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Original Article

RP-HPLC Single Advance Method with Validation Studies for Imipramine Hydrochloride and its Intermediates

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ABSTRACT

Background: A quantifiable method has been developed to determine the key starting material, its intermediates and known impurities in the presence of Imipramine Hydrochloride in the final API of a synthetic laboratory sample. This newly developed Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method is, facile, specific and reliably practical. **Materials and Methods:** The separation column employed was Inertsil ODS-3 C_{18} with a mobile phase comprised of (A) 0.1% OPA (pH adjusted to 3.2) and (B) acetonitrile. Mobile phase A is a 100% buffer solution. Acetonitrile was used as mobile phase "B" and mobile phase B was used as 100% organic Solvent. The analytes were detected at 220 nm using a UV detector where the flow rate of the mobile phase was kept at 1.0 mL min^{-1} and the gradient program was set as T/% B: 0/30, 5/30, 10/80, 12/80, 15/30, 20/30 with a fixed flow rate of 1.0 mL min^{-1}. **Results:** According to the regulatory standards advised by the ICH, the performance of this method is best agreed upon by all the important parameters. The approach used in the present work can be used for process development and determining the purity of associated compounds of Imipramine Hydrochloride, key starting material (2,2-dinitro-1,2-diphenylene ethane), intermediate-1 (2-2-diamino-1,2-diphenyl ethane diphosphate) and intermediate-2 (iminodibenzyl) all in a single method. **Conclusion:** This newly developed and validated method will save time and create ease by preventing the development of different methods for analyzing intermediates and impurity profiling.

Keywords: Imipramine Hydrochloride, API, RP-HPLC, UV detector, Chromatogram, Validation.

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INTRODUCTION

Imipramine Hydrochloride is used to treat depression along with certain anxiety disorders. It is a potent antidepressant agent and is used in veterinary medicine. In humans, the daily dosage can range from 25 mg/day. Imipramine Hydrochloride is also treated with xylazine to make pharmacologic ejaculation in stallions.[1-5] In human blood levels, 150-250 mg mL^{-1} of Imipramine Hydrochloride and its metabolite desipramine represent antidepressant efficacy. The therapeutic calming effect of the Imipramine Hydrochloride drug is relatively lower than the similar drugs in its family. Hence it is effectively used in the treatment of major mental ailments like bipolar depression, dysthymia, agoraphobia, attention deficit and panic disorders.

An API's synthesis involves multiple steps and other materials including solvents, catalysts, Key Starting Material (KSM), intermediates and other reagents. As a precursor, intermediates and KSM play a critical role in the manufacturing of API. KSM is an initial raw material of a drug substance utilized in the synthesis of a drug that serves as an important substantial structural fragment of a drug.[6-9] Further, the KSM is a precursor to convert it into API via intermediate through multistep chemical reactions. The conversion of intermediate to active ingredients can be achieved by further refinement. Therefore, the synthesis of API is usually a complicated and multi-step process that involves numerous chemical transformations and operations for its various intermediates with different physical and chemical properties. During the preparation of the intermediate, the undesired reactions lead to the formation of impurities. Therefore, the purity checks and impurity profiling of starting material and intermediates are important aspects in the synthesis of drug molecules.[10]

Several analytical methods are available for the determination of Imipramine Hydrochloride and its related substances. As S. Strano-Rossi reported, the method for quantification in food supplements reports the limits of detection of 1 to 25 µg g^{-1} and limits of quantification of 50 µg g^{-1} of all compounds. In that, the

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linearity ranges from 50 to 2000 µg g^{-1} for all the analytes with correlation coefficients>0.99.[11] Whereas S. Lee has developed a liquid chromatography-electrospray ionization-tandem mass method and validated it for the determination of 38 compounds simultaneously where a C18 reversed-phase column has been employed using as a buffer aqueous 2 mM ammonium formate and acetonitrile as a solvent.[12] The Limits of Detection (LOD) observed for this method are 0.004 to 0.455 µg mL^{-1} and the quantification limits were observed in the range of 0.012 µg mL^{-1} to 1.5 µg mL^{-1}. We developed a new RP-HPLC technique that can determine the KSM, intermediates, impurities and final API of a finished molecule, in a single method.[13] In that, we present a single method for the analysis of Imipramine Hydrochloride and all known impurities along with other components involved in its synthesis process. For that, we developed a single method that aims to enhance efficiency, short method duration with minimal resources. In this newly developed method, we studied the parameters such as linearity, the limit of quantification, the limit of detection, precision, accuracy, recovery, robustness and specificity and validated them as per ICH guidelines.[14-16]

However, we have not performed the synthesis work involved in obtaining the final product, but we have particularly opted for the four-step synthetic route in which the first step is the conversion of p-Nitrotoluene into 2,2'-Dintro-1,2-diphenylethane (c) in the presence of isoamyl formate. The -NO_2 group of 2,2'-Dintro-1,2-diphenylethane was reduced using Raney Ni as a catalyst to obtain 2,2'-Diamino-1,2-diphenylethane diphosphate (b). In the third step, the compound (b) was then heated at a high temperature to convert it into iminodibenzyl (a). In the fourth step, iminodibenzyl was treated with 3-Chloro-N,N-dimethyl propyl-1-amino as a base and finally, Imipramine Hydrochloride (a) was obtained by the acidic treatment with HCl.

MATERIALS AND METHODS

Materials

The KSM was purchased from Fisher Scientific whereas Intermediates, Final API and all other chemical solvents were purchased from Merck which comes along with a Certificate of Analysis where the finest purity of chemical compound is defined. The KSM, intermediates and API samples were injected separately at different concentrations (ppm) to confirm the retention time and monitor the value of the area of every individual chromatogram (validation parameters) hence it was observed that there was no degradation in any of the chromatograms was in the 20 min of run time. Also, in the chromatogram of mix system suitability, no degradation was observed in the intraday analysis because the fresh solution was prepared and injected. It is not recommended to store the system suitability stock solution for a longer time however during analysis it was observed that mixed SST solution was only stable for 24 hr. when stored at 5°C. The same mix SST was injected after 24 hr, degradation of the API and intermediates started and many unknown peaks (byproducts) were observed. Whereas the individual standard stock solution of KSM, intermediates and API of different concentrations was stable for more than 30 Days when stored at 5°C. 2,2-dinitro-1,2-diphenylene ethane (KSM) was procured from Fisher Scientific. The intermediates 2-2-diamino-1,2-diphenyl ethane diphosphate and iminodibenzyl, the API Imipramine Hydrochloride and the HPLC grade acetonitrile, methanol, orthophosphoric acid (88%) were procured from Merck, India. Milli Q water was purchased from Millipore India.

Methods

Preparation of mobile phase

The mobile phase "A" consists of 1.0 mL orthophosphoric acid in 1 L Milli Q water with pH adjusted to 3.2 using triethyl amine and filtered through 0.45 µ filter paper. Hence the mobile phase A is 100% buffer solution. Acetonitrile was used as mobile phase "B" and mobile phase B was used as 100% organic solvent. Both the mobile phase solutions were stored at room temperature and used for the analysis.

High-pressure liquid chromatographic method

Samples were analyzed on HPLC (Waters Alliance 2695) equipped with a 2489 UV detector [Waters Corporation, Milford, MA, USA]. An Inertsil ODS-3 C18 column (250 mmx4.6 mm, 5 µm) was employed for separation. The flow rate of the mobile phase was kept at 1.0 mL min^{-1} and the gradient program was set as T/%B: 0/30, 5/30, 10/80, 12/80, 15/30, 20/30. The column temperature was kept at 25°C. The injection volume was 10 µL and the detector wavelength was fixed at 220 nm.

Preparation of standard, sample solutions and solvent mixture.

The solvent mixture was prepared with a ratio of Milli-Q water and acetonitrile (50:50). This solvent mixture was used as diluent for standard and sample solutions. A stock solution of Imipramine Hydrochloride was prepared by dissolving 50.01 mg of Imipramine Hydrochloride standard in 100 mL volumetric flask using solvent mixture to make concentration of five hundred parts per million (500 ppm=500 µg mL^{-1}), further dilution was made by pipetting out 10 mL from stock solution in 50 mL volumetric flask to obtain a standard solution of one hundred parts per million (100 ppm=100 µg mL^{-1}). Similarly, 2,2'-dinitro-1,2-diphenylethane stock solution was prepared by dissolving 50.06 mg of it in a 100 mL volumetric flask with the solvent mixture to make the concentration of five hundred parts per million (500 ppm=500 µg mL^{-1}), further dilution was made by pipetting out 10 mL from stock solution in 50 mL volumetric flask to obtain a standard solution of one hundred parts per million (100 ppm=100 µg mL^{-1}). 2-2-diamino-1,2-diphenyl ethane diphosphate the stock solution was also prepared by dissolving 50.05 mg in 100 mL volumetric flask with the solvent mixture to

make the concentration of five hundred parts per million (500 ppm=500 µg mL^{-1}) further dilution was made by pipetting out 10 mL stock solution in 50 mL volumetric flask to obtain a standard solution of one hundred parts per million (100 ppm=100 µg mL^{-1}). Also, the stock solution of iminodibenzyl was prepared by dissolving 50.11 mg of it in a 100 mL volumetric flask with solvent mixture of concentration (500 ppm=500 µg mL^{-1}) further dilution was made by pipetting out 10 mL from stock solution in 50 mL volumetric flask to obtain a standard solution of one hundred parts per million (100 ppm=100 µg mL^{-1}. These standard solutions were freshly prepared and injected during analysis.

RESULTS

Method development

The chromatogram of mix system solutions having a composition of a) Imipramine Hydrochloride, b) 2-2-diamino-1,2-diphenyl ethane diphosphate, c) 2,2'-dinitro-1,2-diphenylethane and d) iminodibenzyl is represented in Figure 1. The retention time in the case of mix solution system was observed at 5.089, 9.966, 14.528 and 15.479 min, respectively for a, b, c and d components. All peaks show resolutions of more than 10 (USP Limit: NLT 2.00).

The separate chromatograms of the starting material, intermediate 1 and 2 and final API Imipramine Hydrochloride are given in the Supplementary information (Figures S1-S4). The chromatogram of the main Active Pharmaceutical Intermediate (API) Imipramine Hydrochloride (a) shows a tailing factor of 1.24 (USP Limit: NMT 2.00). The intermediate-1, 2-2-diamino-1,2-diphenyl ethane diphosphate (b) shows a tailing factor of 1.10 (USP Limit: NMT 2.00). The key starting material i.e., 2,2'-dinitro-1,2-diphenylethane (c) shows tailing factor 1.04 (USP Limit: NMT 2.00) and the intermediate-2, i.e., iminodibenzyl (d) shows the tailing factor 1.05 (USP Limit: NMT 2.00).

DISCUSSION

Validation study

A validation study was conducted, to monitor the factors such as linearity, precision, accuracy, specificity, solution stability and robustness. These validation parameters have been adopted from the International Conference on Harmonization (ICH) and are summed up below.

Linearity

The key starting material, intermediate 1 and 2 and the API solutions containing 10, 30, 50, 80, 100, 120 and 150 µg mL^{-1} standard concentration corresponding to 10, 30, 50, 80, 100, 120 and 150% of the target values, respectively were tested for their linearity test and the plots are given in Supporting information Figures S5-S8. The International Conference on Harmonization (ICH) Q2A and Q2B guidelines describe the validation of analytical methods, including the determination of linearity. According to these guidelines, a correlation coefficient (R2) value of 0.99 or above is acceptable for linearity All the linearity results give a good agreement with the peak area and concentration of Imipramine Hydrochloride, 2-2-diamino-1,2-diphenyl ethane diphosphate, 2,2'-dinitro-1,2-diphenylethane and iminodibenzyl. The combined data recorded for the linearity study for each component is shown in Table 1.

The regression coefficient value for the starting material (2,2'-din itro-1,2-diphenylethane) was found to be 0.9986 indicating good linearity over a wide concentration range. The linearity of intermediate-1 (2,2'-Diamino-1,2-diphenylethane diphosphate) and intermediate-2 (iminodibenzyl) recorded for the samples of the same standard concentrations show regression coefficients 0.9994 and 0.9960 respectively. Similarly, the linearity of the final API (Imipramine Hydrochloride) under the same above-mentioned conditions gives the regression coefficient value of 0.9982 which further indicates that there is nearly no

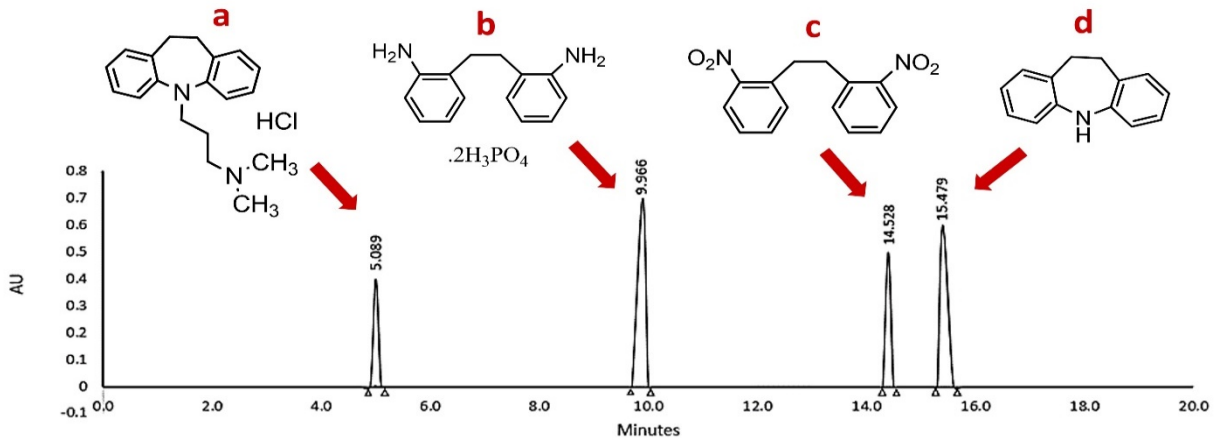

Figure 1: Chromatogram of mix system suitability.

change in peak area response with a change in concentration in the range of 10-150 µg mL^{-1} (Figures 2-5).

Precision

To perform the precision study, the five repeated injections of a standard solution have been run and the relative standard deviation for the peak area of starting material 2,2'-dinitro-1,2-diphenylethane, intermediate-1 i.e., 2-2-diamino-1,2-diphenyl ethane diphosphate, intermediate-2 Iminodibenzyl and the API Imipramine Hydrochloride had found to be 0.08%, 0.19%, 0.07% and 0.08%, respectively. According to guidelines of International Conference on Harmonization (ICH) Q2A and Q2B and United States Pharmacopeia (USP) precision for assay should not be more than 2%. The results obtained for the current analysis are shown in (Tables 2-5).

Accuracy

The accuracy of the method has developed by adding a known quantity of analytes with three different concentrations at three levels of LOQ i.e., 80%, 100% and 120% (w/v). Each of the levels has been prepared in triplicates and its analysis was performed as per the set method. According to the International Conference on Harmonisation (ICH) guidelines Q2A, Q2B and Q2R2, the accuracy of an analytical method should be 95% or above. All four components i.e., key starting material, intermediate-1 and 2 and final API show 94-102% recovery (Tables 6-9).

Table 1: Linearity data of final API, intermediate-1, key starting material and intermediate-2.

		Peak Area			
Level	Concentration (in ppm)	Imipramine Hydrochloride	2-2-diamino-1,2-diphenylethane diphosphate	2,2'-dinitro-1,2 diphenylethane	Iminodibenzyl
10%	10	289775	341669	302504	479887
30%	30	946885	1086170	989893	1582619
50%	50	1464258	1685914	1526820	2436071
80%	80	2359095	2707492	2470153	3918317
100%	100	2896700	3324505	3043308	4802216
120%	120	3342111	3983409	3524669	5344775
150%	150	4036376	4811897	4279780	6455382
Correlation coefficient		0.9982	0.9994	0.9986	0.9960
Slope		26829.62	31907.10	28436.11	42716.32
Intercept		120284.14	91658.98	109291.63	276153.87

Table 2: Precision Study of 2,2'-dinitro-1,2-diphenylethane.

Sl. No.	Name	Injections	Retention Time (min)	Area	Plate Count	Tailing
1	2,2'-dinitro-1,2-diphenylethane	1	14.488	3024658	70059	1.04
2	2,2'-dinitro-1,2-diphenylethane	2	14.558	3024473	71323	1.05
3	2,2'-dinitro-1,2-diphenylethane	3	14.539	3024771	70562	1.04
4	2,2'-dinitro-1,2-diphenylethane	4	14.539	3022338	70683	1.05
5	2,2'-dinitro-1,2-diphenylethane	5	14.530	3028972	70186	1.05
Mean			14.531	3025042		
Std. Dev				2413.4135		
% RSD				0.08		

Table 3: Precision Study of 2-2-diamino-1,2-diphenylethane diphosphate.

Sl. No.	Name	Injections	Retention Time (min)	Area	Plate Count	Tailing
1	2-2-diamino-1,2-diphenyl ethane diphosphate	1	9.966	3310594	74975	1.10
2	2-2-diamino-1,2-diphenyl ethane diphosphate	2	9.978	3310416	76224	1.10
3	2-2-diamino-1,2-diphenyl ethane diphosphate	3	9.968	3325313	74284	1.10
4	2-2-diamino-1,2-diphenyl ethane diphosphate	4	9.972	3313429	76501	1.11
5	2-2-diamino-1,2-diphenyl ethane diphosphate	5	9.964	3319083	75156	1.11
Mean			9.970	3315767		
Std. Dev				6383.3706		
% RSD				0.19		

Table 4: Precision Study of Iminodibenzyl.

Sl. No.	Name	Injections	Retention Time (min)	Area	Plate Count	Tailing
1	Iminodibenzyl	1	15.403	4764749	64180	1.05
2	Iminodibenzyl	2	15.479	4761589	63928	1.05
3	Iminodibenzyl	3	15.459	4770700	63256	1.05
4	Iminodibenzyl	4	15.458	4766143	63276	1.05
5	Iminodibenzyl	5	15.454	4767778	62576	1.05
Mean			15.450	4766192		
Std. Dev				3395.2614		
% RSD				0.07		

Table 5: Precision Study of Imipramine Hydrochloride.

Sl. No.	Name	Injection	Retention Time (min)	Area	Plate Count	Tailing
1	Imipramine Hydrochloride	1	5.025	2872460	7449	1.23
2	Imipramine Hydrochloride	2	5.071	2870487	7486	1.24
3	Imipramine Hydrochloride	3	5.033	2873160	7717	1.23
4	Imipramine Hydrochloride	4	5.022	2875293	7652	1.23
5	Imipramine Hydrochloride	5	5.036	2869299	7327	1.24
Mean			5.037	2872140		
Std. Dev				2339.0850		
% RSD				0.08		

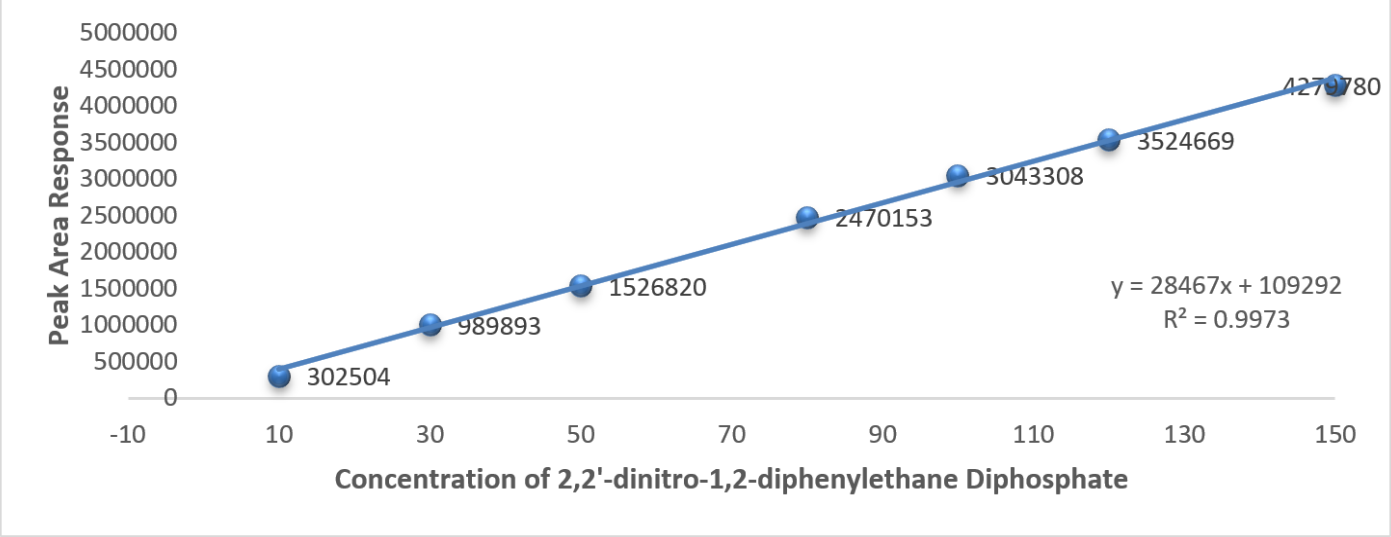

Figure 2: Linearity of 2,2′-dinitro-1,2-diphenylethane (KSM).

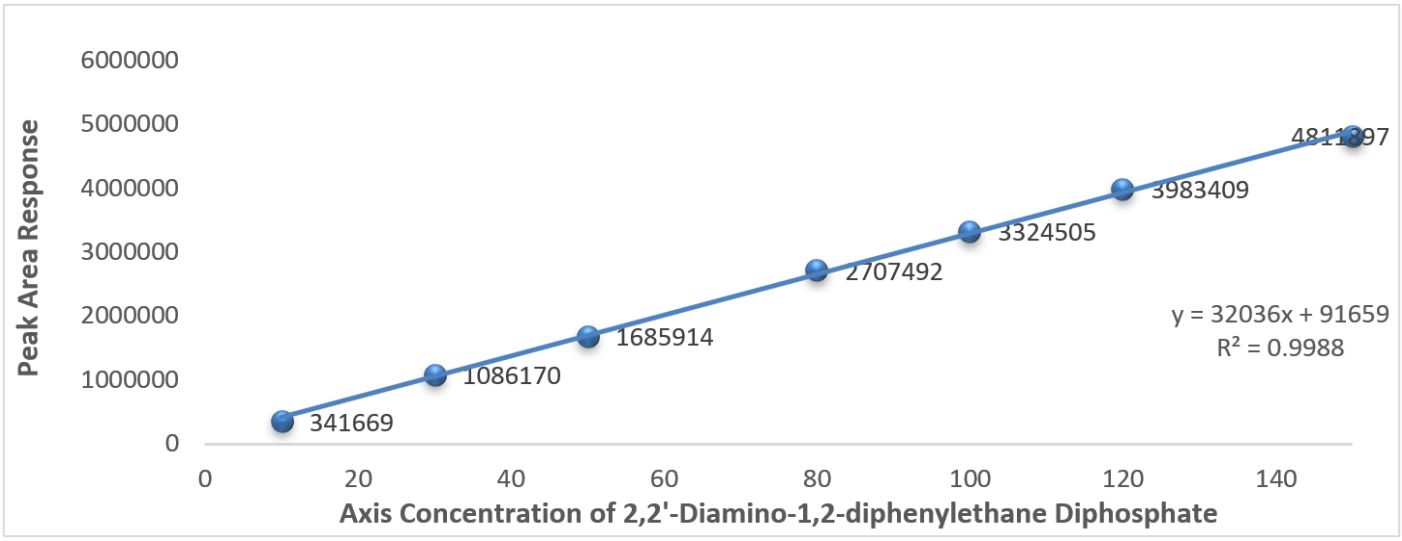

Figure 3: Linearity of 2,2′-Diamino-1,2-diphenylethane diphosphate.

Table 6: Accuracy Study of 2,2′-dinitro-1,2-diphenylethane.

Level	Solution	Concentration (in ppm)	Area obtained after spiking	% Recovery	Mean % Recovery
80%	Test-1	80	2412342	97.65961866	97.80205922
	Test-2		2419379	97.94449979	
100%	Test-1	100	3031503	99.61209973	99.61778433
	Test-2		3031849	99.62346894	
120%	Test-1	120	3570318	101.2951287	101.0061796
	Test-2		3549949	100.7172305	

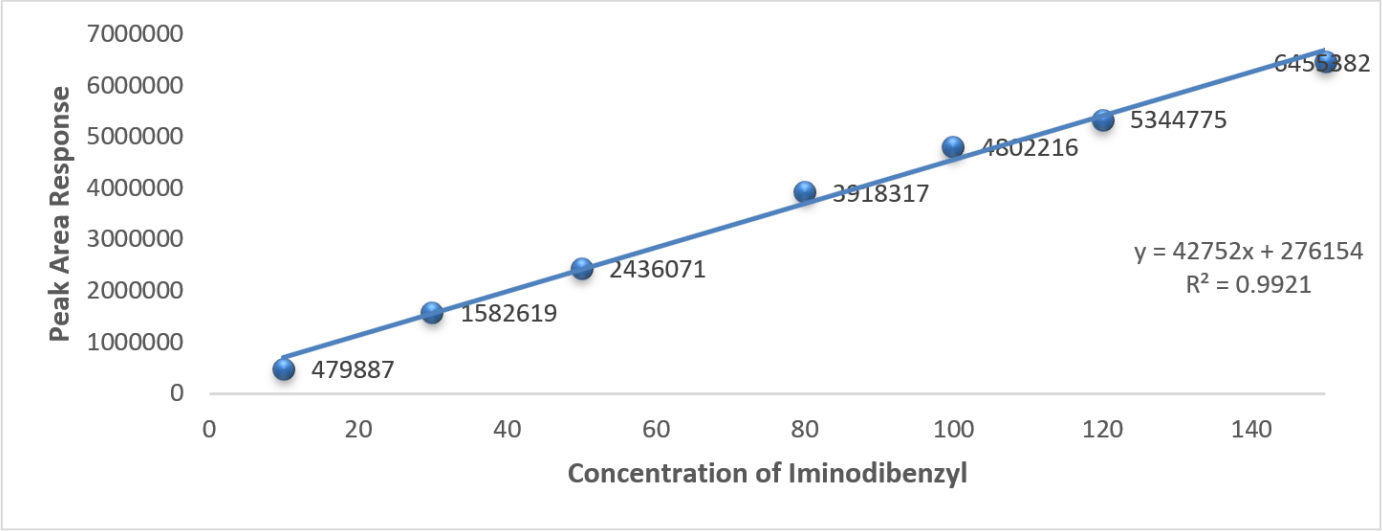

Figure 4: Linearity of Iminodibenzyl.

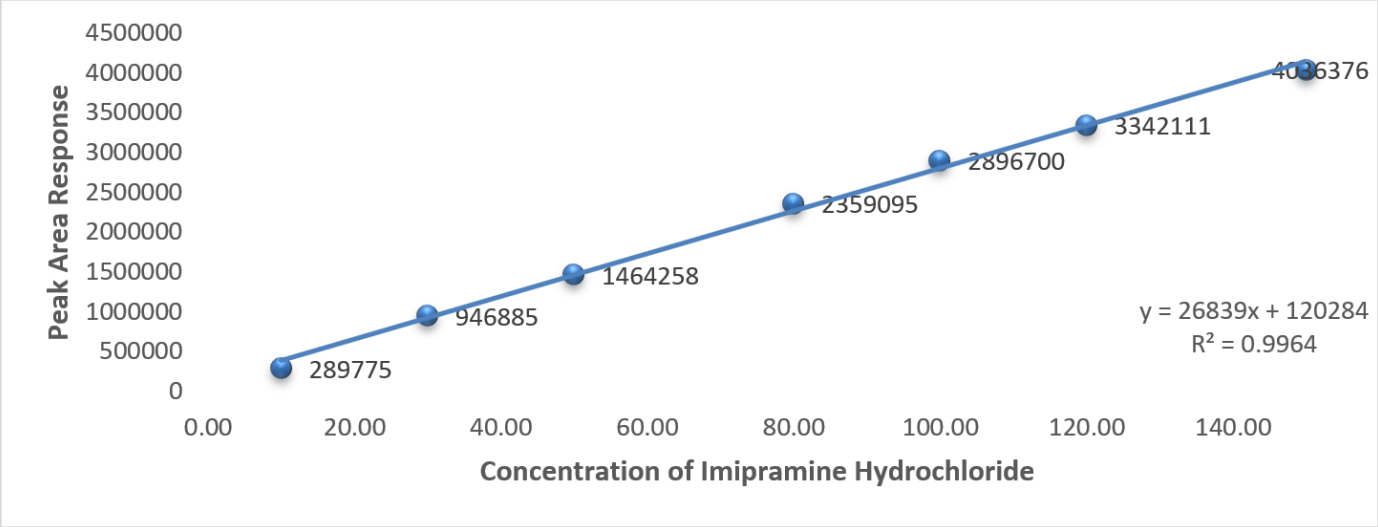

Figure 5: Linearity of API imipramine hydrochloride.

Specificity

Specificity was determined by injecting individual analytes. According to the United States Pharmacopeia (USP), the resolution between all the components in the sample should be 2 or more. There were no interference peaks in diluent during the retention time of Imipramine Hydrochloride, 2-2-diamino-1,2-diphenyl ethane diphosphate, 2,2'-dinitro-1,2-diphenylethane, iminodibenzyl indicating good specificity of the method. The developed method also indicates all the analytes are well separated in the API with a high degree of separation.

Solution stability

The stability of the solution was checked by using the standard test preparations, for that the solution was filled in a tightly capped HPLC vial at 25°C for 48 hr. and there was no detectable change in the observed peak area indicating the stability of the used test solutions. As per ICH there is no such direct limit or timeline for solution stability, for assay solution shall be monitored for about 24 hr and the change in the purity of the sample should not be more than 2% compared to the freshly prepared sample.

Robustness

The low rate of mobile phase and variation in column oven temperature have been monitored to study the robustness of this method. The flow rate of the mobile phase was changed to 0.9 ml min^{-1} and then was altered to 0.8- and 1.0-mL min^{-1}. This effect was investigated at 23°C and 27°C (altered by 2°C) and the outcome was that changing conditions did not affect the performance of the method for Imipramine Hydrochloride, 2-2-diamino-1,2-diphenyl ethane diphosphate, 2,2'-dinitro-1,2-diphenylethane and iminodibenzyl

Table 7: Accuracy Study of 2-2-diamino-1,2-diphenylethane diphosphate.

Level	Solution	Concentration (in ppm)	Area obtained after spiking	% Recovery	Mean % Recovery
80%	Test-1	80	2747375	101.4730607	101.6560344
	Test-2		2757283	101.8390082	
100%	Test-1	100	3314933	99.71207744	99.69945902
	Test-2		3314094	99.6868406	
120%	Test-1	120	4043571	101.5103144	101.2669801
	Test-2		4024185	101.0236458	

Table 8: Accuracy Study of Iminodibenzyl.

Level	Solution	Concentration (in ppm)	Area obtained after spiking	% Recovery	Mean % Recovery
80%	Test-1	80	3712556	94.74874034	94.7800548
	Test-2		3715010	94.81136927	
100%	Test-1	100	4777919	99.49404608	99.43186645
	Test-2		4771947	99.36968683	
120%	Test-1	120	5490615	102.7286462	102.4699169
	Test-2		5462958	102.2111876	

Table 9: Accuracy Study of Imipramine Hydrochloride.

Level	Solution	Concentration (in ppm)	Area obtained after spiking	% Recovery	Mean % Recovery
80%	Test-1	80	2236251	94.79274891	94.84802435
	Test-2		2238859	94.90329978	
100%	Test-1	100	2888000	99.69965823	99.69434184
	Test-2		2887692	99.68902544	
120%	Test-1	120	3387987	101.3726654	101.1779232
	Test-2		3374970	100.983181	

demonstrating the method's robustness if there was no change in the mobile phase components condition.

CONCLUSION

A simple, comprehensive and reproducible method for the simultaneous identification and quantification of Imipramine Hydrochloride, its raw materials, intermediates and other contaminants with high specificity was developed and validated. The proposed method is the first reported method to determine Imipramine Hydrochloride along with its starting material, intermediate and associated impurity in a single method. This method is suitable for routine examination of manufacturing samples due to its simplicity and efficiency.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

API: Active Pharmaceutical Ingredient; **RP-HPLC:** Reverse Phase High-Performance Liquid Chromatography; **ICH:** International Conference on Harmonisation, **OPA:** Orthophosphoric acid; **USP:** United States Pharmacopeia; **LOQ:** Limit of quantification; **LOD:** Limit of detection; **NLT:** Not less than; **NMT:** Not more than.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This research project does not require ethics approval as it does not involve human subjects and therefore, consent to participate is not applicable.

SUMMARY

Imipramine Hydrochloride is a class of tricyclic API that is commonly used to treat various mental health disorders in addition a low dose is prescribed to treat multiple types of nerve pain. In our current study, we have developed a single advanced RP-HPLC method to determine the intermediate and known impurities in the Imipramine Hydrochloride sample. The method is easy, specific and reliable. The separation column is Inertsil ODS-3 C18, with a mobile phase of 0.1% OPA and acetonitrile. All the analytes are detected at 220 nm using a UV detector. The method meets the ICH Q3 D Guideline and can be used for process development and determining the purity of associated compounds of Imipramine Hydrochloride, its key starting material (2,2-dinitro-1,2-diphenylene ethane), intermediates (2-2-diamino-1,2-diphenyl ethane diphosphate and Iminodibenzyl) in a single method. This approach saves time and simplifies the analysis of intermediate, raw material, purity and impurity profiling.

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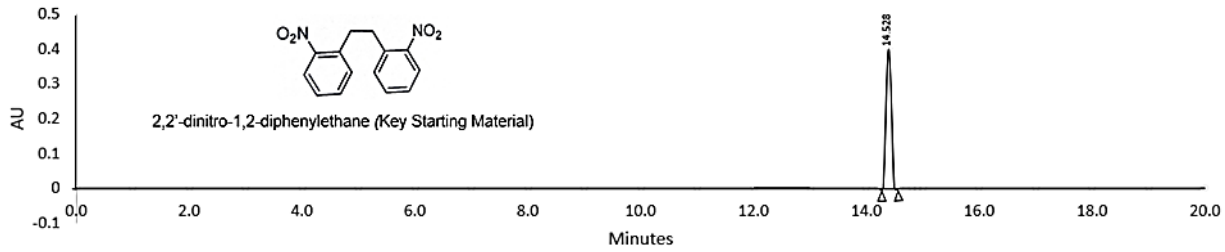

Figure S1: Chromatogram of key starting material 2,2′-dinitro-1,2-diphenylethane.

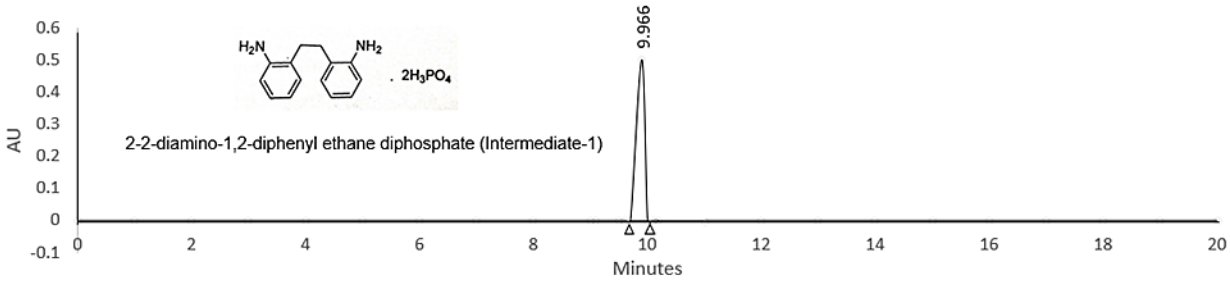

Figure S2: Chromatogram of intermediate-1 i.e. 2-2-diamino-1,2-diphenyl ethane, diphosphate.

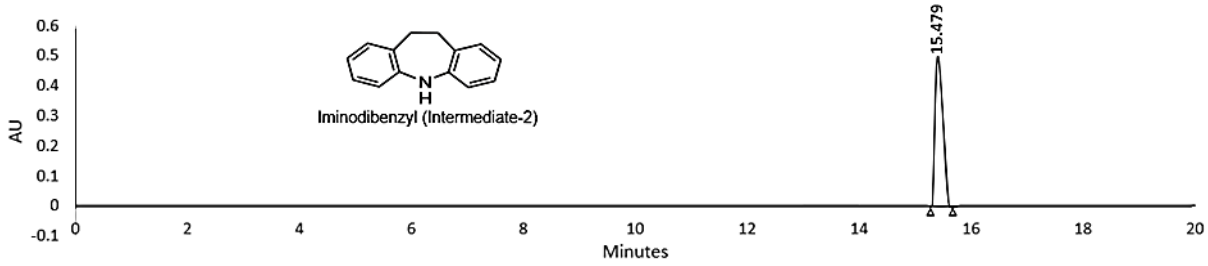

Figure S3: Chromatogram of intermediate-2 i.e. iminodibenzyl.

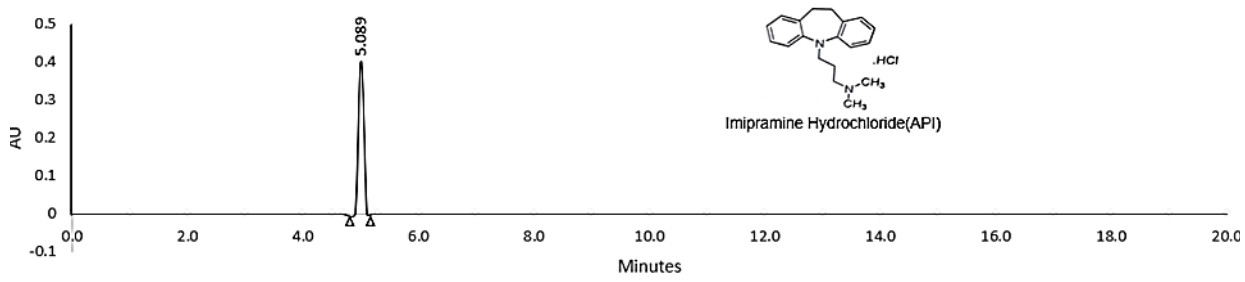

Figure S4: Chromatogram of API imipramine hydrochloride.

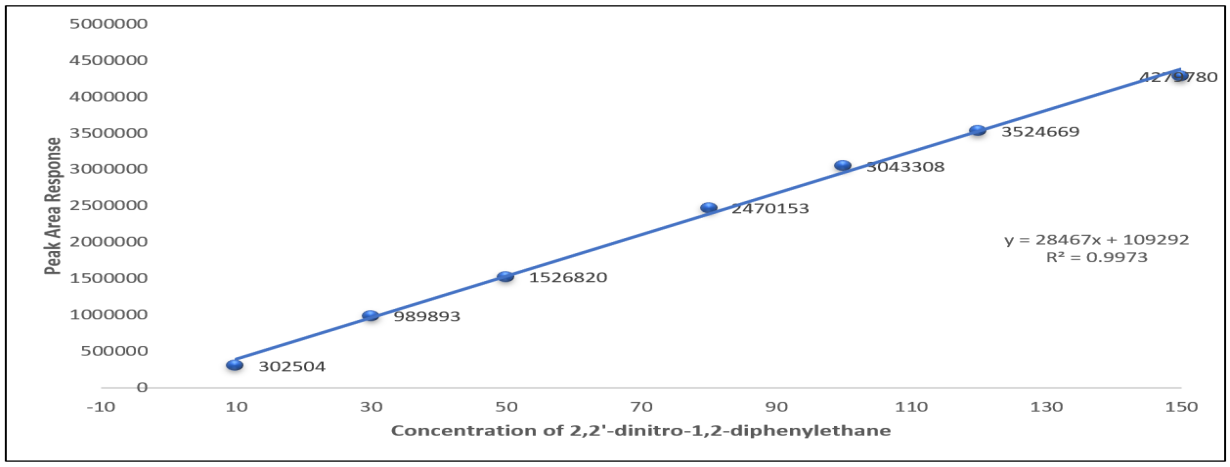

Figure S5: Linearity of 2,2′-dinitro-1,2-diphenylethane (KSM).

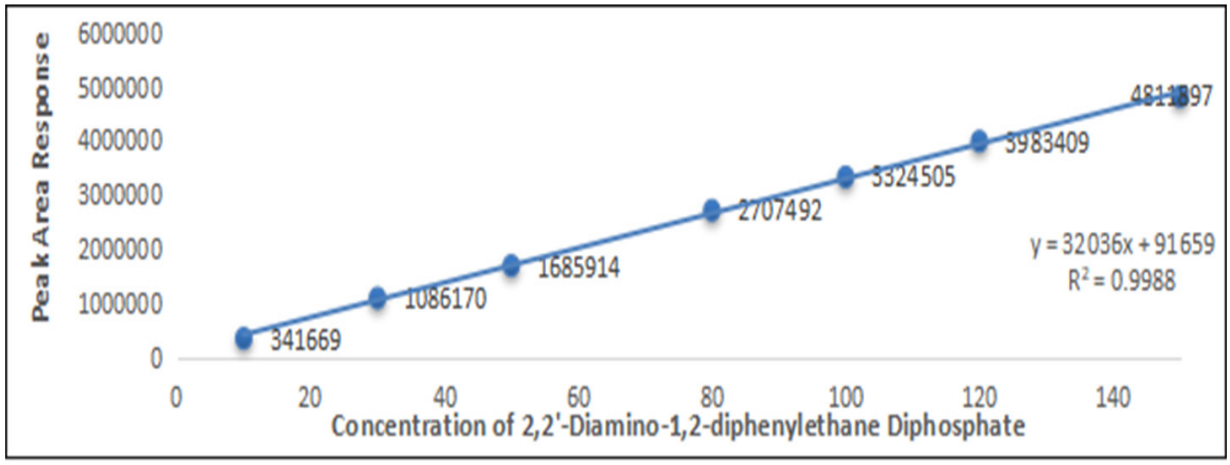

Figure S6: Linearity of 2,2′-Diamino-1,2-diphenylethane diphosphate.

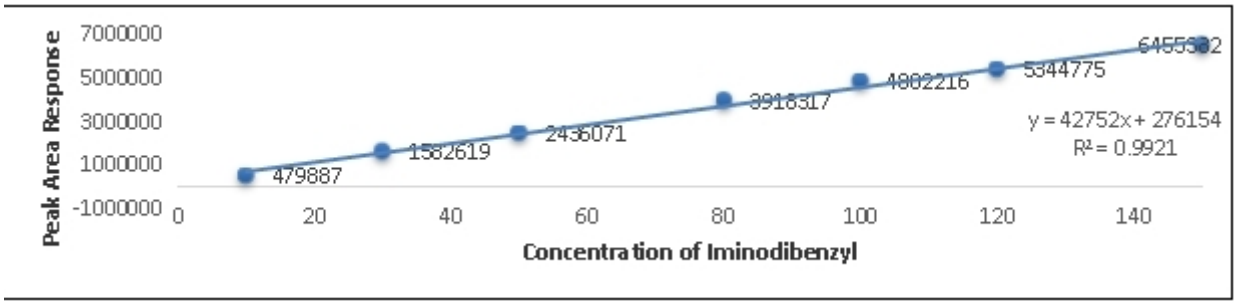

Figure S7: Linearity of iminodibenzyl.

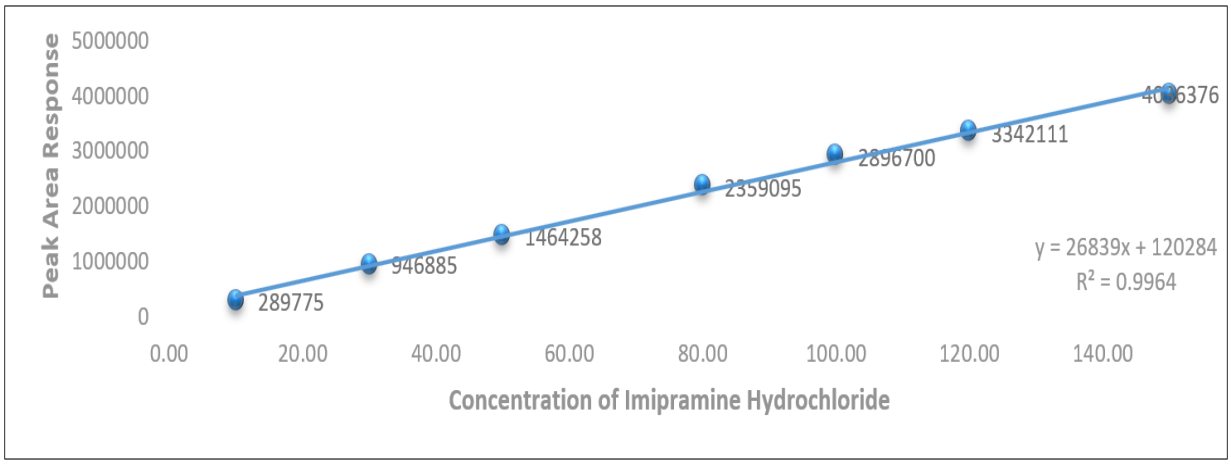

Figure S8: Linearity of API imipramine hydrochloride.