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A validated high performance liquid chromatographic method for analysis of nicotine in pure form and from formulations

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Abstract

A reverse phase HPLC method using C_{18} column has been developed for the quantitative estimation of nicotine in the bulk material and formulations (extended release and immediate release dosage forms). The method is specific to nicotine (RT $\sim$4.64 min, asymmetry $\sim$1.75), and can resolve analyte peak from excipient interferences. It is linear (coefficient of variation $\sim$0.9993), accurate (average recovery $\sim$100%), and passed all the system suitability requirements. Applicability of the method was evaluated in analysis of drug-excipient compatibility samples, commercial dosage form (such as nicotine gum) and two novel in-house formulations.

Keywords: Nicotine; HPLC method; Bulk analysis; Extended release formulations; Immediate release formulations; Dissolution; Analysis of nicotine gum

1. Introduction

Smoking is one of the largest single preventable causes of ill health in the world [1], which leads to loss of personal as well as national income through various associated diseases. Nicotine is the principle alkaloid in tobacco and is responsible for causing dependence due to its psychoactive properties and capacity to induce self-administration behavior. Nicotine abstinence results in precipitation of various withdrawal symptoms like irritability, anxiety, and decrease in concentration which forces smoker to continue smoking [2]. Nicotine replacement therapy (NRT), aimed at reducing withdrawal symptoms, has a greater success potential than any other methodology known so far. NRT helps the smoker to overcome these withdrawal symptoms by providing nicotine in therapeutic doses in a tapering manner over a period of time [3,4]. Currently, NRT consists of four nicotine products (gum, patch, spray, and inhaler), which have specific advantages. Novel formulations of nicotine have been developed in the investigators laboratory, which are targeted at smoking cessation [5].

Nicotine (Fig. 1) is a weak, diacidic base having two p*K*a values and is highly soluble in solvents

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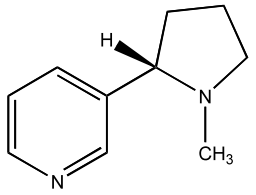

Fig. 1. Structure of nicotine.

such as water, methanol, acetonitrile, chloroform, and petroleum ether. Literature reports colorimetric [6,7], spectrophotometric, and chromatographic methods for analysis of nicotine. Amongst these, chromatographic methods have been widely used [8–12]. Pichini et al. [13] has reported application of solvent optimization software in method development that uses C_{18} column for isolation and quantitation of nicotine present in serum of smokers as well as spiked serum samples. The method successfully isolated various peaks and mobile phase consisted of ion-pairing reagent. Gas chromatographic methods are cited in literature, however, Thompson et al. has reported inability of such methods to quantitate labile compounds such as nicotine 1′-N-oxide [14]. Gas chromatography is the official method for determination of chromatographic purity of nicotine in United States Pharmacopoeia [15]. Ion-pairing and ion-suppression methods have been most commonly employed for the analysis of weakly basic drugs such as nicotine [16], however, they have certain disadvantages. Methods based on ion-pairing principle usually exhibit poor selectivity, and often result in band broadening due to inadequate buffering action or dissociation of ion-pairs [17]. Nicotine, being a weak base has a p*K*a value in the alkaline range. In order to fully suppress the ionization of nicotine, mobile phase pH should be selected in the range of 8–9, at which most of the silica-based phases are unstable. Under the conditions of uncontrolled ionization, a strong polar interaction of nicotine with residual silanol groups on the silica surface often results in band broadening and poor efficiency [18]. Use of triethanolamine is, therefore, suggested by some workers [19] while others have used both amine and ion-pairing agents simultaneously [15]. In addition, costly detector systems such as electrochemical detector (ECD) or hazardous chemicals like barbituric acid [6,20], potassium cyanide [12], and bromine [7] have been used in these methods. Examples of these methods include HPLC coupled with mass detector [21], HPLC coupled with glassy carbon detector [22], ion-pair HPLC using various sulphonic acid sodium salts [13,23], and combination of reverse phase and ion-exchange chromatography [8].

The methods described above were essentially bioanalytical in nature. They have been used either to support pharmacokinetic studies on nicotine, determination of free base and its metabolites in various physiological fluids such as saliva [24,25], plasma/serum [26,27], urine [28], and nicotine determination from tobacco samples etc. These methods though highly specific and sensitive for nicotine, may not yield precise and accurate results during in vitro analysis of nicotine dosage forms.

Nicotine dosage forms (gum and patch) are official in USP [15]. USP mentions chromatographic method for nicotine polacrilex gum (assay) and transdermal patch (release studies and uniformity of dosage). These methods use mobile phase additives such as surfactants (sodium dodecyl sulfate), amines (triethylamine, dioctylamine), and sulfonic acid esters (sodium 1-decanesulfonate and dodecane sulfonate). At the same time, Pharmacopoeia mentions different chromatographic parameters for various analyses involving nicotine products. The method developed in our laboratory has a specific advantage over a USP method that, it does not use any of the above-mentioned mobile phase additives. In addition, single method can be adopted for wide variety of analyses such as in vitro release studies, content uniformity, selection of excipients for a formulation based on drug-excipient analysis etc. that is not the case with the reference method.

We used a base-deactivated C_{18} column wherein the residual silanol groups have been deactivated by special procedures. This kind of packing material requires minimal or no mobile phase additives such as amines, ion-paring agents etc. Base-deactivated silica columns are widely used worldwide for analyses involving basic drugs such as nicotine mainly for superior quality of analysis and economic advantage when compared with

ordinary C_{18} columns. The proposed method has been developed and fully validated with a specific aim of having a method, which is simple in operation, cost-effective, avoids hazardous chemicals and able to analyze bulk material, uniformity of dosage, in vitro release samples, and to detect minute changes that may take place during drug-excipient compatibility study.

The spectrum of applications covered by the method will definitely help readers to use this method in routine analysis of nicotine as well as conducting various studies on the formulations.

2. Experimental

2.1. Instrumentation

A Shimadzu HPLC system equipped with LC-10ATVP Pump, DGU-14AM on-line degasser, SIL-10-ADVP auto injector, CTO-10-ASVP column oven, and SPD-10AVP-UV-VIS detector was utilized. The second instrument, Shimadzu HPLC system equipped with LC-10ATVP pump, DGU-14A on-line degasser, SIL-10-ADVP auto injector, CTO-10AVP column oven and SPD-M10AVP-PDA detector was used for determining peak purity. Shimadzu CLASS-VP software (Version 5.03) was used for data acquisition and system suitability calculations. The chromatographic conditions are outlined in Table 1. In addition, Branson 3510 ultrasonic bath, Mettler Toledo AG-245 electronic balance, and Millipore Filtration assembly were used in the study. Water used throughout the HPLC analysis was prepared by reverse-osmosis using USF ELGA system. Dissolution studies were conducted in USP 24 dissolution apparatus (Electrolab, India).

Table 1
Chromatographic parameters for determination of nicotine

Parameter	Condition
Method	Reverse phase high performance liquid chromatography
Column	Hypersil C_{18} BDS (Thermo Hypersil, UK) 250 × 4.6 mm, and 5 μm particle size
Flow rate	1 ml/minute
Detection	UV detector, 259 nm PDA detector, 200–800 nm for peak purity testing
Column temperature	35 °C
Injection volume	20 μl
Mobile phase	Phosphate buffer (pH 6.8; 10 mM):methanol (35:65% v/v)

2.2. Materials

(-) Nicotine was purchased from Sigma Chemicals, USA (Lot no. 108H1312). Similarly nicotine hydrogen tartrate was also procured from Sigma chemicals, USA (Lot no.100K3250). Both nicotine and nicotine hydrogen tartrate were used after performing initial assay by non-aqueous method as given in USP 24 [15].

Potassium dihydrogen orthophosphate GR was obtained from Hi Media Laboratories Ltd., Mumbai, India, and HPLC grade methanol was obtained from Rankem, Punjab, India.

Two in-house formulations (NIPER ER and NIPER IR) were evaluated using the present method. NIPER ER contained 8.75 mg of nicotine hydrogen tartrate salt (equivalent to 2.8 mg of nicotine) per tablet in a matrix of bioadhesive polymers and NIPER IR contained 2 mg of nicotine per tablet along with other excipients [5]. The excipients used for the development of NIPER ER and NIPER IR were obtained from commercial sources and were used as such. Table 2 gives the composition of NIPER ER and NIPER

Table 2
Composition of nicotine in-house formulations

Ingredients	
NIPER IR	NIPER ER
Nicotine hydrogen tartrate	Nicotine hydrogen tartrate
Flowlac	Flowlac
Avicel PH-102	Metolose 90SH100
Pearlitol SD100	Carbopol 974P
L- HPC	Polycarbophil
Magnesium stearate	Magnesium stearate
Polyplasdone	Talc
Ac-Di-Sol	
Aspartame	
Menthol	
Aerosil	

IR formulations. For practical purposes, the in-house formulations were prepared using equivalent amount of nicotine hydrogen tartrate dihydrate. Marketed NRT formulations such as nicotine gum (Nicorette® containing 2 mg nicotine, Lot BL 516, expiry March 2003 and Nicorette® containing 4 mg nicotine, Lot BL 086, expiry April 2003) were purchased from the retail pharmacies.

2.3. Method validation

The developed method was validated for the parameters like linearity, range, precision, reproducibility, specificity, accuracy, recovery, filter validation, and system suitability as described below.

2.3.1. Linearity

A stock solution of nicotine 500 μg/ml was prepared by dissolving 156.25 mg of accurately weighed nicotine hydrogen tartrate dihydrate (equivalent to 50 mg of nicotine) in 100 ml of phosphate buffer (pH 6.8). This was labeled as solution "A". Various dilutions were prepared in duplicate using solution A in the concentration range of 2–40 μg/ml in phosphate buffer (pH 6.8). The samples were filtered through 0.45 μm nylon filter and injected on column in duplicate. Areas for four injections were determined and graph prepared. Slope and intercept were estimated.

2.3.2. Precision

2.3.2.1. Repeatability. Repeatability of the method was checked by analyzing six replicate samples of nicotine (at the 100% assay concentration i.e. 28 μg/ml) and calculating percent relative standard deviation (% R.S.D.).

2.3.2.2. Intermediate precision. Intermediate precision of the method was checked by repeating the entire procedure for 3 consecutive days and calculating the R.S.D. between 3 days for area, slope, and intercept.

2.3.3. Specificity of method for dissolution studies

To determine the specificity of the method in presence of excipients, a polymer matrix (100 mg) consisting different excipients present in final formulation was prepared in 100 ml of mobile phase. 20 μl of this solution was injected on column after filtration through 0.45 μm nylon filter and peak response was recorded.

2.3.4. Accuracy and recovery studies

The developed analytical method was validated for its accuracy in determining the drug content from solution and from the excipient blend. Two different procedures were used for analyzing drug solution and excipient blend as given below.

2.3.4.1. Drug solution (assay). For demonstrating accuracy of an analytical method, three concentration levels of drug solution (80, 100, and 120% of assay concentration) were prepared in triplicate and analyzed.

2.3.4.2. Recovery from excipient blend (assay by spiking). Recovery studies from excipients blend was carried out by spiking a specified amount of drug solution (80, 100, and 120% of assay concentration) in 100 mg of excipient matrix in a small vial. The solutions were prepared in methanol in triplicate. The samples were mixed thoroughly using vortex mixer and allowed to dry in a dark place. The dried blend was then quantitatively transferred to 50 ml volumetric flask avoiding losses during transfer and 35 ml of phosphate buffer was added. The solution was sonicated for 30 min in an ultrasound bath. The volume was adjusted to 50 ml using buffer and the solutions were filtered through 0.45 μm nylon filter. 20 μl of this solution was injected in duplicate. Percentage of drug recovered was calculated using a standard curve prepared on the same day. Similarly, the changes, if any, in retention time and peak shape were also recorded in the presence and absence of excipient blend.

2.3.5. Filter validation

Filter validation was performed by analyzing solutions at 2 and 40 μg/ml (the lowest and the highest concentration of the linearity curve). The

solutions were analyzed in duplicate after filtration through 0.45 μm nylon filter. The results were compared with the unfiltered sample injected at the same concentration levels and the amount retained by the filter was calculated.

2.3.6. System suitability

Data from five injections (at 100% assay concentration) was utilized for calculating system suitability parameters like capacity factor, asymmetry, number of theoretical plates and area using CLASS-VP software.

3. Analysis of nicotine formulations

Utility of the proposed method was evaluated by performing analysis of drug-excipient compatibility samples. This exercise was carried out to select various excipients for formulation development. The developed analytical method was also tested for analysis of nicotine from the in-house formulations. An immediate release formulation NIPER IR (2 mg of nicotine per tablet) was evaluated for content uniformity while extended release formulation NIPER ER containing 2.8 mg of nicotine per tablet was evaluated for content uniformity and in vitro dissolution.

3.1. Drug-excipient compatibility study

Accurately weighed amounts of drug and excipients in 1:1 ratio were taken in 5 ml glass vials and mixed well. Water equivalent to 5% w/w (10 μl) was added and mixed thoroughly. The samples then stored at 40 °C and 75% RH in stability chambers for 3 weeks. Similarly, another set of samples were prepared, screw capped and stored at 4 °C in the refrigerator. Isothermally stressed samples were visually evaluated after first, second and third week of storage to note any physical change in the mixture and compared with the control samples. After 3 weeks, all the samples (both stressed and control) were quantitatively analyzed by the proposed HPLC method. Similarly, another set of vials were prepared without adding water and subjected to the stress conditions.

Samples were dissolved in 50 ml HPLC methanol and kept aside for 15 min. Volume was made upto 100 ml with phosphate buffer such that the concentration is approximately 1 mg/ml. This solution was suitably diluted to 50 ml with phosphate buffer (28 μg/ml). The solutions were filtered through 0.45 μm nylon membrane and areas were determined. Amount of nicotine present in the mixtures was determined using standard curve.

3.2. Content uniformity

An accurately weighed tablet was triturated in a mortar and the contents were transferred quantitatively to a 10 ml volumetric flask. The contents were dispersed in 8 ml of methanol with the help of sonication for 10 min and the volume was made up to mark with methanol. 1 ml of this solution was diluted to 10 ml with phosphate buffer (pH 6.8) in a 10 ml volumetric flask. The samples, after filtration through a 0.45 μm nylon filter were subjected to HPLC analysis. The experiment was repeated on five additional tablets.

3.3. Dissolution studies

Dissolution study with NIPER extended release formulation ($n = 6$) was carried out on a modified USP II dissolution apparatus using a rotating paddle method (50 RPM). Phosphate Buffer pH 6.8 (100 ml) maintained at 37 ± 0.5 °C was used as dissolution medium. The samples (5 ml) were withdrawn at the predetermined time and replaced with an equivalent amount of fresh medium. The samples were filtered through 0.45 μm nylon membrane filter and analyzed using validated HPLC method. The cumulative percent drug release was plotted against time to determine the release profile.

3.4. Analysis of nicotine gum

An accurately weighed nicotine gum was cut into four pieces and transferred to 250 ml stoppered conical flask. The contents of the gum were extracted in 50 ml of phosphate buffer (pH 6.8) and 50 ml of *n*-hexane under vigorous stirring or

till the contents were fully dispersed. The aqueous layer was separated and washed the precipitate with additional 50 ml of phosphate buffer (pH 6.8). Combined the washings and filtered through 0.45 μm nylon membrane filter. 20 μl of this solution was injected and response recorded. Amount of nicotine present per unit of gum was calculated from the standard curve. The analysis was repeated on five additional units.

Another set of analysis was conducted on 4 mg nicotine gum as per the USP method [15]. The chromatographic conditions used were as given by USP except for the replacement of 30-cm column with 25-cm column.

4. Results and discussion

Fig. 2 represents chromatogram of nicotine obtained by the developed method. Nicotine elutes at retention time of 4.64 min with an asymmetry value of 1.75.

4.1. Linearity

Table 3 describes regression statistics obtained for various analytical tests. The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to concentration of analyte within a given range. The linearity of the method was observed in the expected concentration range, demonstrating its suitability for analysis. The goodness of fit (R^2) was found to be 0.9993.

4.2. Precision

The precision of an analytical method is the degree of agreement among the individual test results when the method is applied repeatedly to multiple sampling of homologous sample. Repeatability refers to the use of analytical procedure within a laboratory over a short period of time using the same analyte with the same equipment and is expressed as the percent R.S.D. The method passed the test for repeatability as determined by

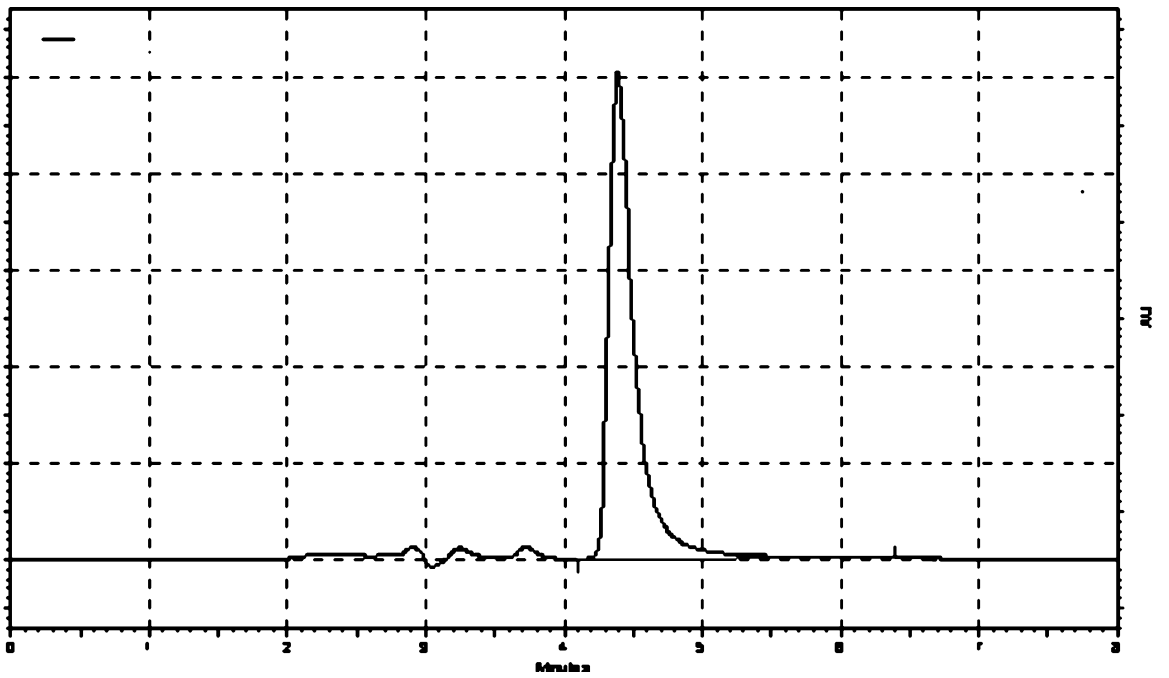

Fig. 2. Representative chromatogram of nicotine using a developed method.

Table 3
Regression statistics for nicotine

Parameter	Target concentration (μg/ml)	Range (μg/ml)	Goodness-of-fit (R^2)	Slope	Intercept
Assay, release, and compatibility study	28	2–40	0.9993	4876.6	−174.94

percent R.S.D. (1.38%) of the area of the peaks of six replicates injection at 100% assay concentration.

Intermediate precision involves estimation of variations in analysis when a method is used within laboratories, on different days, by different analysts, and on different equipments. The intermediate precision was studied by preparing the standard curve for 3 different days, and the results of interday variation are given in Table 4. The method passed the test for intermediate precision as percent R.S.D. of the slope and intercept obtained with 3 different days were within the limits of 2%.

4.3. Specificity

Specificity is the ability of an analytical method to assess unequivocally the analyte in the presence of components that are present in the sample matrix. The representative chromatogram (Fig. 3) of excipient blend shows that excipients do not interfere with the drug peak indicating specificity of the method for nicotine.

4.4. Accuracy

The accuracy of an analytical method is the closeness of test results obtained by the method to the true value. It can be determined by application of analytical procedure to an analyte of known purity (for a drug substance) or by recovery studies, where known amount of standard is spiked in the placebo (for the drug product). The results of accuracy studies from solution and excipient matrix are shown in Table 5, and it is evident that method is accurate within desired range.

Table 4
Intermediate precision

Concentration (μg/ml)	Intra-day variation (% R.S.D.)	Inter-day variation (% R.S.D.)
2	2.0	1.99
4	1.74	2.21
8	0.95	1.63
16	0.69	0.51
32	0.64	1.21
40	1.24	0.48

4.5. Filter validation

The R.S.D. obtained at higher concentration (0.08%) and lower concentration (1.27%) indicates suitability of the nylon filter for the filtration of the dissolution sample, as the R.S.D. is less than 2%.

4.6. System suitability testing

System suitability test are an integral part of chromatographic methods and are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed [29]. The results of system suitability are given in Table 6. All the values for the system suitability parameters are within the acceptable range.

4.7. Applicability of method

The developed method was successfully applied for the assay of nicotine in mixtures containing various excipients, NIPER extended release, immediate release, and marketed formulations. The results of drug-excipient compatibility study revealed absence of co-elution at the retention time of nicotine (peak purity >0.9997). There was no significant degradation of nicotine in mixtures at the end of 3-week storage period (average assay $>$ 95%). The content uniformity results are shown in

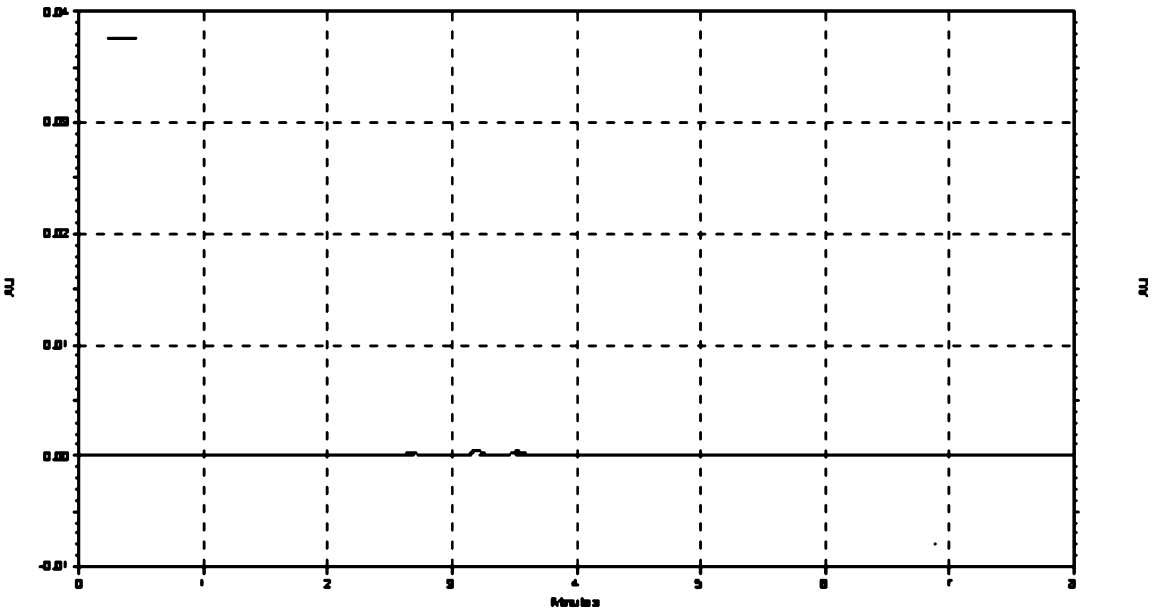

Fig. 3. Chromatogram of excipient blend.

Table 5
Accuracy/recovery data for nicotine

Parameter	Concentration level (μg/ml)	Recovery	R.S.D. (%)
Assay	80	101.33	0.37
	100	102.14	1.11
	120	100.89	1.94
Assay (Spiking)	80	97.76	0.55
	100	98.21	1.05
	120	100.59	1.50

Table 7, which demonstrates the suitability and wide applicability of the developed method. Dissolution samples are also analyzed using a developed method and dissolution profile is shown in Fig. 4.

The proposed method was also compared with the reference method [15] with respect to assay of marketed formulations and linearity. Linearity was determined as per the chromatographic method given by USP and the results are given in Table 8.

5. Conclusion

A simple HPLC method using a C_{18} type column was developed for the analysis of nicotine in bulk material, extended, and immediate release formulations. The method was specific for nicotine and was validated with respect to various analy-

Table 6
System suitability parameters

Parameter	Maximum	Minimum	% R.S.D.	Status
Asymmetry	1.60	1.58	0.559	Passed
Capacity factor	0.45	0.43	1.853	Passed
Theoretical plates	1871.25	1780.24	1.708	Passed
Area	146 958	146 328	0.179	Passed

Table 7
Content uniformity of nicotine gum and NIPER formulations

Sr. No.	Product	Nicotine label claim (mg/unit)	Amount found (mg)	Recovery (%)	% R.S.D.
1	NIPER IR	2.0	2.02	101.00	4.83
2	NIPER ER	2.8	2.81	100.35	2.71
3	Nicotine gum	2.0	1.99	99.5	4.01

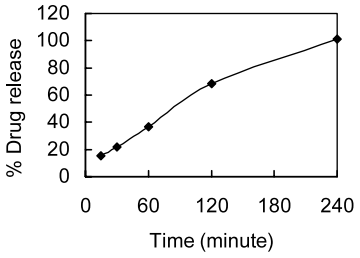

Fig. 4. Dissolution profile of NIPER ER formulation.

tical parameters. The method was found suitable for the analysis of in-house nicotine formulations as well as marketed dosage forms.

The proposed method had specific advantages over the USP method. The elution time of nicotine under the proposed method is much less than the reference method indicating suitability for rapid determination of nicotine from bulk/formulations. Though, the theoretical plates for proposed method were less as compared with USP method, however, they qualified the pharmacopoeial requirement. Also, the peak shape of nicotine was reasonably good and principal peak was well separated from the mobile phase interferences. There was no co-elution of excipients/tartrate salt at the retention time of nicotine under the proposed method as indicated by peak purity index. Although, both the methods gave acceptable assay values with percent R.S.D. much lower for USP method than proposed method, the goodness of fit (R^2) and other linearity parameters suggested enhanced accuracy and sensitivity of in-house method.

Further, due to the presence of ion-pair reagent, it is expected for reference method to have a longer column equilibration time (>3 h) in order to reduce retention time variation (a normal phenomenon with such methods) while under proposed method, chromatographic conditions stabilized in less than an hour. In such cases, cost considerations acquire a significant dimension. In addition to this, quaternary mobile phase and a solvent mixture of similar composition is mentioned in the USP thereby increasing the complexity of the method. Though, the initial cost of special base-deactivated silica column is slightly higher than the conventional C_{18} reversed phase column, however, the long term benefits (rapid analysis, simple chromatographic conditions, accuracy and sensitivity of system, operational convenience, and cost reduction) offered by the proposed method are

Table 8
Comparison of proposed method vs. USP24 method

Parameter	USP method	Proposed method	USP limits
Retention time (min)	35.97	4.64	Not given
R.S.D. for replicate injections	1.4%	1.38%	NMT 2.0%
Asymmetry (10%)	1.31	1.75	NMT 2.0
Theoretical plates (N)	12127.58	5453.51	NLT 2500
Slope (linearity curve)	33 925	4876.6	Not given
Intercept (linearity curve)	16 567	−174.94	Not given
Goodness of fit (R^2) (linearity curve)	0.9983	0.9993	>0.999
% Assay (R.S.D.)	103.5% (0.14)	99.5 (4.0)	90–120%

significant. Therefore, under the experimental conditions, the proposed method was found to be better than the USP method with respect to above-mentioned parameters.

However, the proposed method should be evaluated for its ability to separate various degradation products of nicotine that may arise during stability studies/stressed conditions on the formulations. Experiments should also be carried out to evaluate stability-indicating potential of this method.

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