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Reserch Article

A RAPID SEPARATION AND QUANTIFICATION OF NICOTINE AND NICOTINE RELATED SUBSTANCES USING UPLC IN PHARMACEUTICAL FORMULATIONS

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ABSTRACT

Objective: The aim of this work was to develop a new RP–UPLC method for the Quantification of Nicotine related substances. Separation was performed; using a simple reversed phase UPLC method with photodiode array detector has been developed and subsequently validated for the determination of Nocotine related substances. Method: The separation was based on the use of a BEH C-18 analytical column (100 × 2.1mm, i.d., 1.7 µm). The mobile phase consists of 10Mm Dibasic Ammonium carbonate in water, pH 8.9 and Acetonitrile. The Chromatographic separation was carry through column temperature at 35˚C with a flow rate of 0.6 ml/min. Quantitation was achieved with UV detection at 260 nm. Results and discussion: with linear calibration curves at concentration ranges 0.7 – 5.4 µg ml−1 for Nocotine related substances. The Limit of detection (µg ml−1) was found to be 0.09, 0.10, 0.13, 0.18, 0.28, 0.30, 0.16 and The Limit of Quantitation (µg ml−1) was found to be 0.26, 0.30, 0.38, 0.53, 0.85, 0.9, and 0.47 for Nicotine related substances (A, B, C, D, E, F and G). The %Relative standard deviation values for intra- and inter-day precision studies were < 1.5% and < 1.3%, respectively. The % Mean recovery of Nicotine related substances determined from a spiked sample ranged from 90% to 110%. Conclusion: The method was demonetized to be Specific, Linear, Precise, Accurate and robust for performing the related substance analysis for product release and stability studies.

Keywords : Nicotine related substances, UPLC, Method development, Validation.

INTRODUCTION

Nicotine is heterocyclic Pyridine derivative, which is present in many biologically active substances; is a chemically 3-[(2S)-1-methylpyrrolidin-2-yl] pyridine (Figure 1), with the Molecular Formula is $C_{10}H_{14}N_2$ and Molecular Weight: 162.23. Nicotine is a hygroscopic, oily liquid that is miscible with water in its base form. Nicotine forms salts with acids; that are usually solid and water soluble. Controlled levels of nicotine are given to patients through gums, dermal patches, lozenges, electronic/substitute cigarettes or nasal sprays in an effort to wean them off their dependence. A literature search has revealed few HPLC methods involving assay and a LC–MS/MS method for the determination of impurities of Nicotine have been published, such as Paper Chromatography of Nicotine and Related Substances (Lee Leiserson et al., 1955), Specific Detection of Anabasine, Nicotine, and Nicotine Metabolites in Urine by Liquid Chromatography – Tandem Mass Spectrometry (Hoofnagle AN et al., 2006), Determination of nicotine in mushrooms by various GC/MS- and LC/MS-based methods (A. Lozano et al., 2012), HPLC-UV method for nicotine, strychnine, and aconitine in dairy products (Joseph E Jablonski et al., 2006), A LC-MS/MS Method for Concurrent Determination of Nicotine Metabolites and Role of CYP2A6 in Nicotine Metabolism in U937 Macrophages: Implications in Oxidative Stress in HIV + Smokers (: Jin, Mengyao et al., 2012)[1-12]. The focus of present's manuscript describes a rapid, stable, precise and accurate gradient reverse phase UPLC method has been applied for the analysis of commercial Nicotine preparations.

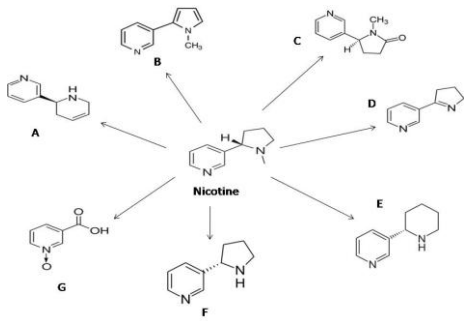

Fig. 1: Nicotine and its related substance, Anatabine (A), β-Nicotyrine (B), Cotinine (C), Myosmine (D), nicotine N-oxide (E), Nornicotine (F), Anabasine (G) chemical structures.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents are at least of analytical grade. Milli-Q-Water was used. Di-Ammonium hydrogen phosphate was purchased from (Merck, Germany). Acetonitrile was of HPLC-grade (J.T. Baker, Holland). 100% purity pharmaceutical grade Nicotine and Nicotine impurities Anatabine (A), β-Nicotyrine (B), Cotinine (C), Myosmine (D), nicotine N-oxide (E), Nornicotine (F), Anabasine (G) procured from Gladwin Pharmaceuticals. Pharmaceutical formulation, Nicotine gum (containing 2mg of Nicotine per patch) was obtained from Indian market.

Equipments and chromatographic conditions

Ultra performance liquid chromatography with mass spectrometry (UPLC/MS) analysis was performed on an ACQUITY SQD and a Waters ACQUITY UPLC-H Class consisting of a A UPLC system (Waters, ACQUITY UPLC H-Class) provided with a Quaternary Solvent Manager (QSM, an automatic injector (FTN - with flow-through needle design), a PDA detector, with an Empower and mass lynx Software's were used. Electro spray ionization (ESI) mass spectra were recorded in the mass range m/z 50–1200 using positive ion mode and the following setting of tuning parameters: target mass m/z = 500, compound stability = 100%, pressure of the nebulizing gas = 490.1 kPa, the drying gas flow rate = 10 L/min and temperature of ion source = 360oC. The selected precursor ions were further analyzed by MS/MS experiments using the isolation width m/z = 4 and the collision amplitude 1.0V. The standards and samples were dissolved in the mobile phase and 5 µL of the solution was injected into the UPLC/MS system. The chromatographic analysis was performed on a 1.7µm particle BEH C18 column; 100×2.1 mm (Waters) kept in a Thermo quest column oven at 35 °C. Final chromatographic conditions were a gradient elution, being solvent A: 10 mM phosphate at pH 8.9 and solvent B: acetonitrile. The phosphate buffer was prepared from Dibasic Ammonium phosphate by adding 20% w/w Ammonium Hydroxide solution to reach the pH 8.9. At t=0 the mobile phase consisted of 95% A and 5% B and it changed with a linear gradient during 4 min to 80% A and 20% B. At min 5 it changed to 55% A and 45% B for 2 min and at t=9 min it returns to the initial conditions (95% A and 5% B) during 1 min remaining at this composition until t=10 min. The flow rate was 0.6 ml-1 and the injection volume was 5µl. UV spectra of all chromatographic peaks were recorded in the range 200–600 nm using a diode-array UV detector with the highest

resolution at 1.2 nm. UV detection was monitored at 260 nm for Nicotine and its related substances, because at this wavelength sensitivity was higher than in other more characteristic wavelengths and it were necessary for the detection of minor substances.

Standard Preparation

Standard solution was prepared by dissolving the respective working standard substances in water to obtain the concentration of 100.0 µg ml−1 for Nicotine.

Sample Preparation

A test solution was prepared containing a mixture of Nicotine and related substances in a concentration ratio corresponding to the recommendations by the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use), Topic Q3A(R2), [13]. The test solution containing 100.0 µg ml−1 Nicotine and 0.36 µg ml−1 of each related compound (corresponding to 0.004 % of the Nicotine) was prepared using standard stock solutions.

Method Validation

In order to study linearity of the response, a series of working standard solutions (0.7, 1.8, 2.9, 3.6, 4.3, 5.4 µg ml−1 for Nicotine and its related substance) were prepared. Three determinations were carried out for each solution. Similarly; (80, 90, 100, 110, 120 µg ml−1) Nicotine standard solution were prepared for assay method. The linearity of the peak area responses versus concentration was studied. The correlation graph was constructed by plotting the peak areas obtained at the optimized conditions.

The quantitation limit (QL) is expressed as: $QL = 10.0\delta/S$

Where,

δ = the standard deviation of the response

S = Slope of the calibration curve

The Detection limit (DL) is expressed as: $DL = 3.3\delta/S$

Where,

δ = the standard deviation of the response

S = Slope of the calibration curve

Precision and accuracy were assessed using three different working standard solutions in the range of 80–120 % of the test concentration of Nicotine. For impurities, in the range, LOQ to 150% of each related compound. Intra-day accuracy and precision were evaluated from replicate analysis (n = 6) of working standard solutions on the same day. Limit of quantification is calculated from signal to noise ratio. To determine limit of quantification, sample blank is injected first and noise is integrated at different intervals at different retention time near the peak of interest.

RESULTS AND DISCUSSION

In previous times LC methods suffered from problems when analyzing basic drugs, such as Nicotine, since these substances strongly interact with polar ends of HPLC column packing materials, causing severe peak asymmetry and low separation efficiencies. High purity silica back bone and advances in bonding technology have alleviated the tailing problem of polar substances in LC to a significant extent. Consequently, for the initial development a BEH C18 100 x 2.1mm, 1.7µm column was used. This packing was selected because it has one of the lowest hydrophobicity and silanol activity as seen in commercial catalogues. The pH value of 8.9, in the mobile phase permitted a low ionization degree and therefore, a higher retention of the analytes. It provides more tools to obtain the separation. The method development process systematically screened columns, modifiers to achieve the best separation of closely eluting impurities, namely, impurity-A and impurity-D, Nicotine and impurity-G. To increase the resolution of closely eluting peaks, a less polar aprotic organic solvent (Acetonitrile) was used. The pH of the buffer is adjusted to 8.9 improve separation between impurity-A and impurity-D, Nicotine and impurity-G. The chromatographic separation was achieved on a Bridged ethylene Hybrid C18 Column, 1.7µm particle size, by using variable mixtures of Buffer and modifier. The Solution-A contains 10mM Di- Ammonium carbonate, pH adjusted to 8.9 using 20% w/v sodium hydroxide solution and Solution-B contains Acetonitrile. The flow rate of the mobile phase was 0.6µml^{-1}. The UPLC gradient program for related compounds method was set as time/% mobile phase-B: 0.01/5, 4/20, 5/55, 7/55, 9/5 and 10/5 with a post run time of 10 minutes. At 35°C column temperature, the peak shape of Nicotine was found symmetrical. In the optimized conditions Nicotine, impurity-A, impurity-D, and impurity-G were well separated with a resolution of greater than 2 and the typical retention times of impurity-A, impurity-B, impurity-G, and Nicotine were about 3.9, 4.1, 5.2, and 5.6 minutes, respectively. The system suitability results for related compounds method are given in Table 3 and the developed UPLC method was found to be specific for Nicotine and its related substances, Anatabine(A), β-Nicotyrine (B), Cotinine(C), Myosmine(D), nicotine N-oxide(E), Nornicotine(F), Anabasine(G).

Method validation

The developed UPLC method was validated in terms of linearity, accuracy, precision, LOD, LOQ, robustness and specificity as per ICH guidelines.

Linearity

The calibration plot for related compounds method was obtained over the calibration ranges tested, The correlation coefficient obtained was greater than 0.995 (Fig. 2). The percentage of RSD values for each level are within the limit (<5.0). The %Y-intercept of the calibration curves were within <5.0. These results show that an excellent correlation existed between the peak area and concentration of the Nicotine related substances from Table 1. Linearity calibration plot for assay method was obtained over the calibration ranges tested, that is, 0.05–0.15mg/mL and the correlation coefficient obtained was greater than 0.999. The percentage of RSD values for each level is within 2.0 and %Y-intercept of the calibration curve was <3.0. These results show that an excellent correlation existed between the peak area and concentration of the analyte (Fig. 3).

Limits of Detection and Quantitation

The LOQ was determined as the lowest amount of analyte that was reproducibly quantified above the baseline noise following six injections. The resultant %RSD for these studies was ≤5.0%. The LOQ that produced the requisite precision and accuracy was found to be 0.26, 0.30, 0.38, 0.53, 0.85, 0.9, and 0.47 for Nicotine related substances (A, B, C, D, E, F and G), respectively. The LOD was determined based on signal-to-noise ratios and was determine using an analytical response of three times the background noise. The LOD for Nicotine related compounds were found to be 0.09, 0.10, 0.13, 0.18, 0.28, 0.30, and 0.16, respectively from Table 2 & 3.

Precision and Accuracy

The %RSD at LOQ concentration for Nicotine related substances was below 10.0%. The accuracy/recovery experiments were carried out Nicotine related substances at LOQ concentration in triplicate and the recoveries were found to be well within 90–110%, which demonstrates that the developed method can accurately measure the impurities present in Nicotine. The percentage recovery of Nicotine in assay method was within the limit (98–102) and recovery data shown for both methods in Table 4 & 5.

Robustness

To evaluate robustness of the developed method, the experimental condition were purposely altered and the robustness of the method was investigated in the method parameters such as the flow rate of the mobile phase, pH of Buffer and the column temperature (CT). The System suitability results were presented in Table 6. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators has proven that the method is robust and the data are summarized in Table 7.

Solution Stability

The %Recovery for assay of Nicotine during solution stability experiment was within limit (98–102%). No significant changes (<2%) were observed for the chromatographic responses for the solutions analyzed, relative to freshly prepared standards. The solution stability data are summarized in Table 8. The solution stability experiment data confirm that sample solutions used during assay and related compounds determination were stable up to 48 hours.

Specificity

No interference from any of the excipients was found at retention time of the Nicotine and its related compounds (fig 2-3). In addition, the Chromatogram of Nicotine in the sample solution was found identical to the Chromatogram received by the standard solution at the wavelengths applied for Assay method. Nicotine identified at retention time 5.6 min (m/z 163), Nornicotine 3.1 min (m/z 150), Anatabine 3.9 min (m/z 161), Myosmine 4.1 min (m/z 147), Anabasine 5.2 min (m/z 163), Cotinine 1.9 min (m/z 177), and β-Nicotyrine 6.9 min (m/z 159), nicotine N-oxide 0.7 min (m/z 179) (fig.4). These results demonstrate the absence of interference from other materials in the pharmaceutical formulations and therefore confirm the specificity of the proposed method.

Table 1: Linear Regression Data for Nicotine Related Substances

Concentration (µg/mL)	Nocotine	Nicotine Related substances peak area						
	Peak area	A	B	C	D	E	F	G
0.7	1633	1055	1256	1286	1993	987	197	765
1.8	4233	2765	3216	3244	5022	2745	688	2011
2.9	6801	4461	5411	5531	8126	4231	1022	3312
3.6	8465	5560	6792	6889	10631	5607	1269	4296
4.3	10099	6611	8012	8121	12533	6413	1516	5011
5.4	12374	8199	10155	10223	15833	8411	1904	6411
Correlation Coefficient	0.9997	0.9999	0.9998	0.9998	0.9995	0.9988	0.9986	0.9996
SLOPE	2302	1526	1901	1915	2967	1561	356	1205
RRF	-	0.66	0.83	0.83	1.29	0.68	0.15	0.52
%Y-intercept	1.2	0.9	1.5	2.1	3.4	2.8	1.4	2.2

Table 2: Results for Nicotine Related Compounds Precision at LOQ

Name of the Impurity	Limit of Quantitation							Mean	%RSD
Imp. A	Concentration	0.26 µg ml^{-1}							
	Area	403	413	427	404	419	411	413	2.2
Imp. B	Concentration	0.30 µg ml^{-1}							
	Area	571	544	567	581	533	575	562	3.4
Imp. C	Concentration	0.38 µg ml^{-1}							
	Area	721	733	711	752	735	719	729	2.0
Imp. D	Concentration	0.53 µg ml^{-1}							
	Area	1566	1556	1531	1547	1552	1578	1555	1.0
Imp. E	Concentration	0.85 µg ml^{-1}							
	Area	1402	1395	1421	1432	1438	1424	1419	1.2
Imp. F	Concentration	0.9 µg ml^{-1}							
	Area	326	302	314	334	316	327	320	3.6
Imp. G	Concentration	0.47 µg ml^{-1}							
	Area	562	573	591	547	564	553	565	2.8

Table 3: Results for Nicotine related compounds Precision at LOD

Name of the Impurity	Limit of Detection							Mean
Imp. A	Concentration	0.09 µg ml^{-1}						
	Area	100	132	112	126	101	104	113
Imp. B	Concentration	0.10 µg ml^{-1}						
	Area	153	182	167	188	183	175	175
Imp. C	Concentration	0.13 µg ml^{-1}						
	Area	246	233	253	217	223	249	237
Imp. D	Concentration	0.18 µg ml^{-1}						
	Area	488	533	528	507	503	541	517
Imp. E	Concentration	0.28 µg ml^{-1}						
	Area	451	403	476	487	442	467	454
Imp. F	Concentration	0.3 µg ml^{-1}						
	Area	112	124	101	128	102	114	114
Imp. G	Concentration	0.16 µg ml^{-1}						
	Area	192	177	183	162	194	207	186

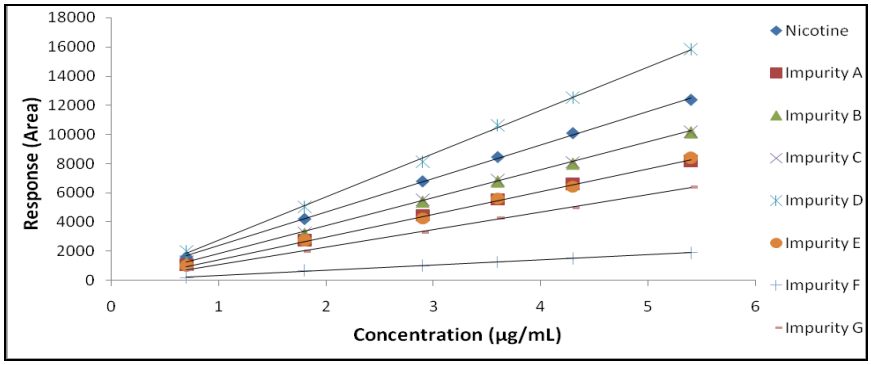

Fig. 2: Calibration plot for Nicotine impurities

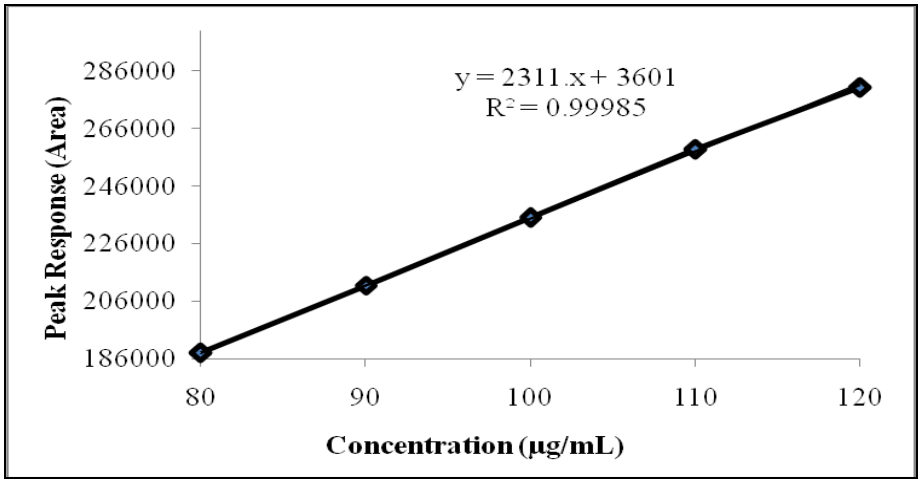

Fig. 3: Calibration plot for Nicotine from Assay method

Table 4: Results for % Recovery of Nicotine from assay method

% Recovery level	Prep.	Imp. A	Imp. B	Imp. C	Imp. D	Imp. E	Imp. F	Imp. G
10% (LOQ)	1	95.4	98.6	97.3	99.4	103.6	103.5	98.8
	2	99.1	95.1	99.7	95.3	96.4	98.3	102.8
	3	102.2	100.3	96.2	101.4	99.2	104.3	99.1
25%	1	99.9	103.2	100.2	100.2	102.2	95.1	96.5
	2	95.9	102.3	102.6	95.7	100.7	103.4	103.4
	3	103.4	98.2	97.1	97.8	96.4	99.7	102.2
50%	1	101.3	103.2	98.8	96.3	102.3	100.3	103.6
	2	104.1	99.1	102.4	99.8	102.1	103.2	96.4
	3	99.6	102.1	100.3	103.4	99.8	99.3	99.8
75%	1	95.1	101.9	103.4	100.2	96.2	96.6	102.2
	2	103.5	96.7	98.3	103.3	95.7	103.4	99.9
	3	97.4	99.2	96.1	101.2	100.4	98.8	102.1
100%	1	96.9	103.3	99.5	96.5	102.4	103.5	99.4
	2	99.9	100.6	102.8	100.7	102.5	103.2	97.6
	3	101.8	96.3	96.4	96.5	103.2	100.1	95.9
125%	1	97.3	98.5	103.6	100.2	99.1	99.6	100.4
	2	98.9	99.9	98.2	100.8	95.3	97.5	103.7
	3	100.3	96.1	96.1	102.3	102.1	99.9	103.2
150%	1	99.3	97.6	95.8	96.7	100.9	104.5	100.7
	2	100.4	102.3	99.9	98.2	96.2	97.3	99.4
	3	96.3	100.1	101.3	95.9	97.2	99.1	103.8
Mean		99.4	99.7	99.3	99.1	99.7	100.5	100.5
%RSD		2.7	2.5	2.6	2.6	2.8	2.8	2.5

Table 5: Results for % Recovery of Nicotine from assay method

Prep.	% Recovery level				
	80%	90%	100%	110%	120%
1	99.2	99.9	100.1	98.5	100.2
2	99.8	100.3	99.7	99.8	99.2
3	100.3	99.4	98.8	99.1	99.7
Mean	99.8	99.9	99.5	99.1	99.7
%RSD	0.6	0.5	0.7	0.7	0.5

Table 6: System suitability Results for robustness study

Robust parameter	System Suitability Results				
	RT[a]	Tailing[b]	Plates[c]	Resolution[d]	% RSD[e]
Normal	5.6	1.4	15,634	2.7	0.62
Flow rate – 0.55 ml-1	5.6	1.3	16,031	2.6	0.77
Flow rate – 0.65 ml-1	5.7	1.5	15,339	2.9	0.81
Buffer pH – 8.8	5.7	1.4	15,844	2.7	0.73
Buffer pH – 9.0	5.6	1.4	15,511	2.7	0.71
CT – 33°C	5.7	1.4	15,976	2.7	0.52
CT – 37°C	5.6	1.4	15,488	2.7	0.84

a Retention time (RT - min) for Nicotine Peak; b Tailing factor for Nicotine Peak; c Plate count for Nicotine Peak; d Resolution between Impurity G and Nicotine peaks; e % RSD for Nicotine peak from 6 replicate injections

Table 7: % Assay for Nicotine and % content of its related compounds results from robustness study

Robust parameter	Assay	Related Compounds						
	Nicotine	Imp. A	Imp. B	Imp. C	Imp. D	Imp. E	Imp. F	Imp. G
Normal	99.8	99.4	99.7	99.3	99.1	99.7	100.5	100.5
	% Difference							
Flow rate – 0.55 ml-1	0.3	3.1	2.2	3.3	1.3	2.4	1.7	3.8
Flow rate – 0.65 ml-1	0.2	2.8	4.2	1.8	2.7	3.8	3.4	2.1
Buffer pH – 8.8	0.5	2.9	3.5	2.6	3.8	1.9	2.8	3.6
Buffer pH – 9.0	0.4	3.7	2.6	3.6	4.2	3.2	2.2	2.2
CT – 33°C	0.6	2.7	3.1	2.9	3.3	1.6	1.9	2.6
CT – 37°C	0.4	4.1	1.9	1.5	2.2	2.4	3.7	3.1

Table 8: % Assay for Nicotine and % content of its related compounds results from Solution Stability

Solution Stability study hour	Assay	Related Compounds						
	Nicotine	Imp. A	Imp. B	Imp. C	Imp. D	Imp. E	Imp. F	Imp. G
Initial (0 hour)	99.9	98.8	99.2	97.5	100.5	100.8	98.6	99.7
	% Difference							
1 hour	0.1	1.8	2.2	2.1	2.4	3.2	2.6	2.3
6 hours	0.1	2.6	4.2	2.2	1.2	1.6	3.2	1.6
12 hours	0.2	3.2	3.5	3.1	1.1	1.7	1.5	1.1
24 hours	0.2	2.1	2.6	2.4	2.5	3.2	1.8	2.8
48 hours	0.3	2.2	3.1	1.3	1.4	2.9	2.9	1.8

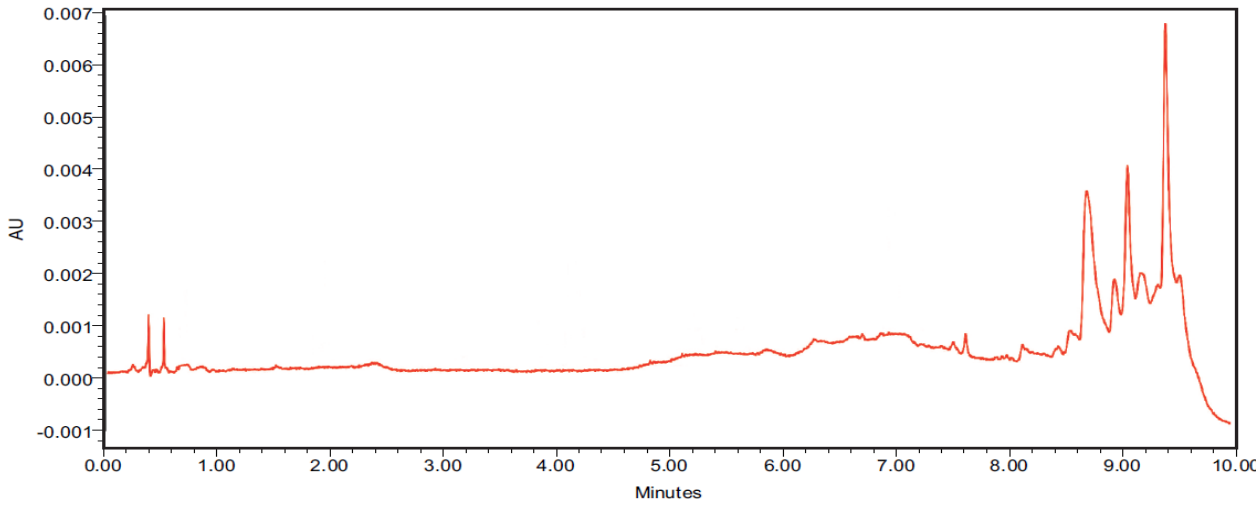

Fig. 4: Typical chromatograms for Placebo

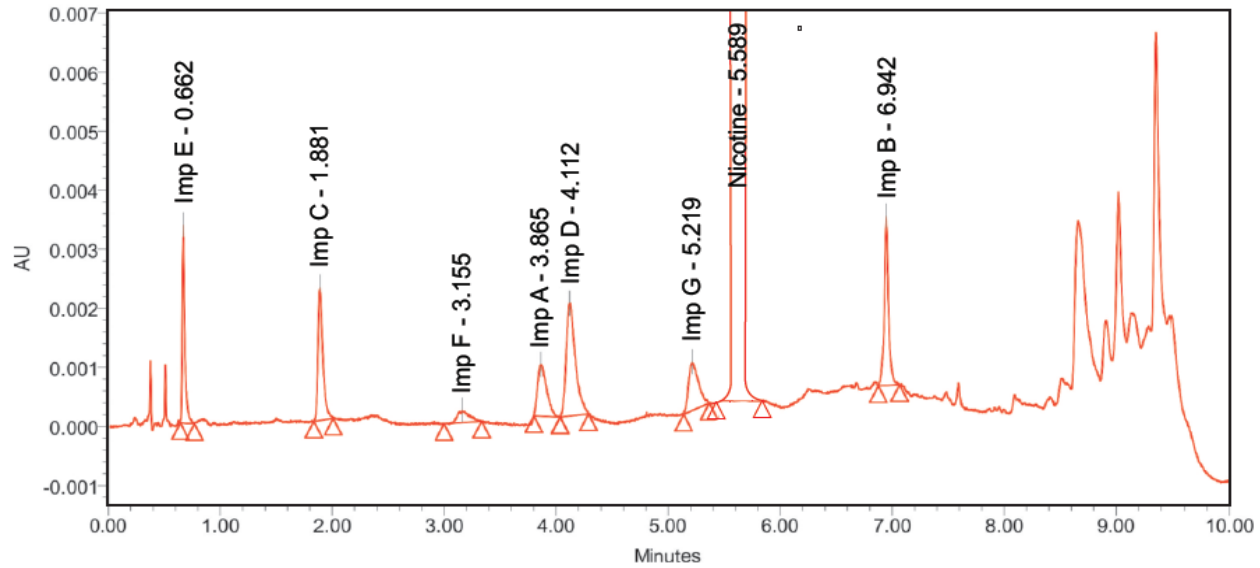

Fig. 5: Typical chromatograms for Spike sample

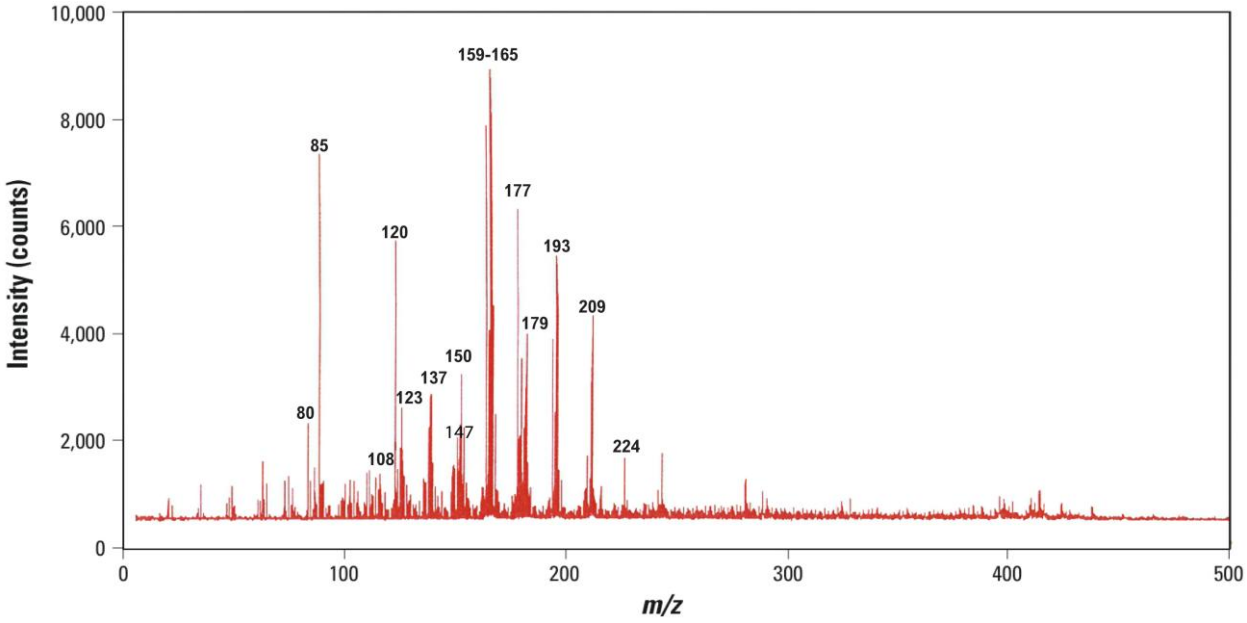

Fig. 6: Typical chromatograms of mass spectrum for Nicotine and tits related compounds from Spike sample

CONCLUSIONS

Unlike the gas chromatographic and HPLC procedures, the instrument is simple and affordable. The importance lies in the chemical reactions upon which the procedures are based rather than upon the sophistication of the instrument. This aspect of UPLC analysis is of major interest in analytical pharmacy since it offers distinct possibility in the quantification of a particular component in complex dosage formulations. The reagents utilized in the proposed methods are cheaper, readily available and the procedures do not involve any critical reaction conditions or tedious sample preparation. The method is unaffected by slight variations in experimental conditions such as pH and reagent concentration. Moreover, the methods are free from interference by common additives and excipients. The wide applicability of the new procedures for routine quality control is well established by the quantification of Nicotine related substances in pure form and in pharmaceutical preparations.

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