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Validation of a reverse phase high-performance liquid chromatography method for the detection of major components and related substances in Nicardipine Hydrochloride Injection

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Abstract: A simple, feasible, isocratic elution and stable reversed-phase high performance liquid chromatography was established and verified. Chromatographic conditions were XY-C18, 4.6x150mm, 5μm column; column temperature 50ºC. Mobile phase was (A) 0.025 mol/L sodium perchlorate solution, added 1ml triethylamine, and adjust the pH value to 2.0 with perchloric acid solution; (B) acetonitrile-methanol (70:30). The elution method was gradient elution; The flow rate was 1.0mL/min; the injection volume was 10μL and the wavelengths were 239nm and 258nm. The developed method had been validated according to the ICH guidelines and the system suitability, specificity, LOD, LOQ, linearity, range, accuracy, precision, durability and solution stability of the proposed method had been verified. The relationship between concentration and peak area was linear in the concentration range, with an $R^2 > 0.995$. The linear relationship was good in the range of impurity concentration and the R^2 was more than 0.999. The accuracy and repeatability meet the specified criteria. The method had good durability.

Keywords: Nicardipine Hydrochloride Injection, HPLC, Validation, Stability-indicating

INTRODUCTION

Calcium channel blockers are prescribed for the treatment of hypertension, angina, and certain cardiac arrhythmias. They decrease blood pressure by directly and specifically relaxing smooth muscle cells, particularly those that constrict blood vessels (Al-Ghannam *et al.*, 2019) aids in the preservation of blood circulation to the heart and diminishes the frequency and intensity of angina episodes. Calcium channel blockers (CCBs) can be used either alone or in conjunction with angiotensin-converting enzyme (ACE) inhibitors or b-blockers (Domenic *et al.*,2001). Nicardipine hydrochloride is a calcium channel-blocking drug of the dihydropyridine type. It works by preventing the entry of calcium ions into the cardiac and smooth muscle cells, without affecting the levels of calcium in the blood(Huang *et al.*,2013). It exhibits a high degree of specificity towards blood vessels. The injection is administered for the immediate management of anomalous hypertension and hypertensive emergencies during surgical procedures. International studies have demonstrated that nicardipine has effectively reduced perioperative blood loss (Allison et al., 2019; Hafeez et al., 2019). Currently, high performance liquid chromatography (HPLC) is the most extensively employed approach. To enhance the regulation of the inherent quality of nicardipine hydrochloride injection, we utilized high performance liquid chromatography to analyse the primary components and associated substances. Additionally, we validated the methodology to enhance the manageability of drug quality and offer guidance for appropriate clinical drug utilization.

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MATERIALS AND METHODS

Chemicals and reagents

Nicardipine Hydrochloride Injection was provided from Fuan Pharmaceutical Group Ningbo Team Pharmaceutical, Co., Ltd. Nicardipine Hydrochloride working standard, sorbitol and impurity I were provided by National Institutes for Food and Drug Control. Impurity B, Impurity C, impurity A3, impurity A4 and impurity A5 were bought from QCS. Impurity D and impurity E were from Beijing Kangpaisen Pharmaceutical Technology Co., Ltd. Sodium perchlorate monohydrate and triethylamine (AR grade) were purchased from Chengdu Cologne Cosmetics Co., Ltd. Perchloric acid (AR) was from Tianjin Zhengcheng Chemical products Co., Ltd. Acetonitrile and methanol were obtained from Adamas-beta Company. fig. 1 is structures of Nicardipine Hydrochloride, impurities B, C, D, A3, A4, A5 and I.

Instrumentation

Agilent 1260VWD, 1260DAD and Shimadzu LC-2050C separation module; Light stabilization chamber (SHH-100GD-2F from Yongsheng Instrument co;ltd); Vacuum oven (DHG-9123A from Shanghai Jing Hong Laboratory Instrument Co., Ltd.) . PH meter (Mettler toledo, FE28). Balance (Mettler toledo, XS205DU).

Chromatographic Conditions

Column: XY-C18, 4.6x150mm, 5μm; column temperature: 50ºC; mobile phase: (A) 0.025 mol/L

sodium perchlorate solution, added 1ml triethylamine and adjust the pH value to 2.0 with perchloric acid solution; (B) acetonitrile-methanol (70:30). Prior to use, the mobile phase underwent filtration using a 0.22μm nylon membrane filter and was degassed. The elution method employed was gradient elution. table 1 presents the parameters for gradient elution. The rate of flow was 1.0 millilitres per minute, and the volume injected was 10 microliters. The substance of interest was observed at a wavelength of 239nm and 258nm.

Table 1: Mobile phase gradient elution table

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0	70	30
15	70	30
55	35	65
60	35	65
62	70	30
70	70	30

Solution

Diluent: Methanol-water (50:50). Stock and Standard Solution. Stock solution: measured out about 10 mg of the nicardipine hydrochloride reference standard, weighed it precisely, transferd the substance into a 50 ml volumetric flask of brown colour, then add a solvent to dissolve it and further dilute it till reaching the specified mark. Standard solution: took 2 ml of the stock solution, put it in the 200 ml brown volumetric flask, added diluent to dissolve and diluted to the mark. Preparation of two sections concurrently.

Sample Solution. Took this product and injected the sample directly.

Placebo Solution. Took approximately 500 mg of sorbitol, precisely measured its weight, and transferred it to a 10 ml volumetric flask. Dissolved the sorbitol in water and diluted it up to the mark on the flask. Shook the mixture thoroughly to achieve homogeneity.

Impurity B stock and reference solution. Stock solution: measured out about 10 mg of impurity B reference standard, weighed it precisely, transferd the substance into a 50 ml volumetric flask of brown colour, then add a solvent to dissolve it and further dilute it till reaching the specified mark. Reference solution: took 5ml of the stock solution, put it in the 200 ml brown volumetric flask, added diluent to dissolve and diluted to the mark. Preparation of two sections concurrently.

Impurity C stock and reference solution. Stock solution: measured out about 10 mg of impurity C reference standard, weighed it precisely, transferd the substance into a 50 ml volumetric flask of brown colour, then added a solvent to dissolve it and further dilute it till reaching the specified mark. Reference solution: took 5ml of the stock solution, put it in the 200 ml brown volumetric flask, added diluent to dissolve and diluted to the mark. Preparation of two sections concurrently.

Impurity D stock and reference solution. Stock solution: measured out about 10 mg of impurity D reference standard, weighed it precisely, transferd the substance into a 50 ml volumetric flask of brown colour, then added a solvent to dissolve it and further dilute it till reaching the specified mark. Reference solution: took 2ml of the stock solution, put it in the 200 ml brown volumetric flask, added diluent to dissolve and diluted to the mark. Preparation of two sections concurrently.

Impurity A3 stock and reference solution. Stock solution: measured out about 10mg of impurity A3 reference standard, weighed it precisely, transferd the substance into a 50 ml volumetric flask of brown colour, then added a solvent to dissolve it and further dilute it till reaching the specified mark. Reference solution: took 6ml of the stock solution, put it in the 200 ml brown volumetric flask, added diluent to dissolve and diluted to the mark. Preparation of two sections concurrently.

Impurity A4 stock and reference solution. Stock solution: measured out about 10 mg of impurity A4 reference standard, weighed it precisely, transferd the substance into a 50 ml volumetric flask of brown colour, then added a solvent to dissolve it and further dilute it till reaching the specified mark. Reference solution: took 6ml of the stock solution, put it in the 200 ml brown volumetric flask, added diluent to dissolve and diluted to the mark. Preparation of two sections concurrently.

Impurity A5 stock and reference solution. Stock solution: measured out about 10 mg of impurity A5 reference standard, weighed it precisely, transferd the substance into a 50 ml volumetric flask of brown colour, then added a solvent to dissolve it and further dilute it till reaching the specified mark. Reference solution: took 3ml of the stock solution, put it in the 200 ml brown volumetric flask, added diluent to dissolve and diluted to the mark. Preparation of two sections concurrently.

Impurity I stock and reference solution. Stock solution: measured out about 10 mg of impurity I reference standard, weighed it precisely, transferd the substance into a 50 ml volumetric flask of brown colour, then added a solvent to dissolve it and further dilute it till reaching the specified mark. Reference solution: took 5ml of the stock solution, put it in the 200 ml brown volumetric flask, added diluent to dissolve and diluted to the mark. Preparation of two sections concurrently.

Optimization of detection wavelength

It was found that under the detection condition of 239nm wavelength, the correction factor of impurity A5 was 5.0, which was quite different from the correction factor (1.0) in the registration standard for imported drugs of this product, and the correction factor of impurity I was also large (1.9). Therefore, under the same concentration (4

μg/mL), the full band (200~400nm) ultraviolet spectral scanning of impurity A5, impurity I and nicardipine hydrochloride reference solution showed that the absorbance of impurity A5, impurity I and nicardipine hydrochloride was quite different at the original detection wavelength of 239nm, so the correction factor of impurity A5 and impurity I was far away from 1. By analyzing the ultraviolet spectra of plasm A5, impurity I and nicardipine hydrochloride, it was found that the absorbance of impurity A5, impurity I and nicardipine hydrochloride was basically the same at about 258nm wavelength, so the 258nm wavelength was used to verify impurity A5 and impurity I.

Forced degradation studies (Ngwa et al., 2010)

Forced degradation testing is helpful to design scientific and reasonable impurity analysis and detection technology, and is very important to ensure the safety of clinical medication(Khandare *et al.*,2019). It is necessary to conduct a mandatory degradation test for the detection methods of related substances, which will help to examine and comprehend the stability of drugs, degradation pathways and degradation products(Kogawa *et al.*,2016). The test also aims to identify the specificity of analytical procedures for related chemicals, particularly degradation products. (Wu *et al.*, 2024)

Acid Degradation. Accurately measured 8ml of the sample solution of nicardipine hydrochloride injection, placed it in a 10ml brown volumetric flask, added 0.5ml of 1mol/L HCL to break for 10 h in an environment with laboratory temperature, then added 0.5ml of 1mol/L sodium hydroxide solution to neutralize, diluted with diluent to the mark, shook well.

Alkali Degradation. Accurately measured 8ml of the sample solution of nicardipine hydrochloride injection, placed it in a 10ml brown volumetric flask, added 0.5ml of 1N sodium hydroxide solution to break for 2 hours in an environment with laboratory temperature, then added 0.5ml of 1N HCL to neutralize, diluted with diluent to the mark, shook well.

Oxidation Degradation. Accurately measured 8ml of the sample solution of nicardipine hydrochloride injection, placed it in a 10ml brown volumetric flask, added 1ml of 3% H_2O_2 solution to break for 4 hours in an environment with laboratory temperature, diluted with diluent to the mark, shook well.

Thermal degradation refers to the process of breaking down or deteriorating due to exposure to high temperatures. To examine the effects of heat, the nicardipine hydrochloride injection was exposed to a temperature of 105 for a period of 10 hours. Afterward, a precise measurement of 8ml of the sample solution was taken and placed in a 10ml brown volumetric flask. The solution was prepared by adding diluent to the mark and vigorously shaking the mixture..

Photolytic Degradation. Accurately measured 8ml of sample solution Nicardipine Hydrochloride Injection (placed under illumination (Ultraviolet 254nm) for 24 h) and placed it in a 10ml volumetric flask. Diluted to the mark and shook well to obtain the solution.

Method validation

The verification of the system's applicability, specificity, LOD, LOQ, linearity, range, accuracy, precision, durability, and solution stability (Wu *et al.*, 2024).

2.7.1. System Suitability. Injected a blank solvent for control. Injected five consecutive injections of reference solution. The relative standard deviation (RSD) of the retention time for the impurity peaks was less than or equal to 1.0% and the RSD of the peak area for the impurity was less than or equal to 2.0%.

2.7.2. Specificity. Each contaminant in the sample was individually identified in the reference solution to accurately pinpoint its position. The possible impact of placebo solutions on the main components was assessed by comparing them to sample solutions. Furthermore, in the strong deterioration testing, it is necessary for the spacing between the main peak and neighboring impurity peaks to be equal to or greater than 1.5, and for the purity factor of the main peak to be equal to or greater than 990. Under various conditions, the difference between the external standard content and purity of the degraded sample was not more than 5%.

LOD and LOQ. An appropriate amount of nicardipine hydrochloride reference solution and impurities I, B, C, D, A3, A4, A5 reference solution were transferred into a brown volumetric flask. And the quantitative limit solution was prepared to be tested with 0.03μg of nicardipine hydrochloride, 0.05μg, 0.025μg, 0.04μg, 0.06μg, 0.03μg, 0.3μg and 0.25μg of impurities B, C, D, A3, A4, A5 and I per milliliter. Accurately measured 5ml of the quantitative limit solution, placed it in a 10 ml brown volumetric flask, diluted with diluent to the mark, shook well. It was used as the detection limit solution. When the samples were injected into the detector, the quantitative limit was 6 consecutive injections, the detection limit was 3 consecutive injections, and the SN was not less than 10 and 3.

Linearity. For the preparation of a linear stock solution at a wavelength of 239 nm, the following steps were followed: 2mL of nicardipine hydrochloride stock solution, 2mL of impurity D stock solution, 5mL of impurity B stock solution, 5mL of impurity C stock solution, 6mL of impurity A3 stock solution and 6mL of impurity A4 stock solution were accurately measured.

These solutions were then combined in a 100mL brown volumetric flask and diluted to the mark with methanol. The flask was thoroughly shaken. To prepare the linear stock solution at a wavelength of 258 nm, precisely measured 2mL of the nicardipine hydrochloride stock solution, 3mL of the impurity A5 stock solution and 5mL of the impurity I stock solution. These were then combined in a 100mL brown volumetric flask and diluted with methanol up to the mark. The flask was thoroughly shaken. The linear solution was generated by taking an accurate measurement of 5mL of the linear stock solution (at 239 nm and 258 nm) and placing it in a 10mL brown volumetric flask to create a 100% linear solution. From this, additional test solutions were prepared at 20%, 50%, 100%, 150%, and 200% levels. Plotted the calibration curve correlating the concentration of the analyte with the corresponding peak region. Utilised Origin software to compute the slope, intercept, and correlation coefficient.

Accuracy. The index is mainly reflected by the recovery rate and evaluated by 100% of the limit concentration. Transfer the required volume of nicardipine hydrochloride and impurity B, C, D, A3, A4, A5 and I standard solution to a brown volumetric bottle and diluted with sample solution to the mark. The concentrations of nicardipine hydrochloride, impurity B, C, D, A3, A4, A5 and I were 2μg/mL, 5μg/mL, 5μg/mL, 2μg/mL, 6μg/mL, 6μg/mL, 3μg/mL and 5μg/mL, respectively. Six recovery solutions were prepared in parallel. The recovery and % RSD of the given parameters were determined.

Precision. In order to ensure the reproducibility of the experimental results, it is recommended to utilize six aliquots of the sample solution for testing purposes. The standard solution should contain 2μg/mL nicardipine hydrochloride, 5μg/mL impurity B, 5μg/mL impurity C, 2μg/mL impurity D, 6μg/mL impurity A3, 6μg/mL impurity A4, 3μg/mL impurity A5 and 5μg/mL impurity I impurity mixture. The % RSD of the recoveries of 6 sections were determined. For the purpose of achieving more accurate results, a different experimenter was substituted in the same laboratory throughout the intermediate precision tests. The intermediate precision test sample was prepared using the same procedure as the repeatability test, with 6 samples prepared simultaneously.

Robustness. The study sought to evaluate the durability of the method by altering the experimental and chromatographic parameters, including the column type, the flow rate (1.0±0.1mL/min), column temperature (50±2ºC), different concentration of sodium perchlorate (0.025±0.002mL/min), different percentage of triethylamine (0.1%±0.01%), the pH value of different buffer salts (2.0±0.2) and different organic phase (acetonitrile-methanol) ratio of mobile phase B (70±2: 30±2).

Sample and standard solution stability. The sample solution of nicardipine hydrochloride should be made and then stored in dark conditions at a temperature of 2-8ºC for particular time intervals, specifically 0h, 7h, 13h and 37 h. The impurity reference substance should be made as a mixed solution and then stored in dark settings at a temperature of 2-8ºC for defined time intervals. These periods include 0 hours, 14h, 20h and 44h. Performed an initial analysis and comparison examination of multiple time intervals for the two suggested alternatives. The ratio of peak area to 0h peak area of the mixed solution of impurity reference substance at different time was determined. The difference between the content of sample solution at different time and the content at 0h.

RESULT

Forced degradation

Fig. 2 displayed a chromatogram illustrating the presence of impurities in the localization solution and the degradation of the sample solution under external stress.

Acid Degradation. Under the condition of adding 0.5 ml 1 mol/L hydrochloric acid for 10 h, the difference between external standard content (residual rate) and purity (area normalization method) of degraded samples under various conditions was 0.636%, and impurities I was generated.

Alkali Degradation. The sample exhibited excellent stability and minimal degradation after being subjected to a 2 h period under the circumstance of adding 0.5 ml of a 1 mol/L sodium hydroxide solution, the difference between external standard content (residual rate) and purity (area normalization method) of degraded samples under various conditions was 0.136%. Furthermore, a single unidentified contaminant is produced.

Oxidation Degradation. Under the condition of adding 1 ml 3% hydrogen peroxide solution for 4 hours, the difference between external standard content (residual rate) and purity (area normalization method) of degraded samples under various conditions was 0.501% and impurities I and unknown impurities were generated.

Thermal Degradation. It can be seen that the sample solution has no impurity degradation at 105ºC for 10 hours, the difference between external standard content (residual rate) and purity (area normalization method) of degraded samples under various conditions was 0.509%, and impurities A5, A3, A4, I and unknown impurities were generated.

Photocatalytic Degradation. The sample was stable under the condition of utraviolet 254nm for 24 hours, the difference between external standard content (residual rate) and purity (area normalization method) of degraded samples under various conditions was 0.389%, and impurities I was generated.

Table 2: System suitability test results

Impurity	Check item	1	2	3	4	5	Average value	RSD/%
			239 nm					
B	Peak area	105080	105053	104906	104736	104631	104884	0.2
	Retention time	22.972	22.916	22.916	22.892	22.860	22.9112	0.2
C	Peak area	212454	212362	212392	211802	211915	212185	0.2
	Retention time	34.120	34.111	34.127	34.123	34.114	34.119	0.02
D	Peak area	72578	72256	72252	72106	72187	72275.8	0.3
	Retention time	18.212	18.191	18.211	18.216	18.205	18.207	0.06
A3	Peak area	211340	211044	211058	210522	210612	210915	0.2
	Retention time	21.803	21.781	21.795	21.797	21.785	21.792	0.05
A4	Peak area	210140	210059	209694	209210	209344	209689	0.2
	Retention time	25.056	25.035	25.049	25.045	25.034	25.044	0.04
Nicardipine hydrochloride	Peak area	63623	63613	63594	63447	63447	63544	0.2
	Retention time	28.304	28.269	28.273	28.254	28.232	28.266	0.1
			258 nm					
A5	Peak area	40415	41170	40756	40816	40246	40681	0.9
	Retention time	16.904	16.903	16.876	16.973	16.774	16.850	0.4
I	Peak area	63581	63520	63434	64366	63462	63673	0.7
	Retention time	25.849	25.842	25.786	25.691	25.666	25.767	0.4
Nicardipine hydrochloride	Peak area	26994	27061	26697	27320	27008	27016	0.9
	Retention time	27.972	27.955	27.905	27.823	27.796	27.890	0.3

Table 3: Precision of impurities content

Number		1	2	3	4	5	6	Mean	%RSD
				Impurity B (239 nm)					
Content (%)	Repeatability	0.024	0.020	0.023	0.020	0.023	0.023	0.02	8.2
	Intermediate precision	0.026	0.025	0.025	0.025	0.026	0.024		
				Impurity C (239 nm)					
Content (%)	Repeatability	0	0	0	0	0	0	0	0
	Intermediate precision	0	0	0	0	0	0		
				Impurity D (239 nm)					
Content (%)	Repeatability	0	0	0	0	0	0	0	0
	Intermediate precision	0	0	0	0	0	0		
				Impurity A3 (239 nm)					
Content (%)	Repeatability	0.059	0.059	0.058	0.059	0.059	0.059	0.06	6.2
	Intermediate precision	0.052	0.052	0.052	0.052	0.053	0.053		
				Impurity A4 (239 nm)					
Content (%)	Repeatability	0.009	0.008	0.009	0.008	0.009	0.009	0.008	6.5
	Intermediate precision	0.008	0.008	0.008	0.008	0.009	0.008		
				Impurity A5 (258 nm)					
Content (%)	Repeatability	0.103	0.104	0.109	0.105	0.105	0.106	0.10	3.9
	Intermediate precision	0.101	0.097	0.099	0.099	0.097	0.099		
				Impurity I (258 nm)					
Content (%)	Repeatability	0.135	0.133	0.134	0.135	0.134	0.136	0.13	1.3
	Intermediate precision	0.137	0.138	0.136	0.133	0.133	0.133		
				Other single impurity (239 nm)					
Content (%)	Repeatability	0.011	0.012	0.010	0.012	0.011	0.011	0.01	6.0
	Intermediate precision	0.011	0.012	0.011	0.011	0.012	0.012		
				Total impurity					
Content (%)	Repeatability	0.358	0.356	0.360	0.351	0.358	0.361	0.35	2.0
	Intermediate precision	0.351	0.347	0.342	0.344	0.345	0.345		

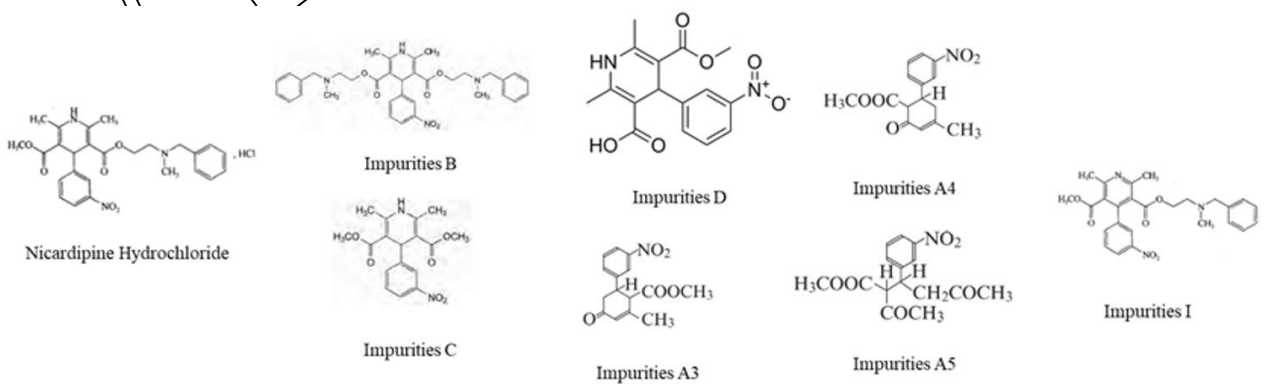
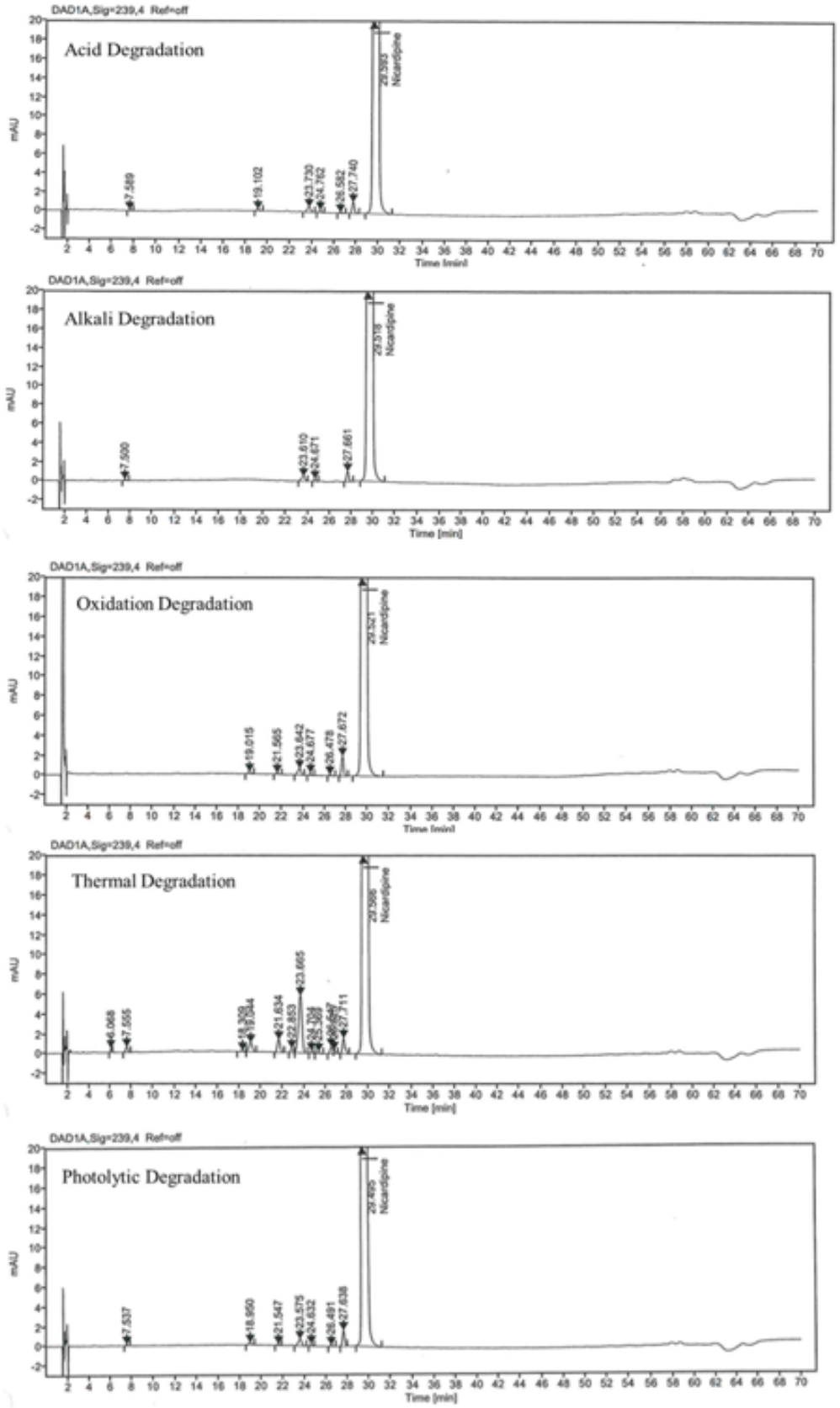


Fig. 1: Structures of Nicardipine Hydrochloride, impurities B, C, D, A3, A4, A5 and I.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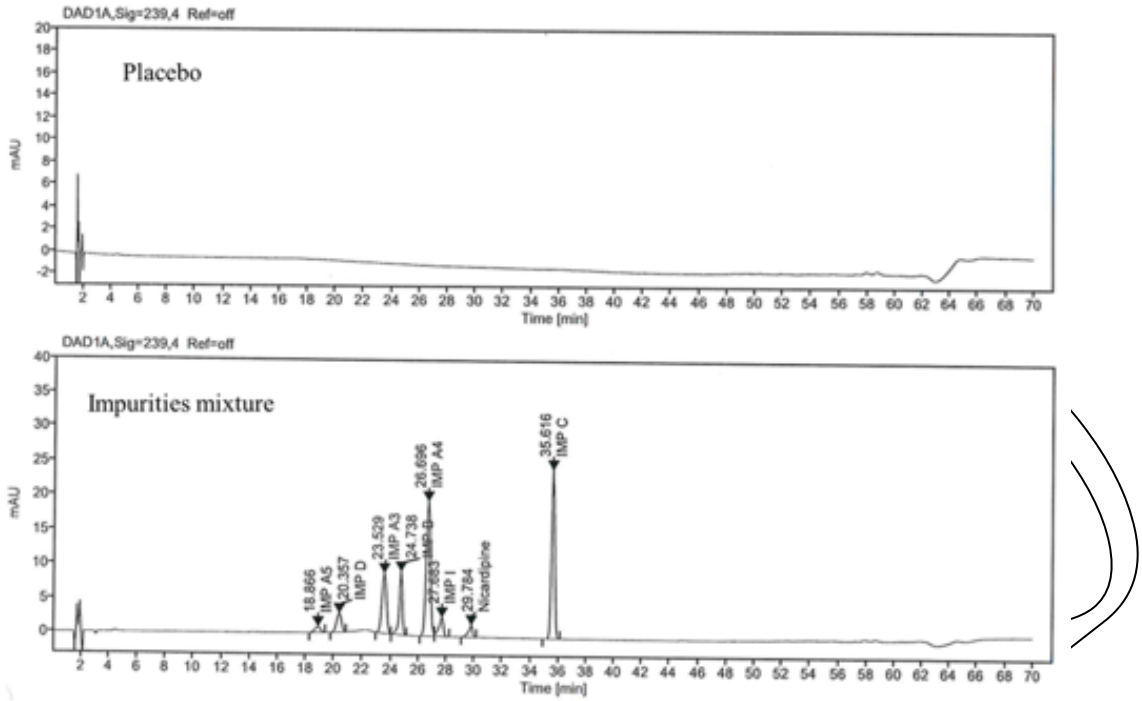

Fig. 2: Degradation chromatogram and impurity reference locating chromatogram.

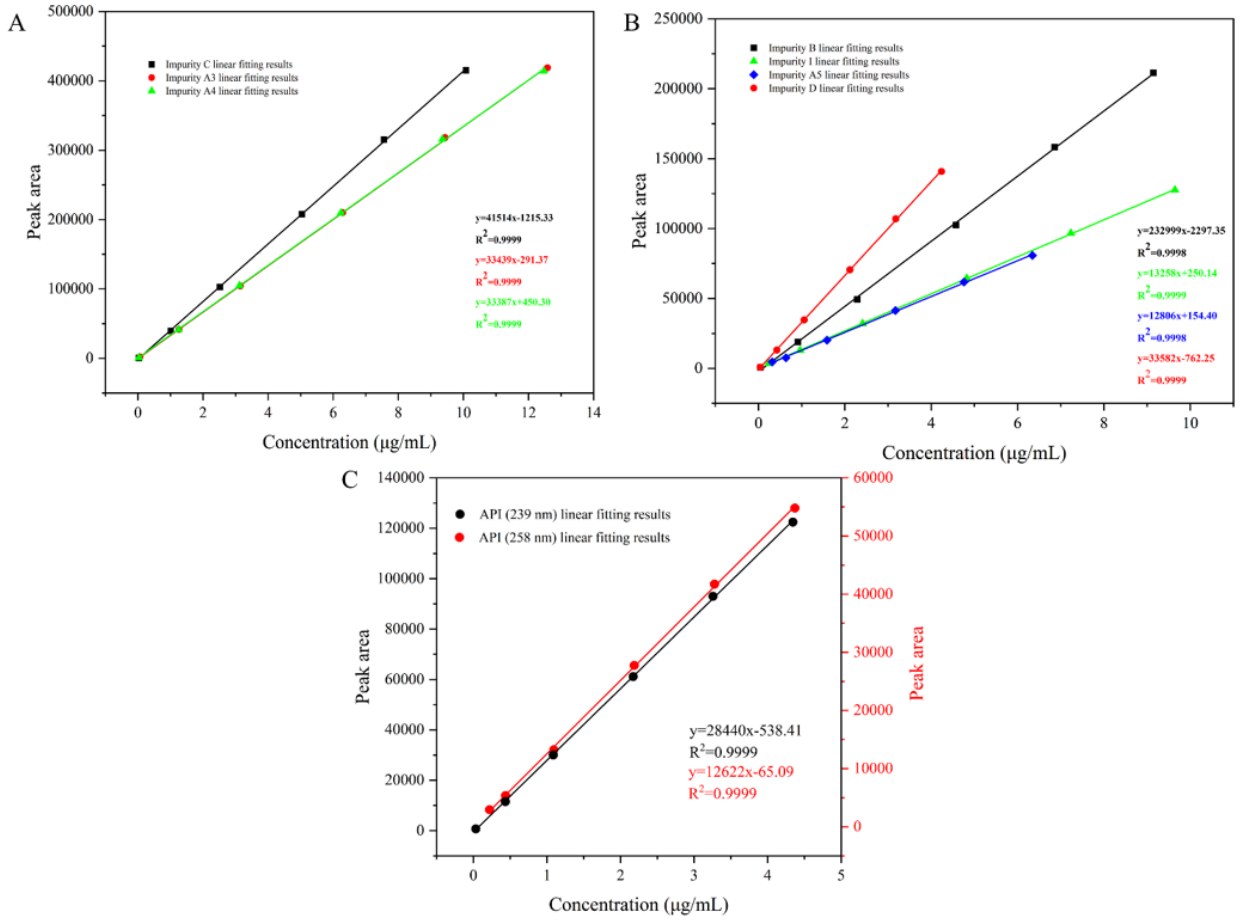

Fig. 3: (A) Linear results of impurities C, A3, A4 (B) Linear results of impurities B, I, A5, D (C) Linear results of principal component.

Table 4: Results of impurity Limit of Quantitation (239 nm)

Number	Concentration of LOQ (µg/mL)	Peak area	%RSD	RT (min)	%RSD	S/N of LOQ
		Impurity B (239 nm)				
1	0.0457	664	7.1	22.695	0.1	12.80
2		596		22.660		11.55
3		685		22.690		12.92
4		564		22.670		11.29
5		623		22.650		12.41
6		638		22.635		14.94
	Equivalent to sample concentration (%)			0.005		
		Impurity C (239 nm)				
1	0.0252	609	6.6	34.135	0.05	11.53
2		620		34.133		11.29
3		579		34.148		10.83
4		570		34.154		10.69
5		512		34.149		10.88
6		572		34.173		12.87
	Equivalent to sample concentration (%)			0.003		
		Impurity D (239 nm)				
1	0.0424	851	6.5	18.322	0.1	10.48
2		984		18.277		12.74
3		908		18.296		12.28
4		972		18.294		11.88
5		937		18.301		12.59
6		1022		18.278		15.37
	Equivalent to sample concentration (%)			0.004		
		Impurity A3 (239 nm)				
1	0.0629	1999	2.5	21.778	0.05	27.94
2		1892		21.789		26.51
3		1954		21.799		26.47
4		2030		21.808		26.64
5		1969		21.800		28.23
6		1945		21.795		32.04
	Equivalent to sample concentration (%)			0.006		
		Impurity A4 (239 nm)				
1	0.0312	869	9.2	25.025	0.05	13.74
2		936		25.031		14.07
3		1035		25.041		15.53
4		1061		25.041		15.37
5		1014		25.032		15.60
6		855		25.014		16.05
	Equivalent to sample concentration (%)			0.003		
		Nicardipine hydrochloride (239 nm)				
1	0.0326	697	5.2	28.121	0.04	15.16
2		615		28.101		12.79
3		624		28.115		13.17
4		605		28.131		12.60
5		628		28.109		14.14
6		647		28.109		16.24
	Equivalent to sample concentration (%)			0.003		

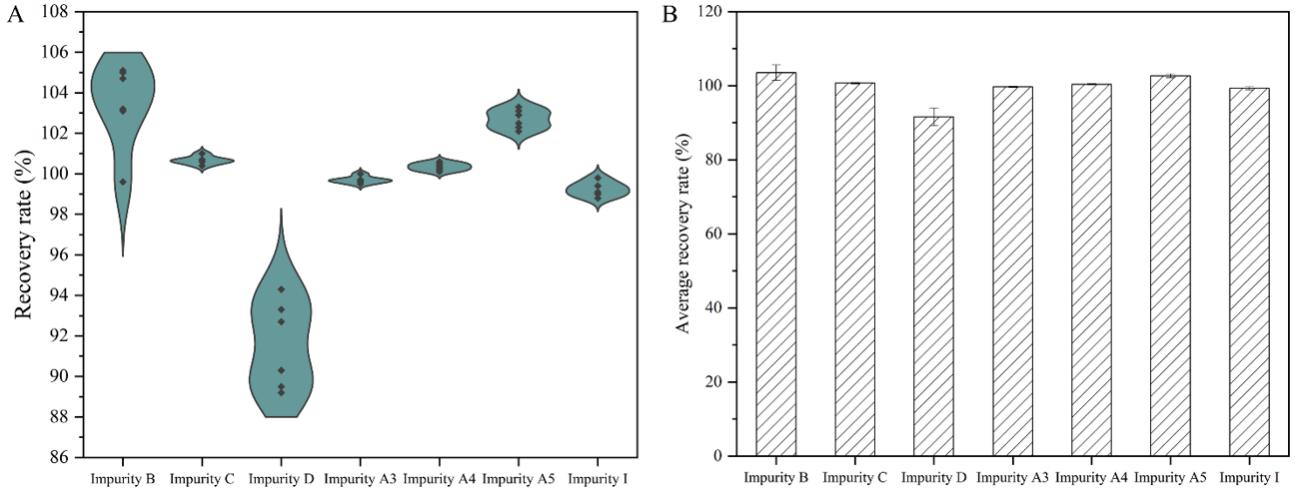

Fig. 4: (A) Results of impurity recovery rate. (B) Results of impurity average recovery rate.

Table 5: Results of impurity Limit of Quantitation (258 nm)

Number	Concentration of LOQ (μg/mL)	Peak area	%RSD	RT (min)	%RSD	S/N of LOQ
			Impurity A5 (258 nm)			
1	0.3173	4584	7.6	16.884	0.2	22.96
2		4502		16.839		11.64
3		4255		16.837		13.33
4		4133		16.795		11.53
5		3720		16.837		10.72
6		4042		16.845		13.09
Equivalent to sample concentration (%)					0.03	
			Impurity I (258 nm)			
1	0.2411	3526	2.8	25.678	0.07	24.99
2		3669		25.669		12.66
3		3573		25.661		14.85
4		3521		25.657		13.67
5		3517		25.668		13.29
6		3370		25.631		15.33
Equivalent to sample concentration (%)					0.02	
			Nicardipine hydrochloride (258 nm)			
1	0.2186	2942	5.3	27.810	0.05	21.87
2		2837		27.789		11.54
3		2654		27.795		12.64
4		3071		27.808		12.83
5		2811		27.823		10.96
6		2730		27.788		13.04
Equivalent to sample concentration (%)					0.02	

Table 6: Results of Impurity Limit of Detection

Name	Concentration of LOD (μg/mL)	S/N			Equivalent to sample concentration (%)
Impurity B (239 nm)	0.0229	9.95	11.01	11.53	0.002
Impurity C (239 nm)	0.0126	5.20	5.93	7.56	0.001
Impurity D (239 nm)	0.0212	3.19	5.18	3.95	0.002
Impurity A3 (239 nm)	0.0315	12.82	14.27	17.66	0.003
Impurity A4 (239 nm)	0.0156	6.20	7.30	9.37	0.002
Impurity A5 (258 nm)	0.1586	4.96	4.76	7.08	0.02
Nicardipine hydrochloride (239 nm)	0.0163	8.80	7.11	8.95	0.002
Impurity I (258 nm)	0.1206	5.28	5.00	7.70	0.01
Nicardipine hydrochloride (258 nm)	0.1093	4.26	4.17	6.62	0.01

Method validation results

System Suitability. table 2 displayed the results of the system suitability test. The system suitability data met the acceptable standards. The relative standard deviation (RSD) of the impurity peak area for five injections of the reference solution did not exceed 2.0%. Similarly, the RSD of the impurity peak retention time for five injections of the reference solution was below 1.0%. The HPLC system was suitable for analysis.

Specificity. The retention time of each impurity peak in the mixed solution was identical to that of the corresponding impurity peak in the located solution. Based on the forced degradation data presented in Table S1 and fig. 2, it is evident that the placebo does not cause any disruption to the primary peak and impurity peak in the sample solution. In all circumstances, the separation of impurities and major peaks had a resolution exceeding 1.5 and the purity factor of the main peak was higher than 990. Under various conditions, the difference between the external standard content and purity of the degraded sample was not more than 5%, indicating that the specificity of the method was good.

Linearity. Fig. 3 demonstrated that the primary constituents and associated compounds exhibited a linear relationship throughout the specified range. Plot the calibration curve that represents the relationship between the concentration of the analyte and the corresponding peak area. The test demonstrated a high degree of linearity, as evidenced by a correlation coefficient (R2) of 0.9999, which above the threshold of 0.995. The impurities exhibited a high degree of linearity, as indicated by the R2 values exceeding 0.995. Additionally, the intercept was found to be within 10% of the response value at the maximum limit of 100%. All of the outcomes satisfied the specified criteria. It showed that the linear relationship was good.

Accuracy. The results in fig. 4 and table S2 showed that the recovery rates of impurities at 100% concentration levels met the criteria, the method had high accuracy (Moberly *et al.*, 2020; Zhang *et al.*, 2021). (The acceptance requirements for this test are as follows: the recovery rate must fall within the range of 85.0% to 110.0% and the relative standard deviation (RSD) must not exceed 10.0%).

Precision. From the results in table 3, it can be seen that the precision of impurities met the criteria. Acceptance criteria (Repeatability and Intermediate precision): X indicates the content of impurities in the solution of the sample. X <0.05%, not counting RSD; 0.05% ≤X < 0.10%, RSD ≤10.0%; 0.10% ≤X <0.50%, RSD ≤ 5.0%; X ≥0.50%, RSD ≤2.0%. Acceptance criteria (Precision): X indicates the content of impurities in the solution of the sample. X <0.05%, not counting RSD;0.05% ≤X < 0.10%, RSD ≤30.0%;0.10% ≤X <0.50%, RSD ≤ 15.0%; X ≥0.50%, RSD ≤7.5%.

LOD and LOQ. The acceptance criteria for LOQ and LOD were met, as indicated by the findings presented in Table 4-6. The acceptance criteria for LOQ were as follows: the RSD of the peak area should not exceed 10.0%, and the S/N should be equal to or more than 10. For LOD, the S/N should not be less than 3.

Robustness. Table S3 showed that under different chromatographic conditions, when the chromatographic conditions change (the pH 2.2 of buffer salts, organic phase (acetonitrile-methanol) ratio of mobile phase B (68: 32), column temperature 52°C, flow rate 0.9 mL/min), the separation of impurity A3 and impurity D was not good. In the chromatographic columns of different manufacturers, the resolution of impurity A5 and impurity D was less than 1.2, and the separation of impurity B and impurity A4 was not good. The sample solution met the criteria (Acceptance criteria: X indicates the content of impurities in the solution of the sample. X < 0.05%, the difference between the content of sample solution at different chromatographic conditions and the content at normal conditions is not compared; 0.05% ≤X < 0.10%, the difference ≤ 0.05%; 0.10% ≤X < 0.50%, the difference≤ 0.10%; X ≥0.50%, the difference≤ 0.20%. To sum up, the above chromatographic conditions need to be strictly controlled.

Solution Stability. table S4-S5 and table 10 results showed that after the mixed solution of impurity reference substance were placed for 44 h, its peak area is 95% - 105% compared with 0h, which proved were stable within 44 hours. The sample solution stable within 37 hours and met the criteria (Acceptance criteria: X indicates the content of impurities in the solution of the sample. X < 0.05%, the difference is not compared; 0.05% ≤X < 0.10%, the difference ≤0.05%; 0.10% ≤X < 0.50%, the difference ≤0.10%; X ≥0.50%, the difference≤ 0.20%; When new impurities appear, the difference≤ 0.05%).

DISSCUSION

In existing literature reports, most methods had only used single wavelength detection for the compound of nicardipine. For example, Fernandes and LM HT *et al.* (2003) mentioned in their study that using HPLC method to detect the injection level of nicardipine in plasma .. Although this method can detect the level of nicardipine to some extent, the recovery rate was low and the separation degree between compounds in the chromatogram was low. Kharad *et al.* (2011) proposed an isocratic HPLC method for detecting nicardipine (but this method could only detect the nicardipine compound and could not detect its accompanying impurities. At present, there are few suitable methods to simultaneously detect and achieve good separation of nicardipine and its related substances. At the same time, there are also few research reports on the related substances in nicardipine injection. This is because the maximum absorption wavelength of some related substances in nicardipine is different, which makes it difficult to effectively detect them with a single wavelength. In response to the limitations of existing research and detection methods for nicardipine injection, this article developed a dual wavelength HPLC method to determine the main components and impurities in nicardipine hydrochloride injection. This study validated the entire method and achieved good results. The main component and related substances in nicardipine injection were well separated, and the method was able to isolate 7 related substances, which is currently not available in other methods. This will help us better understand and use nicardipine injection in clinical practice, ensuring its clinical safety and effectiveness. Through forced degradation experiments, it was found that nicardipine injection is unstable and degrades more violently under thermal conditions, providing some basic suggestions for the daily storage and clinical use of the injection.

However, the technology used in this study also has certain limitations, that is, the stability of this method still needs to be further improved. For example, when there are significant changes in the mobile phase ratio and pH, it may cause a decrease in the separation degree of some impurities. In addition, the detection time of this method is 70 min, which will reduce efficiency and increase detection costs in daily testing. Shortening the testing time is very necessary, especially in the production of pharmaceutical companies, which is particularly important. From the chromatogram, there were basically no other impurities detected after 35 min, so shortening the detection time is very feasible.

CONCLUSION

This study describes the development of a reversed-phase The principal components and impurities in nicardipine hydrochloride injection were determined by HPLC

method with optimal conditions established by dual wavelengths. The HPLC method for optimal conditions developed in the study was validated with system suitability as required, and the sample was found to be unstable and more violently degraded under thermal conditions by forced degration experiments. There was no interference from placebo in the specificity validation, and impurities achieved good separation, all above 1.5. The concentration of impurities and the peak area was linear with r^2 > 0.995. The repeatability results were good, and the %RSD met the requirements. The reference solution is stable within 44 hours under the refrigeration condition of 2~8 °C, and the test solution is stable within 37 hours under the refrigeration condition of 2~8°C. Therefore, this method is suitable for the determination of related substances in nicardipine hydrochloride injection. When certain chromatographic conditions change, poor resolution of some impurities can be caused, so it is necessary to strictly control the buffer pH, column temperature, flow rate, the ratio of acetonitrile to methanol in mobile phase B and the chromatographic column.

high-performance liquid chromatography (HPLC) method that fulfils the necessary criteria for specificity, system application, limit of quantification, detection limit, linearity, precision and accuracy. The approach should be assessed in accordance with the regulations set by the International Council for the Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The results of the technique validation were deemed acceptable. Hence, this technique is appropriate for the identification of nicardipine hydrochloride and its contaminants.

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