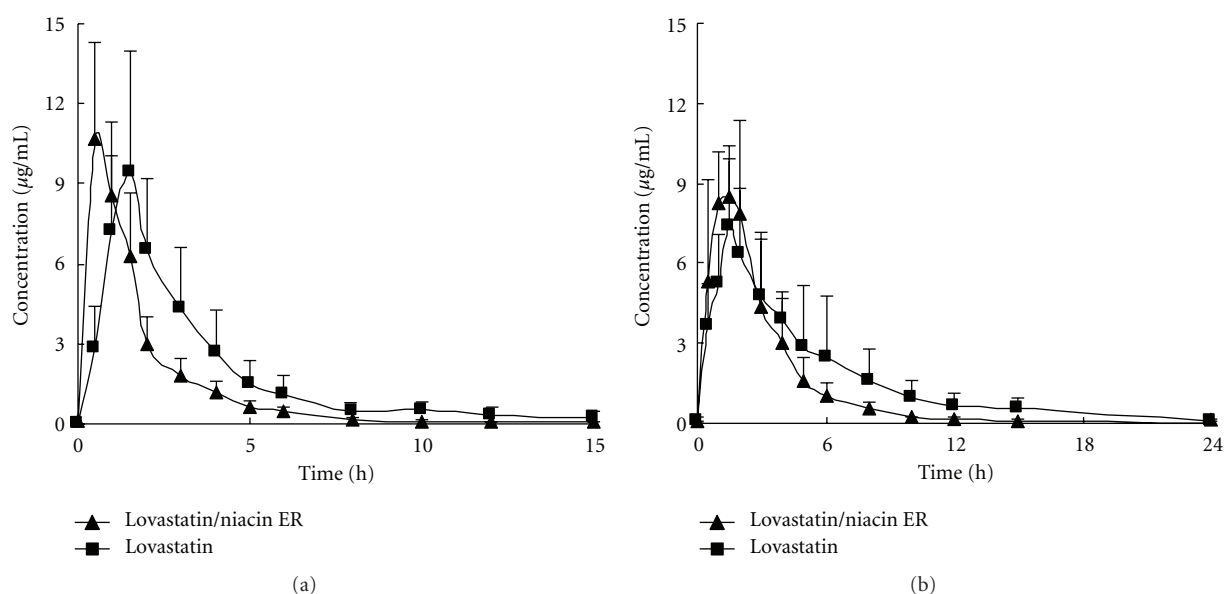
FIGURE 4: Plasma concentration-time curve of lovastatin after single- and multiple-dose administration of lovastatin/niacin ER (500 mg/20 mg) and lovastatin (20 mg) (mean ± SD) ((a) single-dose; (b) multi-dose).

particularly during ER niacin coadministration. This high variability in conjunction with the small number of subjects and apparently little or no effect on the exposure to the parent drugs makes it difficult to establish cause relationships for the potential interactions.

Lovastatin and ER-niacin in a fixed-dose combination (Advicor) is approved for the treatment of dyslipidemia [12]. In single-dose studies of ADVICOR, rate and extent of niacin and lovastatin absorption were bioequivalent under fed conditions to that from NIASPAN (niacin extended-release tablets) and Mevacor (lovastatin) tablets, respectively. After administration of two ADVICOR 1000 mg/20 mg tablets, peak concentrations averaged about $18\,\mu\mathrm{g/mL}$ and occurred about 5 hours after dosing. And peak lovastatin concentrations averaged about 11 ng/mL and occurred about 2 hours after dosing. It was shown that coadministration of NA and lovastatin did not significantly influence $C_{\max}$ and AUC_{0-t} of lovastatin, NA, NUA, and total urinary recovery of niacin and metabolites. Although both drugs are extensively metabolized, genetic variation in those isoenzymes has been surveyed in an ethnically diverse population [9, 10]. Compared with Advicor, the pharmacokinetic profile of NA and lovastatin was similar. But in our study, the mean increase in NA, NAM, and NUA was 30 times, when the dose was changed from 500 mg to 750 mg, but no big difference when the dose was changed from 750 mg to 1000 mg. It was indicated that there was a liver enzyme saturation phenomenon in NA metabolism [11, 12] in Chinese healthy volunteers at the range of 750–1000 mg NA. Meanwhile, the recommended dosage of lovastatin/ER-niacin tablets may be better at the range of 350–500 mg for Chinese volunteers.

In the study of Advicor [12], it was also shown that a 22 to 25% decrease in lovastatin $C_{\max}$ was observed, when it was coadministered with NA. Lovastatin appears to be incompletely absorbed after oral administration. Because of extensive hepatic extraction, the amount of lovastatin reaching the systemic circulation as active inhibitors after oral administration is low (<5%) and shows considerable interindividual variation. Peak concentrations of active and total inhibitors occur within 2 to 4 hours after Mevacor administration. But, in our study, there was no significant difference on pharmacokinetic parameters of $C_{\max}$, $\mathrm{AUC}_{(0-24)}$ between single use and coadministration and 90% CI of both $C_{\max}$ and $\mathrm{AUC}_{(0-24)}$ were at the range of 100–125% that is typically established for bioequivalence, considering the small sample size and moderate variability. But it was interesting that the pharmacokinetic parameters for 500 mg, 700 mg, and 1000 mg dose of lovastatin were 10.25 ± 3.12 ng/mL, 10.45 ± 3.85 ng/mL, and 12.67 ± 6.09 ng/mL for $C_{\max}$ and 29.85 ± 10.25 ng·h/mL, 33.33 ± 9.86 ng·h/mL, and 37.25 ± 13.61 ng·h/mL for $\mathrm{AUC}_{(0-24)}$. It was indicated that lovastatin may have the nonlinear pharmacokinetic profile in Chinese healthy volunteers, when it was coadministered with NA. It also suggested the metabolism of NA and lovastatin may exit competitive in human body.

On the basis of these results, no dose adjustment for lovastatin should be necessary when lovastatin is administered in combination with sustained-release niacin. The same would be true for the ER niacin. These statements would be true if indeed the patient was equal to a subject displaying near mean plasma levels of drug in this study. Given the wide variability in the results for all the drugs when administered together, caution in the form of close monitoring of the patient by blood tests and safety evaluations would be reasonable.

The treatment emergent adverse events that occurred during coadministration were similar in incidence and severity to those reported during the administration of sustained-release niacin alone or lovastatin alone and to those reported in registration documents, that is, the package insert. The notable changes from baseline in laboratory parameters after coadministration of sustained-release niacin and lovastatin were also expected from previous experience with sustained-release niacin or lovastatin administered alone. The adverse events observed are similar in type and degree to those observed in clinical trials testing the efficacy of combinations of ER niacin and lovastatin. These results, which demonstrate high intra- and intersubject variability due to high-dose ER niacin, have to be tempered by the fact that the individual medications alone and in combination can increase the risk of myopathy and rhabdomyolysis.

In conclusion, the data suggest that there is small PK drug interaction between ER niacin and lovastatin and that, although this is not considered to be clinically significant, the concomitant use of these drugs should be appropriately monitored, especially during the prescribed niacin titration period.

Authors' Contribution

S. Ying and Y. Jia contributed equally to this study.

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Research Article

Development and Validation of Selective High-Performance Liquid Chromatographic Method Using Photodiode Array Detection for Estimation of Aconitine in Polyherbal Ayurvedic Taila Preparations

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A simple, sensitive, and selective high-performance liquid chromatographic (HPLC) method has been developed and validated for the analysis of aconitine in marketed ayurvedic *taila* (oil) formulations containing roots of *Aconitum chasmanthum*. Chromatography of methanolic extracts of these formulations was performed on C_{18} (5 μm × 25 cm × 4.6 mm i.d.) column using isocratic mobile phase consisting of (65 : 35% v/v) acetonitrile and buffer solution (aqueous 0.01 M ammonium bicarbonate buffer, adjusted to pH 9.6 using 30% ammonia solution) at a flow rate of 1 mL/min and SPD-10 A_{VP} photodiode array (PDA) UV-Visible detector. The analytical reference, aconitine, was quantified at 238 nm. The retention time of aconitine was about 42.54 min. The linear regression analysis data for the calibration plot showed a good linear relationship with correlation coefficient of 0.9989 in the concentration range of 15 to 90 μg/mL for aconitine with respect to peak area. The limit of detection and limit of quantitation values were found to be 0.03 μg/mL and 0.1 μg/mL respectively. Repeatability of the method was found to be 0.551–1.689 RSD. Recovery values from 97.75 to 99.91% indicate excellent accuracy of the method. The developed HPLC method is accurate and precise and it can be successfully applied for the determination of aconitine in marketed ayurvedic oil formulations containing *Aconitum chasmanthum*.

1. Introduction

Aconitum chasmanthum (Family, Ranunculaceae) is a most valuable medicinal plant, widely used in the traditional and folk medicines of a number of countries of south east Asia. The chief chemical constituents of *Aconitum chasmanthum* root are aconitine, mesaconitine, and hypaconitine, and their respective hydrolyzed analogs are called monoester alkaloids, that is, benzoylaconine, benzoylmesaconine, and benzoylhypaconine [1–3]. Aconitine being the major diterpenoid responsible for the biopotency of *Aconitum chasmanthum* is recognized as the reference compound [3, 4]. Many polyherbal oil formulations in Indian and Chinese traditional systems of medicine used for control of skin diseases contain aconitum root as major active ingredient [5, 6]. Polyherbal oil formulations are made with the main objective of incorporating the fat-soluble fraction of the component of herbal drugs to a suitable oil base. Standardization of these formulations in terms of composition is important to ensure quality and safety. Aconitine can be used as analytical reference in the quality control of polyherbal oil formulations containing *Aconitum chasmanthum* [7–10].

There are reports on the application of various analytical methods for isolation and quantitation of aconitine present in *Aconitum chasmanthum*, biological fluids and other botanical sources [10–14]. Its estimation in polyherbal traditional medicines especially oil formulation is challenging. But no reported method deals with estimation of aconitine in complex matrix of ayurvedic *taila* formulations. High-performance liquid chromatography has emerged as an efficient

tool for the phytochemical evaluation of herbal drugs because of its simplicity, sensitivity, accuracy, suitability for high-throughput screening, and so forth [15–21]. Hence it was thought worthwhile to develop a simple chromatographic method for determination of aconitine in ayurvedic *taila* formulations. The method was validated and found to be sensitive and reproducible [22, 23].

2. Material and Methods

2.1. Reference Compound and Reagents. Reference aconitine (95% w/v purity) was purchased from Sigma-Aldrich (Germany). All the chemicals used in analysis were of AR grade except those used for HPLC analyses which were of HPLC grade. All A.R. grade chemicals were procured from S.D. fine chemicals, Mumbai and all HPLC grade solvents were procured from Merck, Mumbai.

2.2. Polyherbal Oil Formulations. Commercial marketed ayurvedic polyherbal oil formulations "*Varnaraksasa taila*" and "*Vipritmalla taila*" which contain *Aconitum chasmanthum* as one of the components were selected for studies. *Vranaraksasa taila* is a polyherbomineral oil containing mercury, sulphur, cinnabar (HgS), realgar (As_4S_4), fine copper powder, orpiment (As_2S_3), *Allium sativum* bulb (family, Alliaceae), and *Aconitum chasmanthum* root (family, Ranunculaceae) digested in mustard seed oil (*Brassica nigra.* Family, Brassicaceae) as per classical Ayurvedic text [5, 6]. *Vipritmalla taila* contains cinnabar (HgO), *Saussurea lappa* root (family, Asteraceae), *Aconitum chasmanthum* root (family, Ranunculaceae), *Ferula asafetida* resin (family, Apiaceae), *Allium sativum* bulb (family, Alliaceae), *Plumbago zylenicum* root (family, Plumbaginaceae), *Valeriana wallichii* root (family, Valerianaceae), and *Gloriosa superba* root (family, Liliaceae) digested in mustard seed oil as per classical Ayurvedic text [5, 6].

Samples of the same formulations in triplicate, manufactured by three different reputed ayurvedic drug manufacturers were collected from retail pharmacies in Indore, Madhya Pradesh, India.

2.3. Preparation of Standard Solutions. The stock solution of 1 mg/mL in methanol was prepared after keeping the purity of reference aconitine into consideration. Solution was filtered through Whatman filter paper (no. 1). Aliquots of stock solution were diluted to 5 mL using methanol to obtain working standards in concentration range from 15 to 90 μg/mL.

2.4. Chromatographic Conditions. The mobile phase consisted of acetonitrile: aqueous 0.01 M ammonium bicarbonate buffer, adjusted to pH 9.6 using ammonia solution (65 : 35% v/v) at a flow rate of 1 mL/min. Before use, the mobile phase was degassed by an ultrasonic bath and filtered using 0.4 μm membrane filter. Separation was performed at room temperature on HPLC system having a pump (Shimadzu LC 10AT$_{VP}$) with 20 μL Rheodyne injector, Phenomenex Luna C$_{18}$ (5 μm × 25 cm × 4.6 mm i.d) column,

and SPD-10 A$_{VP}$ photodiode array (PDA) UV-Visible detector set at 238 nm and equipped with CLASS-VP software (Shimadzu. Kyoto, Japan).

2.5. HPLC Analysis of Ayurvedic Taila Preparations. Oil formulations (10 gm) were homogenated using homogenizer (Scientific instruments ltd, Indore) using methanol (50 mL) in proportion of 1 : 5, w/v at 50°C for 20 min. The mixture was centrifuged at 2000 rpm for 20 min at 4°C and the supernatant was collected. The residue was resuspended in methanol and the extraction was repeated five more times similarly. The supernatants were pooled and concentrated under vacuum at room temperature and made up to a volume of 20 mL using methanol. The extracts were filtered through 0.45 μm filter and HPLC was performed under the conditions optimized for the reference compound. The amount of aconitine was quantified using calibration curves plotted with the reference compound.

2.6. Validation of Method

(a) Calibration Graph (Linearity of the HPLC Method). The calibration curve was obtained at 6 concentration levels of aconitine standard solutions (15–90 μg/mL). The solutions (20 μL) were injected into the HPLC system ($n = 6$) with the chromatographic conditions previously given. The linearity was evaluated by the least-squares regression method.

(b) Limits of Detection and Quantification. For determination of the limit of detection (LOD) and the limit of quantification (LOQ) different dilutions of the standard solution of aconitine were analyzed using mobile phase as the blank. The LOD and LOQ were determined on the basis of signal-to-noise ratio until the average responses were approximately 3 and 10 times the responses of the blank, respectively.

(c) Accuracy (Recovery). The accuracy of the methods was determined by calculating recovery of aconitine by the standard addition method. Known amounts of standard solution of aconitine (at three levels 50%, 100%, 150%) were added to prequantitated sample solutions. The amount of aconitine was estimated by applying values of peak area to the regression equations of the calibration graph. Five replicate samples of each concentration level were prepared.

(d) Method Precision (Repeatability). The precision of the instruments was checked by repeatedly injecting and analyzing ($n = 6$) standard solutions of aconitine (45 μg/mL). The results are reported in terms of relative standard deviation (RSD).

(e) Intermediate Precision (Reproducibility). The intraday and interday precision of the proposed method were determined by analyzing standard solution of aconitine at 3 different concentrations (15, 45, and 90 μg/mL) three times on the same day and on three different days. The results are reported in terms of RSD.

(f) Solution Stability and Mobile Phase Stability. Solution stability in the assay method was evaluated by leaving test solutions of sample and reference standard in tightly capped volumetric flasks at room temperature in the dark for 24 h. The same sample solutions were assayed every 6 h interval in the study period. Mobile phase stability was studied by assaying the freshly prepared sample solutions against freshly prepared reference standard solutions at 6 h intervals up to 24 h. Mobile phase was prepared and kept constant during the study period. The relative standard deviation (RSD) of the assay of aconitine was calculated for the study period during mobile phase and solution stability experiments.

2.7. Statistical Analysis. The statistical analysis was performed using Microsoft Excel 2003.

3. Result and Discussion

The literature revealed that methanol is preferred for extraction of aconitine from *Aconitum chasmanthum* [10–14]. The same was used for extraction of aconitine from oil formulations. It is advantageous as the base oil in all the selected ayurvedic oil formulations was immiscible in this solvent. The immiscibility of oil in solvent will help in reducing number of interfering components in further chromatographic development. Multiple extractions were carried out to ensure complete extraction.

3.1. Development of the HPLC Method. The method development and selection of a suitable mobile phase involved several trials because of the complexity of the chemical composition of the herbals and the affinities of the components towards various solvents. The proportions of the organic and aqueous phases were adjusted to obtain a simple assay method with a reasonable run time and suitable retention time. Further optimization of mobile phase was performed based on resolution, asymmetric factor, and theoretical plates obtained for aconitine. Different mobile phases were tried like methanol: acetonitrile: water (45 : 45 : 10) which gave broad peak for aconitine. Combination of acetonitrile: methanol (10 : 90) was tried which gave broad and tailed peak. A mixture of methanol: water (60 : 40) was tried, which gave unresolved peak at retention time 48.9 min. Under optimized conditions HPLC with C18 column and UV detector at 238 nm using mobile phase (acetonitrile: aqueous 0.01 M ammonium bicarbonate buffer, adjusted to pH 9.6 using 30% ammonia solution (65 : 35%v/v)) gave well-resolved symmetric band for aconitine from its oil formulation (Figure 1). The resolution was found to be 1.7. Retention time was found to be around 42 minutes and aconitine appeared on chromatogram at 42.54 minutes. Retention time of aconitine was found to be of 42.54 minutes.

The method consumes less volume of HPLC solvents. When the same drug solution was injected 6 times, the retention time of the drug was found to be the same (Figure 2).

3.2. Validation of Method. The calibration curve was prepared by plotting the peak area against aconitine concentration; it was found linear in the range of 15–90 μg/mL. The
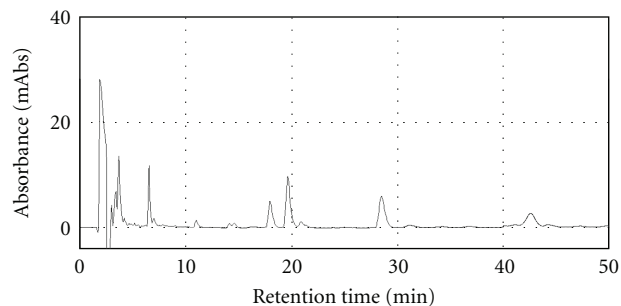

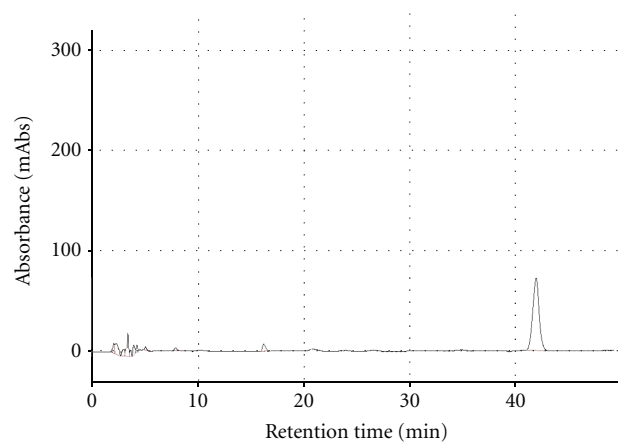Figure 1: HPLC chromatogram of a 20 μL injection of 40 μg/mL reference aconitine at 238 nm.

Figure 2: HPLC chromatogram of a 20 μL injection of the sample of taila preparation at 238 nm.

Table 1: Summary of validation parameters and system suitability parameters.

Parameters	Observations ± % RSD ($n = 06$)
Retention time (min)	42.54 ± 0.33
Tailing factor	1.1 ± 0.1
Resolution	1.7 ± 0.23
Theoretical plates	3213 ± 0.42
Linearity range (μg/mL)	15–90
Correlation coefficient (r^2)	0.9989
Regression equation	$y = 71.5x - 24.5$
Limit of detection (μg/mL)	0.03
Limit of quantification (μg/mL)	0.1
Repeatability (% RSD, $n = 6$)	0.55–1.68

regression equation was found as $y = 71.5x - 24.5$ ($r^2 = 0.9989$), showing excellent linearity (Figure 3). The method was validated in terms of precision, repeatability, accuracy, and other validation parameters (Table 1). The repeatability of the HPLC method and the intermediate precisions for intraday and interday variations are given in Table 2. The LOD value was found to be 0.03 μg/mL, which is the concentration that yields a signal-to-noise (S/N) ratio of 3/1. The LOQ value under the described conditions was 0.1 μg/mL
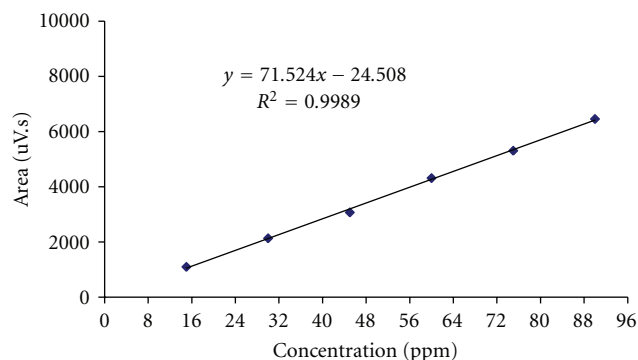
FIGURE 3: Calibration curve of reference aconitine in blank oil sample matrix at 238 nm.

TABLE 2: Precision studies data for aconitine.

Concentration (µg/mL)	Intraday		Interday*	
	Peak area ± SD	% RSD*	Peak area ± SD	% RSD*
15	1101.6 ± 16.7	1.52	1112.0 ± 6.3	0.57
45	3059.9 ± 29.5	0.96	3063.0 ± 29.5	0.96
75	5282.5 ± 42.3	0.80	5292.6 ± 47.8	0.90

* Relative standard deviation (% RSD, $n = 3$).

with an S/N ratio of 10 : 1. This confirmed the sensitivity for quantitation of aconitine in *taila* preparations. Recovery values from 97.7 to 99.9% indicate excellent accuracy of the method (Table 3). The RSD values of assay of aconitine during solution stability, mobile phase stability and robustness studies (Table 4) were within 2.0%. The data obtained in both experiments proves that the sample solutions and mobile phase used during assay were stable up to 24 h.

3.3. HPLC Analysis of Ayurvedic Taila Preparations. Quantitative estimation of aconitine in polyherbal oil formulations given in Table 5 revealed variation in its content in different brands, which indicates the need of standardization of raw material used and uniformity in method of manufacturing to be followed by different ayurvedic manufacturers. The method developed here does not require separation of unsaponifiable matter for quantification as reported for some active ingredients in oil formulation. Oil extract can be directly used for analysis. Avoidance of long and tedious step therefore makes this method more amendable to the high-throughput screening.

4. Conclusion

A method for analysis of *Aconitum chasmanthum* using aconitine as analytical reference in polyherbal oil formulation was developed. Proposed method does not require tedious steps of saponification for separation of fatty acid which are the major interfering component in analysis of oils. Further the method does not require any chemical transformation of active moiety aconitine and it is analyzed as such. The method was found to be simple, precise, specific,

TABLE 3: Accuracy study of the proposed method for aconitine.

Amount detected in sample (µg/mL)	Amount of standard drug added (µg/mL)	Amount of drug recovered (µg/mL)*	% Recovery*
Varnaraksasa taila			
28.3	0	27.6 ± 0.4	97.7 ± 1.6
28.3	14	41.7 ± 0.2	98.6 ± 0.5
28.3	28	56.2 ± 0.3	99.9 ± 0.6
28.3	42	69.2 ± 0.4	98.5 ± 0.5
Vipritmalla taila			
32.2	0	31.8 ± 0.2	98.7 ± 0.6
32.2	16	47.9 ± 0.4	99.4 ± 0.2
32.2	32	65.4 ± 0.3	101.8 ± 0.5
32.2	48	80.4 ± 0.5	100.2 ± 0.7

* Mean value ± relative standard deviation (% RSD, $n = 3$).

TABLE 4: Results of robustness studies.

Parameter	% RSD peak area
Mobile phase composition	1.22
Amount of mobile phase	0.83
Temperature	0.92
Chamber saturation time	0.71
Chamber dimensions	0.43

TABLE 5: Aconitine content found in various *taila* preparations.

Sample	Brand	Aconitine content in oil (mg/100 gm)
Varnaraksasa taila	1	0.27 ± 0.003
	2	0.28 ± 0.005
	3	0.26 ± 0.004
Vipritmalla taila	1	0.31 ± 0.05
	2	0.32 ± 0.07
	3	0.32 ± 0.08

* Mean ± relative standard deviation (RSD, $n = 3$).

sensitive, and accurate. It can be used for routine quality control of polyherbal oil formulations containing *Aconitum chasmanthum*.

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Research Article

A Validated RP-HPLC Method for the Estimation of Pizotifen in Pharmaceutical Dosage Form

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A simple, selective, linear, precise, and accurate RP-HPLC method was developed and validated for rapid assay of Pizotifen in pharmaceutical dosage form. Isocratic elution at a flow rate of 1.0 mL/min was employed on Chromosil C18 (250 mm × 4.6 mm, 5 μm) column at ambient temperature. The mobile phase consists of methanol : acetonitrile in the ratio of 10 : 90 v/v. The UV detection wavelength was 230 nm, and 20 μL sample was injected. The retention time for Pizotifen was 2.019 min. The percent RSD for accuracy of the method was found to be 0.2603%. The method was validated as per the ICH guidelines. The method can be successfully applied for routine analysis of Pizotifen in the rapid and reliable determination of Pizotifen in pharmaceutical dosage form.

1. Introduction

Pizotifen (4-(1-methyl-4-piperidylidine)-9,10-dihydro-4H-benzo-[4, 5]cyclohepta[1, 2]-thiophene); Figure 1(a)) is a benzocycloheptene-based drug used for the prevention of vascular headache including migraine and cluster headache. Alternatively used instead of propanolol, topiramate, valproic acid, and amitriptyline. Pizotifen is reasonably effective, but its use is limited by side effects, principally drowsiness and weight gain, and it is usually not the first-choice medicine for preventing migraines, instead it is used as an alternative when other drugs have failed to be effective [1–3]. Pizotifen is also used as antidepressant or for the treatment of anxiety or social phobia. Animal studies also suggest that pizotifen could be used in the treatment of serotonin syndrome or MDMA overdose in a similar manner to the closely related antihistamine/antiserotonin drug cyproheptadine [4–6]. Pizotifen is contraindicated in gastric outlet obstruction, pregnancy, angle-closure glaucoma, and difficulty in urination [6–8].

Abounassif et al. [9] reported the stability-indicating methods and validated through a study of UV-degraded

solutions contained in quartz cells. RP-HPLC liquid chromatographic method for quantification of Pizotifen malate in pharmaceutical solid dosage forms was reported by Rahman et al. [10]. Here we with reporting precise and accurate RP-HPLC method developed and validated for rapid assay of Pizotifen in pharmaceutical dosage forms.

2. Materials and Methods

2.1. Chemicals and Reagents. The reference sample of Pizotifen (API) was obtained from V.V MED, Hyderabad. The Formulation was procured from the local market. acetonitrile, methanol, ammonium dihydrogen phosphate, triethylamine, and orthophosphoricacid used were of HPLC grade and purchased from Merck Specialties Private Limited, Mumbai, India.

2.2. Instruments. Peak HPLC containing LC 20 AT pump, variable wavelength programmable UV/VIS detector, and Rheodyne injector were employed for the investigation. The chromatographic analysis was performed on a Chromosil C18 column (250 mm × 4.6 mm, 5 μm). Degassing of
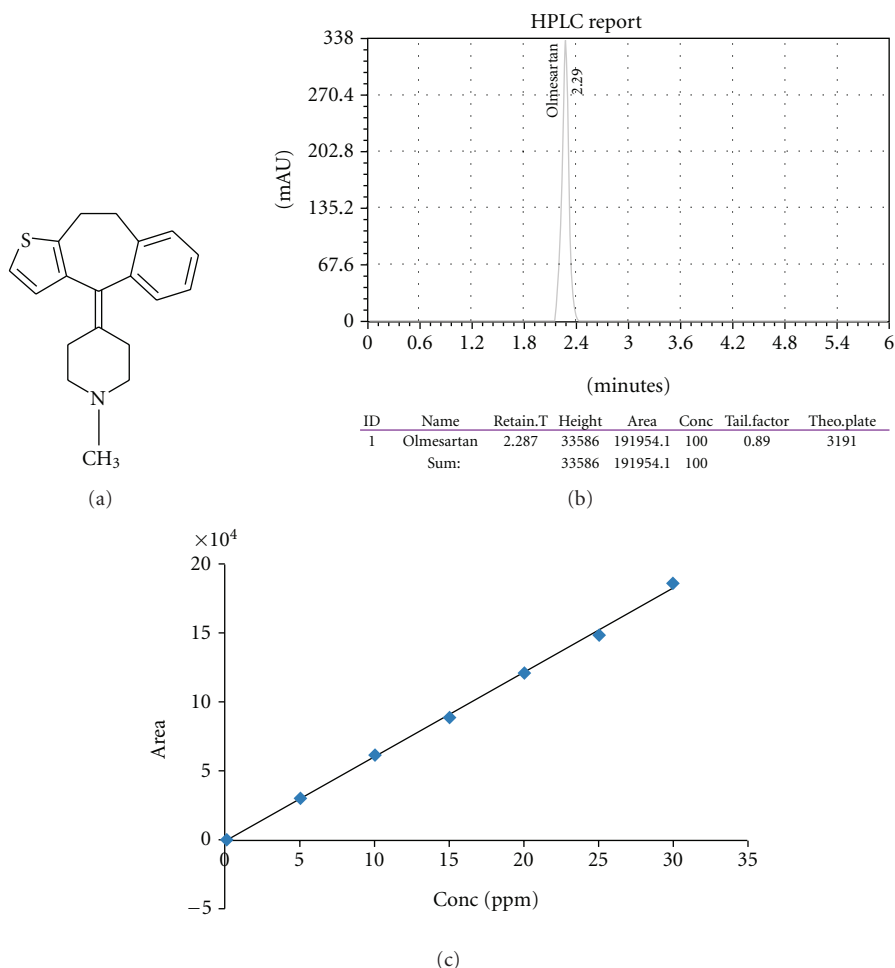
FIGURE 1: (a) Structure of Pizotifen. (b) RP-HPLC Chromatogram of standard solution (c) On *x*-axis: concentration of sample solution, on *y*-axis: peak area response.

the mobile phase was done by using a Loba ultrasonic bath sonicator.

2.3. Chromatographic Conditions.
Proper selection of the stationary phase depends upon the nature of the sample, molecular weight, and solubility. Pizotifen, the selected drug was analyzed by reverse phase columns. Among C8 and C18, Chromosil C18 column (250 mm × 4.6 mm, 5 μm) was selected. Nonpolar compound is very attractive with reverse phase columns. Various combinations of methanol, acetonitrile, orthophosphoric acid ammonium dihydrogen phosphate, and Triethylamine were tested. Mixture of acetonitrile: methanol (90 : 10 v/v) was selected as mobile phase and the pH was adjusted to 5.8 using orthophosphoric acid. Composition of mobile phase on the retention time of Pizotifen was thoroughly investigated. The concentrations of the acetonitrile: methanol (90 : 10 v/v) were optimized to give symmetric peak with short runtime. UV detection wavelength was 230 nm, flow rate was 1.0 mL/min, injection volume was 20 μL, with ambient temperature, runtime was 6 min, and retention time was 2.019 min; the resulting HPLC chromatogram is shown in Figure 1(b).

2.4. Preparation of Standard Solution.
For analysis 100 ppm standard solution was prepared in mobile phase. Required concentrations were obtained from 100 ppm standard solution by proper dilution. pH of the mobile phase was adjusted to 5.8 with orthophosphoric acid and filtered through 0.45 μ nylon filter.

2.5. Preparation of Sample Solution.
The formulation tablets of Pizotifen (Migralin—0.725 mg) were crushed to give finely powdered material. With powder we prepared 70 ppm solution in mobile phase and then filtered it through Ultipor N_{66} Nylon 6,6 membrane sample filter paper.

2.6. Method Development.
For developing the method, a systematic study of the effect of various factors was undertaken by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate wavelength and choice of stationary and mobile phase. The following studies were conducted for this purpose.

2.7. Wavelength Detection.
The spectrum of diluted solutions of Pizotifen in mobile phase was recorded separately on

TABLE 1: Specificity study.

Name of the solution	Retention time in min
Blank	No peaks
Pizotifen	2.01

TABLE 2: Linearity results.

Level	Concentration of Pizotifen in ppm	Mean peak area
Level-1	5.0	30333.1
Level-2	10.0	61990.7
Level-3	15.0	88759.2
Level-4	20.0	121043.1
Level-5	25.0	148510.7
Level-6	30.0	186164.2
		6119.996
Slope intercept correlation coefficient		966.4
		0.998

TABLE 3: Results of Precision Study (Intraday).

Sample	Conc (PPM)	Injection no.	Peaks area	RSD (acceptance criteria 2.0)
Pizotifen	25 ppm	1	152526.6	1.40
		2	151615.4	
		3	152127.8	
		4	153481.7	
		5	150705.3	
		6	156830.7	

TABLE 4: Results of precision study (interday).

Sample	Conc (PPM)	Injection no.	Peaks area	RSD (acceptance criteria 2.0)
Pizotifen	25	1	146180.5	1.07
		2	142312.7	
		3	143153.8	
		4	145268.1	
		5	143858.6	
		6	145253.9	

UV spectrophotometer. The peak of maximum absorbance wavelength was observed. The spectra of the Pizotifen showed that a wavelength was found to be 230 nm. RP-HPLC chromatogram of standard solution is given in Figure 1(b).

2.8. Validation of the Proposed Method. The proposed method was validated as per ICH guidelines. The parameters studied for validation were specificity, linearity, precision, accuracy, robustness, system suitability, limit of detection, limit of quantification, and solution stability.

2.9. Specificity. The specificity of the method was performed by comparing the chromatograms of blank, standard, and sample. It was found that there is no interference due to excipients in the tablet formulation and also that there is good correlation between the retention times of standard and sample. The specificity results are shown in Table 1.

2.10. Linearity. Linearity was performed by preparing mixed standard solutions of Pizotifen at different concentration levels including working concentration mentioned in experimental condition, that is, 25 ppm. Twenty microliters of each concentration was injected in duplicate into the HPLC system. The response was read at 230 nm, and the corresponding chromatograms were recorded. The regressions of the plots were computed by least square regression method. Linearity results are presented in Table 2 and linearity graph in Figure 1(c).

2.11. Precision. Precision of the method was performed as intraday precision and interday precision. To study the intraday precision, six-replicate standard solution of Pizotifen was injected. The percent relative standard deviation (% RSD) was calculated, and it was found to be 1.4, which is within the acceptable criteria of not more than 2.0. Results of system precision studies are shown in Table 3.

For interday precision six-replicate standard solution of Pizotifen was injected on the third day of sample preparation. The percent relative standard deviation (% RSD) was

calculated, and it was found to be 1.07, which is within the acceptable criteria of not more than 2.0. Results of system precision studies are shown in Table 4.

2.12. Accuracy. A known amount of standard drug was added to the fixed amount of preanalyzed tablet solution. Percent recovery was calculated by comparing the area before and after the addition of the standard drug. The standard addition method was performed at 25%, 50%, and 75% level. The percent recovery and % RSD were calculated, and results are presented in Table 5. Satisfactory recoveries ranging from 99.93 to 100.4 were obtained by the proposed method.

2.13. Robustness. Pizotifen at 6 ppm concentration was analyzed under these changed experimental conditions. It was observed that there were no marked changes in chromatograms, which demonstrated that the developed method was robust in nature. The results of robustness study are shown in Table 6.

2.14. System Suitability. System suitability was studied under each validation parameter by injecting six replicates of the standard solution. The system suitability parameters are given in Table 7.

2.15. Limit of Detection and Limit of Quantification for Pizotifen. For this study six replicates of the analyte at lowest concentration were measured and quantified. The LOD and LOQ of Pizotifen are given in Table 8.

3. Discussion on Results

Various combinations of orthophosphosph acid, triethylamine, ammonium dihydrogen phosphate, acetonitrile, and

TABLE 5: Mean percentage recovery.

Level	Amount of Pizotifen spiked (ppm)	Amount of Pizotifen recovered (ppm)	% recovery	Mean % recovery	% RSD	Mean % RSD
25%	5	4.97	99.4			
	5	5.02	100.4			
	5	4.98	99.6		0.53	
50%	10	10.01	100.1			
	10	9.99	99.9	99.92994		0.2603
	10	9.98	99.8		0.15	
75%	15	14.99	99.93			
	15	15.02	100.13		0.101	
	15	15.01	100.06			

TABLE 6: Robustness.

Condition	Mean area	% assay	% difference
Unaltered	144805.5	100.0	0.0
Flow rate at 0.8 mL/min	144720.3	99.94	0.06
Flow rate at 1.2 mL/min	143951.2	99.41	0.59
Mobile phase:			
Acetonitrile : Methanol			
92% 08%	144902.5	100.06	0.06
88% 12%	144763.4	99.97	0.03
pH of mobile phase at 6.0	144275.2	99.63	0.37
pH of mobile phase at 5.6	144358.7	99.69	0.31

TABLE 7: System suitability parameters.

Parameter	Tailing factor	Theoretical plates
Specificity study	1.34	4964.61
Linearity study	1.17	4590.18
Precision study	1.48	4875.34

TABLE 8: LOQ and LOD.

Parameter	Measured volume
Limit of quantification	65 ng/mL
Limit of detection	20 ng/mL

methanol were tested as mobile phase. The concentrations of acetonitrile and methanol were optimized to give symmetric peak with short runtime. A system suitability test was applied to representative chromatograms for various parameters. Ten-point graph was constructed covering a concentration range 5–30 ppm (three independent determinations were performed at each concentration). Linear relationship between the peak area signals of Pizotifen and the corresponding drug concentrations was observed. The calibration curve was obtained for a series of concentration in the range of 05–30 ppm and it was found to be linear. The data of regression analysis of the calibration curves are shown in Table 2. Low values of standard deviation denoted very good repeatability of the measurement. Thus, it was

shown that the equipment used for the study was appropriate and the developed analytical method was consistent. For the intermediate precision a study carried out indicated a RSD of 1.235.

The stability of Pizotifen in standard and sample solutions was determined at ambient temperature ($20 \pm 10°C$). The solutions were checked in triplicate after three successive days of storage, and the data were compared with freshly prepared samples. In each case, it could be noticed that solutions were stable for 48 hrs, as during this time, the results did not decrease below 98%. This denotes that Pizotifen is stable for at least 2 days at ambient temperature. The system suitability parameter values are within the limits. The statistical evaluation of the proposed method revealed its good linearity, reproducibility, and its validation for different parameters.

4. Conclusion

A validated RP-HPLC method has been developed for the determination of Pizotifen in tablet dosage form. The proposed method is simple, rapid, accurate, precise, and specific. Its chromatographic runtime of 6 min allows the analysis of a large number of samples in short period of time. Therefore, it is suitable for the routine analysis of Pizotifen in pharmaceutical dosage forms. So it could be used for the rapid and reliable determination of Pizotifen in tablet formulations.

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