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Development and validation of UPLC methods for the determination of 4-Nitro phthalimide, 5-Bromo phthalide and 5-Amino phthalide, 4-Amino phthalimide contents in Citalopram hydrobromide drug substance

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ABSTRACT

Simple and sensitive Ultra Performance Liquid Chromatography (UPLC) methods were developed, optimized and validated for the determination of 4-Nitro phthalimide, 5-Bromo phthalide and 5-Amino phthalide, 4-Amino phthalimide contents in Citalopram hydrobromide drug substance. All these impurities have been originated from 5-cyanophthalide, a key raw material which is used in the synthetic process of Citalopram. The chromatographic separations were achieved on Acquity UPLC HSS C18 SB, 1.8µm (100 mm x 2.1mm) column maintained at temperature 40°C for the quantification of 4-Nitro phthalimide and 5-Bromo phthalide with a simple mobile phase consisted of 0.01M phosphate buffer at a pH 5.5 and acetonitrile in gradient mode at a flow rate of 0.10 ml/min and monitored at 245nm. Quantification of 5-Amino phthalide and 4-Amino phthalimide were achieved on the same column maintained at temperature 30°C and with same buffer at pH 3.0 and acetonitrile as mobile phase in gradient mode at a flow rate of 0.15 ml/min. These impurities were monitored at 205nm. The analytic methods have been demonstrated through validation experiments. The achieved limit of detection (LOD) values were 1.0, 1.6 ,1.8 and 1.2 ppm, limit of quantification (LOQ) values were 3.0, 5.0, 5.5 and 3.6 ppm and the average accuracy values were 97.9, 102.8, 102.8 & 103.7% for 4-Nitro phthalimide, 5-Bromo phthalide, 5-Amino phthalide and 4-Amino phthalimide respectively.

Key words: Citalopram hydrobromide; 4-Nitro phthalimide; 5-Bromo phthalide; 5-Amino phthalide;4-Amino phthalimide

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INTRODUCTION

Citalopram is an antidepressant drug of the selective serotonin reuptake inhibitor (SSRI) class. It offers similar therapeutic efficacy and a more favourable tolerability profile than the tricyclic antidepressants[1] and was originally created in 1989[2]. Citalopram hydrobromide is a racemic mixture and is chemically designated as 1-[3-(dimethylamino)propyl]-1-(4-fluoro-phenyl)-1,3-dihydroisobenzofuran-5-carbonitrile, monohydrobromide with molecular formula $C_{20}H_{21}FN_2O.HBr$ and having molecular weight 405.30. The chemical structure of Citalopram hydrobromide is given in Fig.1.

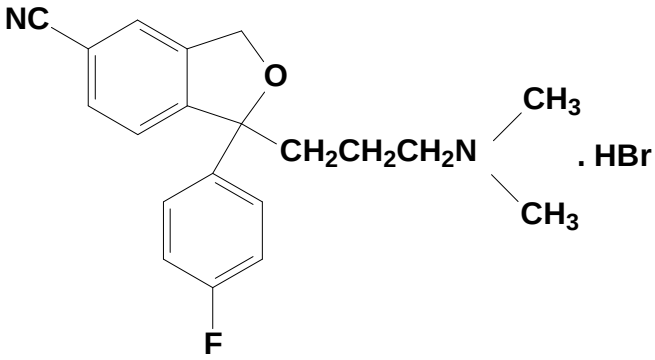

Fig 1 Chemical structure of Citalopram hydrobromide

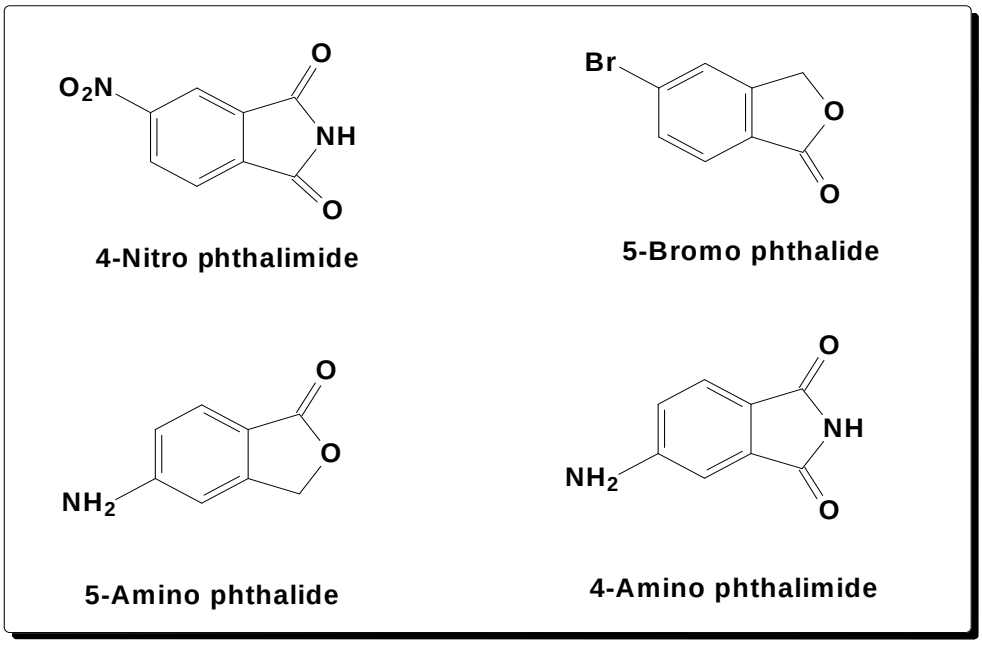

Fig 2 Chemical structures of 4-Nitro phthalimide, 5-Bromo phthalide, 5-Amino phthalide and 4-Amino phthalimide

5-cyanophthalide, a key synthetic raw material used in the preparation of Citalopram. The impurities, 4-Nitro phthalimide (4-NPM), 5-Bromo phthalide (5-BP), 5-Amino phthalide (5-AP) and 4-Amino phthalimide (4-APM) may arise during the synthetic process in the preparation of 5-cyanophthalide. Further, these impurities may carryover in drug substance along with its related substances [7]. These are the impurities which can come under genotoxic category based on their structural alerts [3,4]. The chemical structures of four impurities are given in Fig 2.

As per regulatory aspects and EMEA guidance, testing will be required for all potential impurities from an API's synthetic route possessing structural elements that are alerting for genotoxicity potential using the well-established Ames test for mutagenicity. Citalopram is generally considered safe and well-tolerated in the therapeutic dose range of 10 to 60 mg/day [5]. The impurities should not be present in drug substance or should be less than 25 ppm as per TTC approach based on daily dosage of drug substance [3, 4, 6]. Moreover, there is no specific information is available in the literature on toxicity of 5-Bromophthalide confirming either its carcinogenicity or mutagenicity. Further USP related compound H[7] of citalopram may originates from 5-Bromophthalide. Hence, controlling of 5-Bromophthalide along with three impurities to be monitored in citalopram hydrobromide drug substance. For the sensitivity of impurity levels, we have chosen an analytic liquid chromatographic technique, UPLC instead of HPLC as this technology takes full advantage of chromatographic principles to run separations using columns packed with smaller particles and /or higher flow rates for increased speed, with superior resolution and sensitivity. To the best of our knowledge, determination of these impurities by UPLC has not been reported in literature till date. This paper describes the development, optimization and validation of UPLC methods for these impurities.

MATERIALS AND METHODS

Experimental

Chemicals, reagents and samples

Samples of citalopram hydrobromide and its related substances were procured in APL Research Centre (a unit of Aurobindo Pharma Ltd., Hyderabad.). Analytical grade (AR grade) potassium dihydrogen orthophosphate, orthophosphoric acid, potassium hydroxide, gradient grade acetonitrile and methanol were procured from E.Merck; India. Highly pure milli-Q water was prepared by using millipore purification system.

Ultra performance liquid chromatography (UPLC)

Chromatographic separations were performed on UPLC system with Acquity binary solvent manager, sample manager and PDA detector with Empower pro data handling system [Waters Corporation, MILFORD, MA 01757, USA].

Chromatographic conditions for 4-NPM and 5-BP (Method-I)

The mobile phase A was 0.01M potassium dihydrogen orthophosphate solution adjusted to pH 5.5 using potassium hydroxide solution and mobile phase B was acetonitrile. The analysis was carried out on Acquity UPLC HSS C18 SB, 100 mm long, 2.1 mm i.d., 1.8 µm particle diameter column(Make: Waters), maintained at temperature 40°C. Mobile phase was flushed through the column at a flow rate of 0.10 ml/min and pump was in gradient mode and the program was as follows: Time (min)/ A (v/v): B(v/v); $T_{0.01}$/40:60, T_6/40:60, T_7/25:75,T_{30}/25:75,$T_{30.5}$/40:60,

T_{35}/40:60. The run time for the standard was 7 min (with initial gradient) and the sample was 35 min. The injection volume was 8 µl and the analytes were monitored at 245 nm. A mixture of water : acetontrile in the ratio 70:30 v/v was used as diluent. The retention times of 4-NPM and 5-BP are at about 3.3 and 4.3 min respectively. The resolution between 4-NPM and 5-BP is not less than 4.0 set as system suitability purpose.

Chromatographic conditions for 5-AP and 4-APM (Method-II)

The mobile phase A was 0.01M potassium dihydrogen orthophosphate solution adjusted to pH 3.0 using diluted orthophosphoric acid solution and mobile phase B was acetonitrile. The analysis was carried out on Acquity UPLC HSS C18 SB, 100 mm long, 2.1 mm i.d., 1.8 µm particle diameter column maintained at temperature 30°C. Mobile phase was flushed through the column at a flow rate of 0.15 ml/min and pump was in gradient mode and the program was as follows: Time (min)/ A (v/v): B(v/v); $T_{0.01}$/90:10, T_7/55:45, T_9/30:70,T_{15}/30:70,$T_{15.5}$/90:10, T_{20}/90:10. The run time for the standard and sample was 20 min. The injection volume was 8 µl and the analytes were monitored at 205 nm. A mixture of water : methanol in the ratio 60:40 v/v was used as diluent. The retention times of 5-AP and 4-APM are at about 3.5 and 4.8 min respectively. The resolution between 5-AP and 4-APM is not less than 8.0 set as system suitability purpose.

Preparation of solutions

For 4-NPM and 5-BP

Standard solution

Accurately weigh and transfer 25mg each of 4-NPM and 5-BP standards into a 100 mL volumetric flask, add 5 mL of acetonitrile and sonicated to dissolve, make up to the volume with diluent. Dilute 5 mL of this solution to 100 mL with diluent, and then dilute 5 mL of this solution to 50 mL with diluent and further dilute 5 mL of this solution to 50 mL with diluent. Filter through the 0.22 µ porous membrane.

Sample solution

Accurately weigh and transfer 250 mg of sample into a 50 mL volumetric flask, add 30 mL of diluent and dissolve by sonicating and make up to the volume with diluent. Filter through the 0.22 µ porous membrane.

For 5-AP and 4-APM

Standard solution

Accurately weigh and transfer 25mg each of 5-AP and 4-APM standards into a 100 mL volumetric flask, add 50 mL of methanol and sonicated to dissolve, make up to the volume with methanol. Dilute 5 mL of this solution to 100 mL with diluent, and then dilute 5 mL of this solution to 50 mL with diluent and further dilute 5 mL of this solution to 50 mL with diluent. Filter through the 0.22 µ porous membrane.

Sample solution

Accurately weigh and transfer 250 mg of sample into a 50 mL volumetric flask, add 30 mL of diluent and dissolve by sonicating and make up to the volume with diluent. Filter through the 0.22 µ porous membrane.

RESULTS AND DISCUSSION

Method development and optimization

The objective of this work is to determine very low level concentrations of these four impurities in a single method by using ultra performance liquid chromatography. Method development was initiated by using 0.1% orthophosphoric acid buffer as mobile phase A and acetonitrile as mobile phase B. Gradient programme was run on Acquity BEH C18, 1.7µm (100mm x 2.1mm) column. In this trial, four impurity peaks were resolving with each other, but citalopram peak was interfering with 5-bromo phthalide peak. After that many trails were performed to resolve this problem using different stationary phases like BEH phenyl, C8 and C18 with different phosphate buffers at various pH (2.0-7.0) by varying different gradient programme. In these trails, 0.01M phosphate buffer at pH 3.0, using Acquity UPLC HSS C18 SB, 1.8 µm (100 mm x 2.1 mm), 5-AP and 4-APM peaks were eluted with better resolution and peak shapes and further any of related substances of citalopram and citalopram were not interfering, while the 4-NPM and 5-BP peaks tailing was observed more and also satisfactory resolution was not observed between the peaks. But at pH 5.5, 4-NPM and 5-BP peaks were eluted with better resolution and peak shapes, further any of related substances of citalopram and citalopram was also not interfering. More over the spectral characteristics of 5-AP & 4-APM were similar i.e both are exhibiting wavelength maxima or better response at about 205nm, whereas 4-NPM & 5-BP are at about 245nm. Henceforth, based on these experiments; two separate methods were developed for each of two analytes as described under method-I and method-II. Further, these two methods were optimized with performing various gradient programmes at different column oven temperatures. Finally satisfactory separations were achieved on chromatographic conditions which have been mentioned in method-I and method-II.

Method validation

The optimized methods were established through the validation experiments per the ICH guidelines[8], individually in terms of specificity or selectivity, LOD, LOQ, linearity, accuracy, precision(system precision, method precision and intermediate precision or ruggedness) and stability of sample solution.

Specificity

Specificity is the ability of the method to determine the individual analyte in the presence of other related substances of drug substance. For specificity determination, all the related substances of citalopram including 4-NPM, 5-BP, 5-AP and 4-APM solutions were prepared individually and injected into UPLC as per methodologies to confirm the retention times. After that diluents, solutions of citalopram hydrobromide drug substance, citalopram hydrobromide drug substance spiked with 4-NPM and 5-BP (spiked sample-1), citalopram hydrobromide drug substance spiked with 5-AP and 4-APM (spiked sample-2), citalopram hydrobromide drug substance spiked with all related substances of citalopram including 4-NPM and 5-BP (all spiked sample-1), and citalopram hydrobromide drug substance spiked with all related substances of citalopram including 5-AP and 4-APM (all spiked sample-2) were prepared and injected into UPLC as per methodologies and confirm any co-elution with analyte peaks from respective diluents, any of related substances peaks and the peak homogeneity was verified for each analyte using waters empower software and found to be pure (purity angle should be less than purity threshold). The typical UPLC chromatograms of Citalopram hydrobromide spiked with 4-Nitro

phthalimide / 5-Bromo phthalide and Citalopram hydrobromide spiked with all related substances of Citalopram including 4-Nitro phthalimide / 5-Bromo phthalide, Citalopram hydrobromide spiked with 5-Amino phthalide / 4-Amino phthalimide and Citalopram hydrobromide spiked with all related substances of Citalopram including 5-Amino phthalide / 4-Amino phthalimide are shown in the Fig 3 and Fig 4 respectively. The specificity experiments data is given in Table 1.

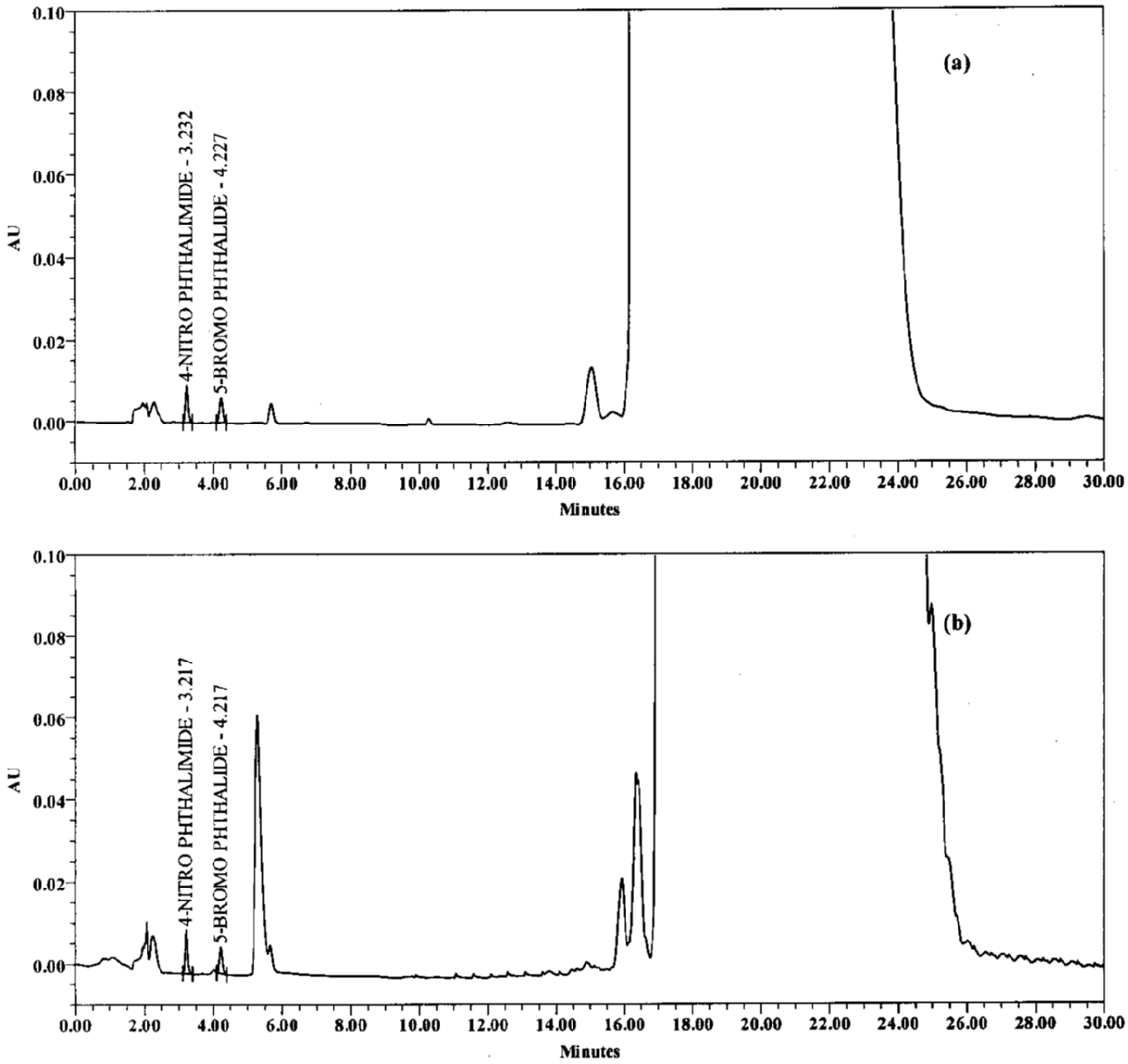

Fig. 3 Typical representative UPLC chromatograms of Citalopram hydrobromide drug substance spiked with 4-Nitro phthalimide and 5-Bromo phthalide (a) and Citalopram hydrobromide drug substance spiked with phamacopoeial impurities including 4-Nitro phthalimide and 5-Bromo phthalide (b)

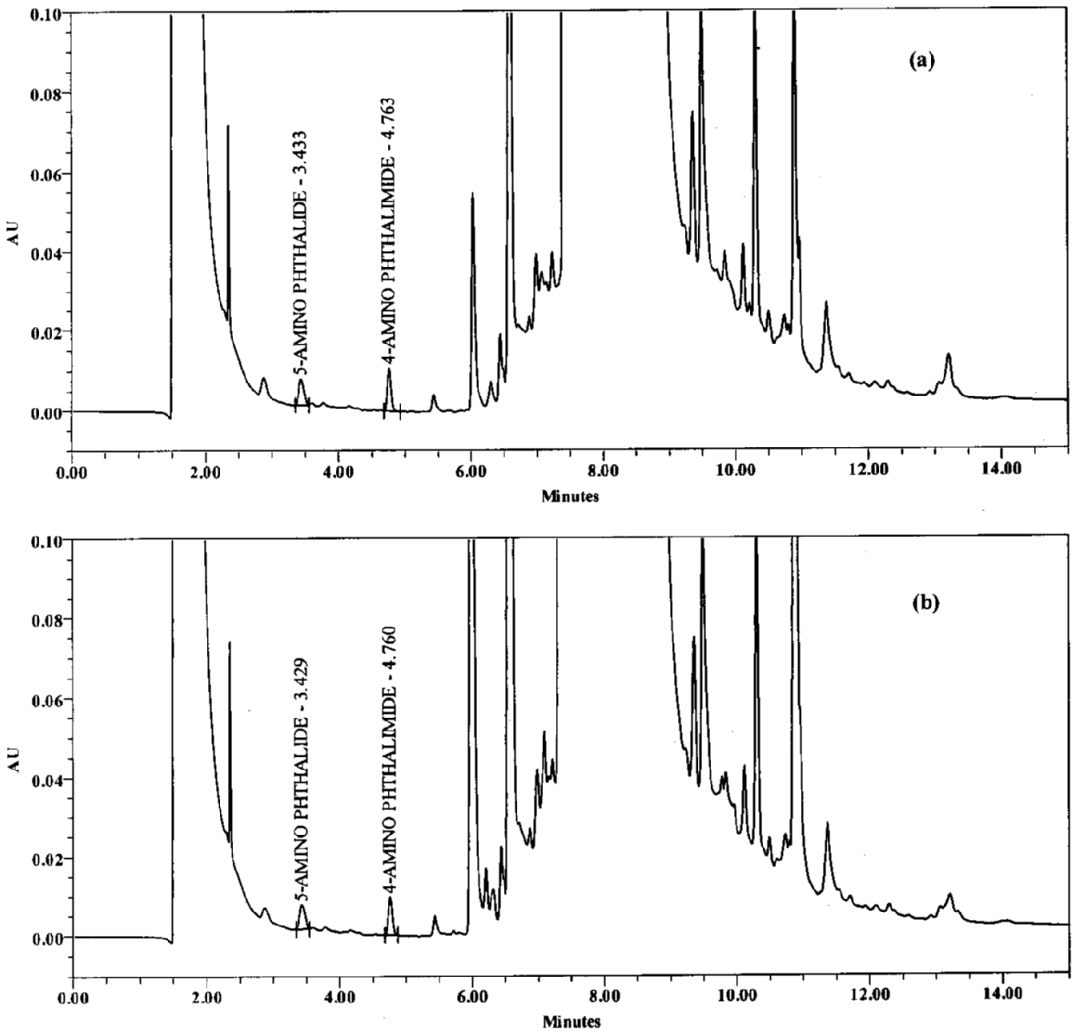

Fig. 4 Typical representative UPLC chromatograms of Citalopram hydrobromide drug substance spiked with 5-Amino phthalide and 4-Amino phthalimide (a) and Citalopram hydrobromide drug substance spiked with phamacopoeial impurities including 5-Amino phthalide and 4-Amino phthalimide (b)

Table 1 Specificity table

			Purity angle	Purity threshold		Purity angle	Purity threshold
Method-I	4-Nitro phthalimide	Spiked sample-1	0.185	0.453	All spiked sample-1	0.240	0.922
	5-Bromo phthalide		0.229	0.626		1.054	1.549
Method-II	5-Amino phthalide	Spiked sample-2	0.616	0.675	All spiked sample-2	0.624	0.676
	4-Amino phthalimide		0.372	0.488		0.382	0.472

LOD and LOQ

For determining the limit of detection (LOD) and limit of quantification (LOQ), signal to noise ratio method was adopted. Signal to noise ratio values were considered from the standard solutions of 4-NPM, 5-BP, 5-AP and 4-APM injected into UPLC as per method-I and II. LOD and LOQ values were predicted by using standard concentration(S) and signal to noise ratio (SN) method by using the formula 3.3 x S/SN for LOD and 10 x S/SN for LOQ. LOQ values were predicted as 2.9, 4.5, 4.8 and 3.3 ppm and LOD values were predicted as 1.0, 1.5, 1.6 and 1.1 ppm for 4-NPM, 5-BP, 5-AP and 4-AMP respectively. The LOD and LOQ solutions were prepared at about predicted concentration levels and analyzed six times for checking the precision and the results are tabulated in Table 2.

Linearity

The linearity of the detector was determined by preparing a series of solutions using 4-NPM, 5-BP, 5-AP and 4-APM at concentration levels from about LOQ level, 10 to 40 ppm. The data was subjected to statistical analysis by using a linear-regression model. The statistical evaluations like slope, intercept, STEYX and correlation coefficient values of linearity data is given in Table 2.

Table 2 Statistical data of linearity and LOD/LOQ

	Method-I		Method-II	
Statistical parameters	4-Nitro phthalimide	5-Bromo phthalide	5-Amino phthalide	4-Amino phthalimide
No. of points covered	5	5	5	5
Concentration range (ppm)	3.0-40.3	4.9-39.5	5.5-39.3	3.6-40.8
Slope	1984	1724	1503	1631
Intercept	-152.11	-800.37	-87.05	116.44
STEYX	697.9	501.0	226.9	599.6
Correlation coefficient	0.9997	0.9998	0.9999	0.9997
Limit of detection(ppm)	1.0	1.6	1.8	1.2
Limit of quantification(ppm)	3.0	5.0	5.5	3.6
Precision for Limit Of Detection (%R.S.D)	17.9	8.9	10.1	10.2
Precision for Limit Of Quantification (%R.S.D)	1.4	0.4	1.4	1.2

Accuracy

Accuracy of the methods was performed by recovery experiments using standard addition technique. The recoveries were determined by spiking 4-NPM, 5-BP, 5-AP and 4-APM at four different levels at LOQ level, 20, 30 and 40 ppm into Citalopram hydrobromide drug substance. These samples were prepared as per respective test procedure and analyzed in triplicate and the percentage recoveries were calculated. The % recovery values for analytes ranged from 95.5 - 99.5, 101.0 - 105.0, 100.6 - 103.8 and 98.4 - 106.9 and the average % recovery of four levels (twelve determinations) were 97.9, 102.8, 102.8 and 103.7. The fully validated accuracy results are shown in Table 3.

Table 3 Accuracy data

Accuracy (Average of 3 replicates)	4-Nitro phthalimide			
	LOQ level	20ppm level	30ppm level	40ppm level
Added (ppm)	2.996	20.084	29.774	39.901
Recovered (ppm)	2.981	19.975	28.867	38.122
Recovery (%)	99.5	99.5	97.0	95.5
R.S.D(%)	1.6	2.6	2.0	1.0
5-Bromo phthalide				
Added (ppm)	4.935	19.663	29.150	39.064
Recovered (ppm)	4.983	20.369	29.547	41.028
Recovery (%)	101.0	103.6	101.4	105.0
R.S.D(%)	0.8	0.2	1.1	0.3
5-Amino phthalide				
Added (ppm)	5.483	19.736	29.486	38.787
Recovered (ppm)	5.518	20.494	30.390	40.173
Recovery (%)	100.6	103.8	103.1	103.6
R.S.D(%)	0.5	0.8	0.5	1.0
4-Amino phthalimide				
Added (ppm)	3.662	20.479	30.595	40.247
Recovered (ppm)	3.604	21.883	32.256	41.827
Recovery (%)	98.4	106.9	105.4	103.9
R.S.D(%)	0.5	1.7	0.5	0.3

Precision

System precision was demonstrated by preparing the standard solutions of individual analytes as per respective methodologies and analyzing six replicate injections. Repeatability is the intra-day variation (method precision) and the intermediate precision is the inter-day variation (ruggedness). Method precision was demonstrated by preparing six sample solutions individually using a single batch of citalopram drug substance spiked with 4-NPM / 5-BP and 5-AP / 4-APM separately at a known concentration level (about 25ppm) as per respective methodologies and injected each solution and determined the content of analytes. Ruggedness was demonstrated by following the same procedure as mentioned for method precision experiment by another analyst using another lot of column, different system on different day. Achieved results like %RSD and 95% confidence interval for six determinations and cumulative of twelve determinations are summarized in Table 4.

Solution stability

For the determination of stability of the sample solutions, sample solutions were spiked with 4-NPM / 5-BP and 5-AP / 4-APM at known concentration levels as per respective methodologies and analyzed initially and after every one hour time interval upto 12 hours by keeping the sample cooler temperature at 25°C. The % difference in the peak areas of analytes from initial to different time interval was found to be less than 5.0. The results concluded that sample solutions are stable for 12 hours at room temperature.

Table 4 Precision data

Experiment	4-Nitro phthalimide		5-Bromo phthalide		5-Amino phthalide		4-Amino phthalimide	
System precision (n=6) %RSD 95% Confidence interval(CI)	1.8 ±955		2.4 ±974		0.2 ±83		1.3 ±585	
	MP	IP	MP	IP	MP	IP	MP	IP
Sample-1	24.9	25.0	24.9	24.7	25.6	25.2	26.3	25.7
Sample-2	24.8	24.7	24.9	25.1	25.9	25.1	26.5	25.7
Sample-3	24.7	24.7	24.6	24.7	25.6	25.0	27.3	25.9
Sample-4	24.6	24.8	24.7	24.9	26.2	25.3	26.0	25.6
Sample-5	25.0	24.8	24.7	25.0	25.7	25.4	26.3	25.6
Sample-6	25.2	25.0	24.7	25.0	25.8	25.4	26.3	25.9
Mean	24.9	24.8	24.8	24.9	25.8	25.2	26.5	25.7
SD	0.22	0.14	0.12	0.17	0.23	0.16	0.45	0.14
%RSD	0.9	0.6	0.5	0.7	0.9	0.6	1.7	0.5
95% Confidence interval(CI)	±0.231	±0.147	±0.126	±0.178	±0.241	±0.168	±0.472	±0.147
Overall mean	24.9		24.8		25.5		26.1	
Overall SD	0.2		0.2		0.4		0.5	
Overall %RSD	0.7		0.6		1.4		1.9	
Overall 95% Confidence interval(CI)	±0.108		±0.102		±0.222		±0.311	

MP : Method precision; IP: Intermediate precision (or) Ruggedness

CONCLUSION

Two UPLC chromatography methods were developed, optimized and validated for the determination of 4-Nitro phthalimide / 5-Bromo phthalide and 5-Amino phthalide / 4-Amino phthalimide contents in Citalopram hydrobromide drug substance and the results of various validation parameters demonstrated that the methods are specific, sensitive, linear, precise and accurate and these methods can be introduced into routine use.

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