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Development and validation of analytical methods for the simultaneous determination of nebivolol hydrochloride and hydrochlorothiazide in pharmaceutical dosage forms

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ABSTRACT

A simple, selective, rapid, precise and economical reverse phase high-pressure liquid chromatographic RP-HPLC) and HPTLC methods have been developed for the simultaneous estimation of Nebivolol Hydrochloride and Hydrochlorothiazide for tablet formulations. The retention times of Nebivolol Hydrochloride and hydrochlorothiazide were 3.6 and 2.5 minutes respectively in RP-HPLC method. The linearity of the method was studied over the concentration range of 3 to 18 µg/ml for Nebivolol Hydrochloride and 6 to 36 µg/ml for hydrochlorothiazide. The clear separations of peaks were found for Nebivolol and Hydrochlorothiazide respectively in HPTLC method. The limit of detection and limit of quantitation were found to be 70 ng/spot & 90 ng/spot for Nebivolol and 110 ng/spot & 140 ng/spot for Hydrochlorothiazide respectively. The proposed method could apply for the quantitative determination of Nebivolol Hydrochloride and hydrochlorothiazide in pharmaceutical dosage forms.

Keywords: Nebivolol HCl, Hydrochlorothiazide, RP-HPLC, HPTLC

INTRODUCTION

Nebivolol HCl is chemically known as α,α′-[Iminobis(methylene)]bis[6–fluoro–3,4–dihydro–2H-1–benzopyran–2– methanol] and has been used in the management of hypertension [1]. It is white powder, soluble in methanol and practically insoluble in water [2,3]. Nebivolol HCl is a highly selective β1-blocker with nitric oxide mediated vasodilatory actions and also effects on vascular endothelial function [4,5]. Hydrochlorothiazide is chemically 6-Chloro–3,4– dihydro–2H-1,2,4 benzothiadiazine–7–sulfonamide1,1–dioxide and its a white crystalline powder and practically insoluble in water [6]. It is a thiazide diuretic and increases sodium and chloride excretion in distal convoluted tubule. Nebivolol HCl and hydrochlorothiazide have been formulated in a fixed dose tablet dosage form and used in the treatment of hypertension.

A few reports pointed out analytical methods include UV-spectrophotometry, high performance liquid chromatography (HPLC) and HPTLC for simultaneous quantification of nebivolol HCl and hydrochlorothiazide [7,8]. These present investigations develop a simple, eco-friendly and

economical analytical methods like RP-HPLC and HPTLC for simultaneous quantification of nebivolol HCl and hydrochlorothiazide in tablet dosage form. The analytical method also validated as per ICH guidelines.

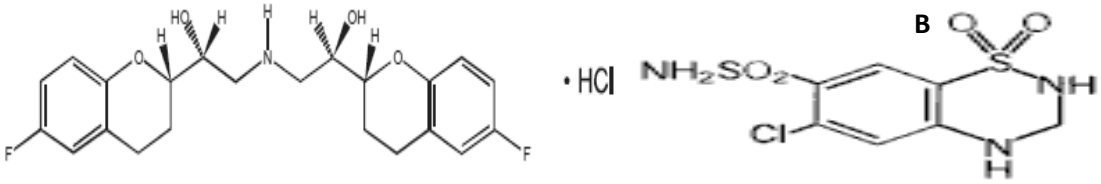

Figure 1: Chemical structure of a) Nebivolol HCl and b) Hydrochlorothiazide

MATERIALS AND METHODS

Materials

Working standards of pharmaceutical grade Nebivolol and Hydrochlorothiazide were obtained as gift samples from Dr.Reddys Laboratories Ltd. India and Lupin Ltd. India respectively. Nebistar H Tablet is composed of the following salts Hydrochlorothiazide (12.5 mg) and Nebivolol (5 mg) was purchased. All chemicals and reagents of analytical grade were purchased from Merck Chemicals, Mumbai, India. High purity deionized water was obtained from Millipore, Milli-Q (Bedford, MA, USA) water purification system.

METHODS

RP-HPLC analysis

Mobile phase

Weighed about 1.36 g of Potassium dihydrogen orthophosphate dissolved in 1000 ml of HPLC water and pH adjusted to 5.5 with orthophosphoric acid and filtered through a 0.45 µ membrane filter and degassed. Buffer and Acetonitrile were mixed in the ratio of 35:65 v/v and filtered through a 0.45 µ membrane filter and degassed.

Preparation of standard solution

Weighed about 50mg of Nebivolol hydrochloride RS and 125mg of Hydrochlorothiazide RS in a two 100ml volumetric flask, dissolve the content and the volume make up with mobile phase. Pipette out 1ml of Nebivolol Hydrochloride (5 µg/ml) and 1ml of Hydrochlorothiazide (12.5 µg/ml) in a 100ml volumetric flask and the volume make up with mobile phase and filtered through a 0.45 µ membrane filter and degassed.

Preparation of sample solution

Twenty tablets were weighed and triturated to a fine powder. A quantity of powder equivalent to 5mg of Nebivolol hydrochloride and 12.5mg of Hydrochlorothiazide was weighed and transferred to a 100ml volumetric flask. The powder was dissolved by sonication with sufficient amount of mobile phase and then made up to the mark with mobile phase. The solution was filtered through a 0.45 µmembrane filter. Pipetted out 5ml of above solution and transferred in 50 ml volumetric flask and volume make up with same solvent so as give a concentration of 5 µg/ml of Nebivolol hydrochloride and 12.5 µg/ml of Hydrochlorothiazide.

VALIDATION PARAMETERS

Linearity

A minimum of five concentrations were recommended for linearity studies. Varying quantities of standard stock solution was diluted with the mobile phase to give concentrations of 3-18 µg/ml of Nebivolol Hydrochloride and 6-36 µg/ml of Hydrochlorothiazide respectively. A calibration curve was constructed for each of the drugs independently by plotting the peak areas against concentrations. There exists a linear relationship in the two graphs showing concentrations ranging from 3-18 µg/ml of Nebivolol Hydrochloride and 6-36 µg/ml of Hydrochlorothiazide respectively. From the data obtained correlation coefficient, y-intercept and slope were calculated to provide mathematical

estimates of the degree of linearity. The relationship between the concentration and response (peak area) should be linear in the specified range and the correlation coefficient should be around 1.000

Accuracy/recovery

The accuracy of the method was determined by recovery experiments. The recovery was performed by adding Nebivolol hydrochloride and Hydrochlorothiazide working standard to placebo in the range of 80% to 120% of test concentration (80%, 100%, and 120% levels) and expressed as % recovered.

Stability of Analytical Solutions

The stability of analytical solutions has been established by injecting the standard solution and sample solution HPLC after 24 and 48 hours by storing the solutions at room temperature. The responses of standard solution and sample solution are measured and the % differences of peak area with respect to initial area value were calculated.

Robustness

The evaluation of robustness should be considered during the development phase and depends on the type of procedure under study. It should show the reliability of an analysis with respect to deliberate variations in method parameters.

HPTLC analysis

Preparation of standard solution

About 25mg of Nebivolol Hydrochloride and 60mg of Hydrochlorothiazide was accurately weighed and transferred to a 100ml volumetric flask dissolve completely with methanol, and further made it to volume with same solvent to get the stock solution. From the above solution take 10ml into 50ml volumetric flask (Stock solution contains 50 µg/ml and 120 µg/ml respectively).

Preparation of sample solution

Twenty tablets were weighed and triturated to a fine powder. A quantity of powder equivalent to 5mg of Nebivolol hydrochloride and 12.5mg of Hydrochlorothiazide was accurately weighed and transferred to 100 ml volumetric flask dissolved with methanol and made the volume with same solvent. From the above solution pipette out 5ml of the above solution in 50 ml volumetric flask made up with same solvent. The resulting solution was filtered through a 0.45µ membrane filterand first few ml of the filter was rejected. It is then suitably diluted to yield 5 µg/ml of Nebivolol Hydrochloride and 12.5 µg/ml of Hydrochlorothiazide which are used for the estimation.

Procedure

Ten microliters of each of the standard and sample solution were spotted on the HPTLC plate using automatic application device. The chromatoplate was then developed in twin trough chamber containing the mobile phase. After development, it was scanned at 215 nm and the peak areas were measured.

Linearity

A calibration curve was constructed for each of the drug independently by plotting the peak areas against concentrations. There exists a linear relationship in the two graphs showing concentrations ranging from 5-25 µg/ml of Nebivolol hydrochloride and 12.5-62.5 µg/ml of Hydrochlorothiazide respectively.

Precision

The precision of an analytical method were carried out by assaying a sufficient no. of aliquots of a homogeneous sample to be able to calculate statistically valid estimate of % RSD.

Recovery (Accuracy)

To ensure the accuracy of the method, recovery studies were carried out by addition of a known quantity of the standard drug to the pre-analysed samples (50,100 and 150%) of target level) and the whole content were reanalysed by the proposed method.

RESULTS AND DISCUSSION

RP-HPLC analysis

The working condition for the HPLC method was established for simultaneous estimation of

Nebivolol hydrochloride and Hydrochlorothiazide in pharmaceutical dosage forms. Various mobile phase systems were prepared and used to provide an appropriate chromatographic separation, but the proposed mobile phase comprising of Buffer: Acetonitrile in the ratio of 35:65 v/v gave a better resolution and sensitivity. The detection was carried out by using UV-Visible detector at 282 nm. System suitability parameters such as tailing factor, theoretical plate and tesolution were calculated and the results are furnished in **Table 1**. The selectivity is established by injecting blank (diluent), Nebivolol and Hydrochlorothiazide sample preparations into the chromatograph were shown in **Figure 2**. It was observed that there is no interference of the peaks in the chromatograms of blank and standard preparation. Hence, the chromatographic method is selective and specific.

Nebivolol and Hydrochlorothiazide standard preparations were subjected to stress conditions/agents (forced degradation studies) such as, 0.1M Hydrochloric acid, 0.1M Sodium hydroxide, and at high temperature (108°C). There is no interference of standard preparation at the RT of Nebivolol hydrochloride and Hydrochlorothiazide standard peak. Peak purity test also performed (Purity angle should be less than purity threshold).The linearity of the HPLC method used for the assay was evaluated by injecting standard concentration of Nebivolol and Hydrochlorothiazide ranging from 3-18 µg/ml (25-150% of target) and 6-18 µg/ml (25-150% of target) respectively. A summary of the data showing the slopes, y-intercepts values are furnished in **Table 2**.

The correlation coefficients for standard preparation of Nebivolol and Hydrochlorothiazide are 0.999 and 0.999. The relationship between the concentration and response (peak area) of Nebivolol hydrochloride and Hydrochlorothiazide is linear in the range examined as all the points fall in a straight line and the correlation coefficients are within the specified limit. The precision for the Nebivolol hydrochloride and Hydrochlorothiazide were evaluated by using homogeneous sample in six times determination for both system precision and method precision (100% target). The data are furnished in **Table 3**. Overall assay of Nebivolol hydrochloride and Hydrochlorothiazide ranges from 99.74% -100.85% with grand mean of 100.29% and 99.82% – 100.94% and grand mean of 100.38%. The percentage relative standard deviation of Nebivolol hydrochloride and Hydrochloro-thiazide are within the specified limit (2%).

Accuracy/ Recovery for the Nebivolol and Hydrochlorothiazide were determined by fortifying sample and standard drug substances at concentration from (80 to 120% of target level. The dataare furnished in **Table 4**. Overall recovery of Nebivolol hydrochloride ranges from 99.13% - 100.09% and grand mean of 99.62% whereas Hydrochlorothiazide ranging from 99.28% – 99.96% grand mean of 99.57%. The % RSD for all recovery values (3 concentrations) are within the range of 2.0%. The percentage relative standard deviation for Nebivolol hydrochloride and Hydrochlorothiazide was found to be 0.2 and 0.3. Hence, the method is accurate in the range of 80% to 120% of test concentration.

The solution stability was evaluated for Nebivolol hydrochloride and Hydro-chlorothiazide standards and sample at assay concentrations. As per method prescribed for the assay of Nebivolol hydrochloride and Hydrochlorothiazide are stable minimum up to 24 hrs. To evaluate method robustness a variety of parameters were deliberately modified such as flow rate, mobile phase ratio and wavelength were evaluated. Therefore, the proposed RP-HPLC method can be utilized for the routine simultaneous estimation of Nebivolol hydrochloride and Hydrochlorothiazide in pharmaceutical dosage form.

HPTLC analysis

The proposed method shows that the chromatographic layer gives the best separation of the two component in the mobile phase consisting of toluene: Ethyl acetate (60:40); other system like Chloroform: Methanol (80:20); where the components move along with solvent front; chloroform: Methanol: Formic acid (8.0:1.5:0.5) where the component (Hydrochlorothiazide) not moved. Finally Chloroform: Methanol: Acetic acid (8.0:1.8:0.2) gave the complete separation with R_f values of Nebivolol hydrochloride and Hydrochlorothiazide were 0.21 ± 0.02 and 0.53 ± 0.02 respectively. Total separation time for both components was reasonably short. The HPTLC

graph of Nebivolol hydrochloride and Hydrochlorothiazide were shown in **Figure 3 and 4**.

The linearity of the HPTLC method used for assay was evaluated by spotting standard concentration of Nebivolol hydrochloride and Hydrochlorothiazide ranging from 5-25 µg/ml and 12.5-62.5 µg/ml respectively. A summary of the data showing the slopes, y-intercept value, P-value are furnished in **Table 5**. The correlation coefficient all assay of Nebivolol hydrochloride and Hydrochlorothiazide were all greater than 0.999. In addition, the analysis of residuals for the assay Nebivolol hydrochloride and Hydrochlorothiazide shows that the values of randomly scattered around zero which show a good fit with the linear model. To evaluate whether the y-intercepts were significantly different than zero, the P- value was determined for each line. If P-value was >0.05 then the intercept was considered statistically equal to zero.

The precision for the Nebivolol hydrochloride and Hydrochlorothiazide were evaluated by using homogeneous sample in six times determination (100% of target) the data are furnished in **Table 6**. Overall assay of Nebivolol hydrochloride ranged from 98.41% to 100.04% with the mean value of 99.37%.Hydrochlorothiazide ranged from 99.23% to 100.41% with the mean value of 99.75%. The accuracy of Nebivolol hydrochloride and Hydrochlorothiazide was determined by fortifying sample and standard drug substances at concentration from 50 – 150% of target level. The data are furnished in **Table 7**. Overall recovery of Nebivolol hydrochloride ranged from 98.88% to 101.29% with the mean value of 99.9%. Hydrochlorothiazide ranged from 99.09% to 101.20% with the mean value of 100.1%. All the above parameters combined with the simplicity and care of operation ensure the use of proposed method in the assay of pharmaceutical dosage form containing this combination.

Table 1: System suitability test (RP-HPLC)

S. No	Parameter	Nebivolol	Hydrochlorothiazide
01	Retention time	3.630	2.517
02	Asymmetry factor	1.29	1.44
03	Capacity factor	0.10	0.08
04	Column efficiency	7293	5485
05	Resolution	7.2	

Table 2: Linearity range of Nebivolol and Hydrochlorothiazide (RP-HPLC)

S. No	Nebivolol		Hydrochlorothiazide	
	Conc. (µg/ml)	Peak area	Conc. (µg/ml)	Peak area
01	3	166.452	6	55.589
02	6	337.309	12	111.152
03	9	506.251	18	165.192
04	12	675.205	24	221.501
05	15	843.759	30	274.755
06	18	1012.511	36	332.892
	Slope	56.368		9.2078
	y-intercept	-1.6121	+0.1502	
	Correlation coefficient (r^2)	0.999998	0.999952	

Table 3: Statistical data for precision (RP-HPLC)

Sample No	System Precision		Method Precision	
	Nebivolol hydrochloride	**Hydrochloro -thiazide**	**Nebivolol hydrochloride**	**Hydrochloro -thiazide**
1	100.86	100.91	99.92	99.99
2	100.62	100.50	100.16	100.14
3	101.15	101.13	99.86	100.07
4	100.52	101.18	99.53	99.29
5	101.11	100.98	99.27	99.61
6	101.68	101.59	99.93	99.13
Mean	100.85	100.94	99.74	99.82
% RSD	0.2	0.2	0.3	0.3
	Nebivolol hydrochloride		**Hydrochlorothiazide**	
Grand mean	**100.29**		**100.38**	
%RSD	**0.2**		**0.2**	

Table 4: Accuracy/ recovery (RP-HPLC)

% Level		Nebivolol	Hydrochlorothiazide
80		99.79	99.28
		99.64	99.82
		99.61	99.41
	Mean	99.68	99.50
	% RSD	0.1	0.3
100		99.13	99.60
		99.87	99.44
		100.09	99.33
	Mean	99.70	99.46
	% RSD	0.5	0.1
120		99.59	99.96
		99.20	99.95
		99.65	99.38
	Mean	99.48	99.76
	% RSD	0.2	0.3
Grand mean		**99.62**	**99.57**
% RSD		**0.3**	**0.2**

Table 5: Analytical performance parameter (HPTLC)

Parameter	Nebivolol hydrochloride	Hydrochlorothiazide
Slope	151.12 ± 2.8254	88.59 ± 1.000
y-intercept	33.593 ± 46.854	98.54 ± 41.48
Correlation coefficient	0.9992	0.9996
p-value of intercept	0.23	0.21
Percentage of intercept at Quantification level	+4.19	+1.60
Limit of detection(ng/spot)	70	110
Limit of quantification (ng/spot)	90	140

Table 6: Precision data (HPTLC)

	Nebivolol hydrochloride	Hydrochlorothiazide
	98.41	99.49
	100.04	99.65
	99.29	99.23
	99.81	100.41
	98.96	99.96
	99.73	99.78
Mean	**99.37**	**99.75**
% RSD	0.5	0.3

Table 7: Accuracy data (HPTLC)

% of target		Nebivolol hydrochloride	Hydrochlorothiazide
50		100.86	100.36
		101.29	100.23
		99.62	101.20
	Mean	100.6	100.6
	%RSD	0.9	0.5
100		99.92	100.21
		99.45	101.06
		100.29	99.88
	Mean	99.9	100.4
	%RSD	0.4	0.6
150		98.88	99.66
		99.12	99.12
		99.65	99.09
	Mean	99.2	99.3
	%RSD	0.3	0.3
Grand mean		**99.9**	**100.1**
%RSD		0.8	0.8

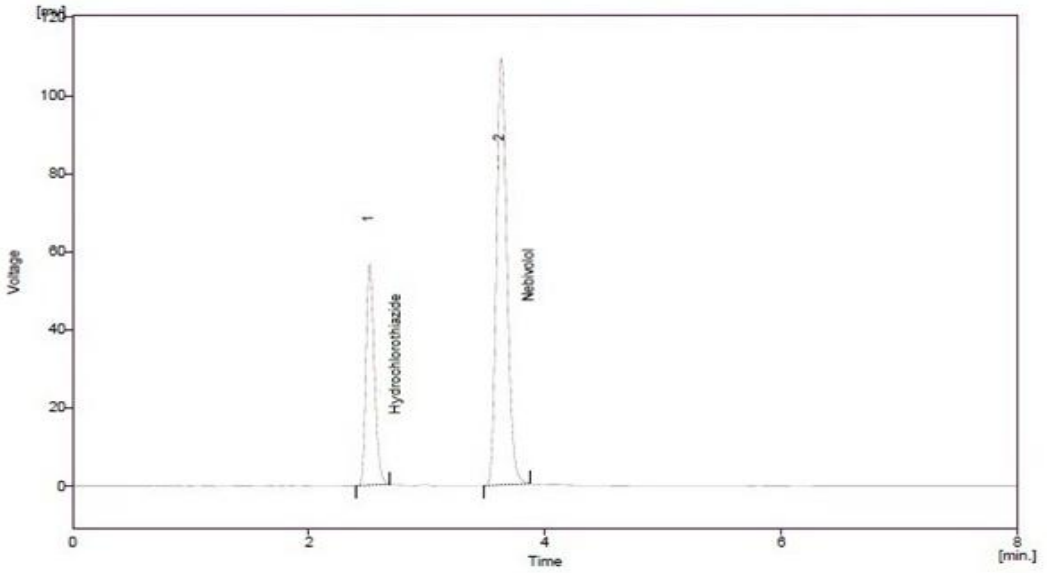

Figure 2: Chromotogram peak of Nebivolol hydrochloride and Hydrochlorothiazide

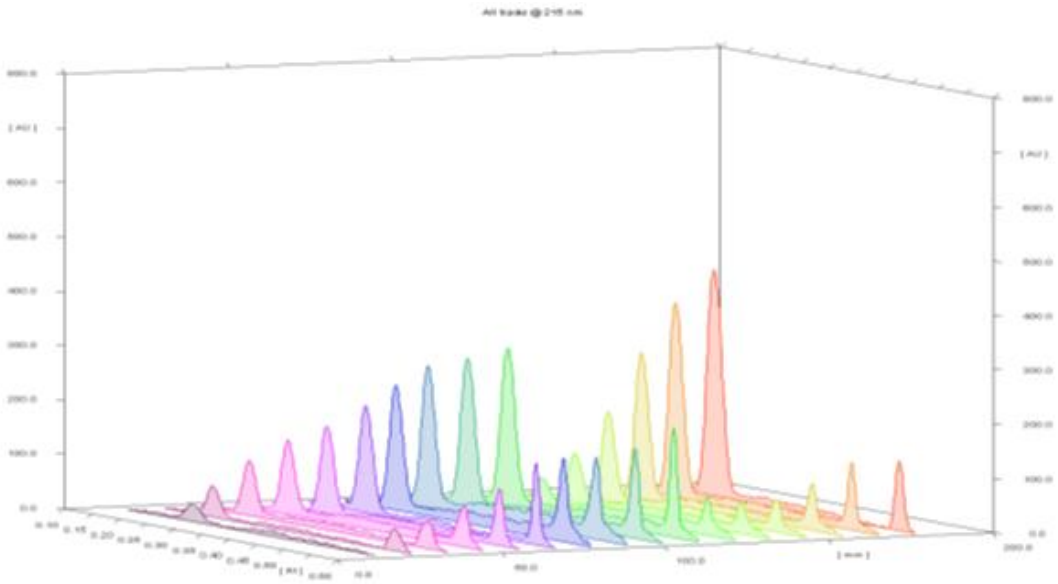

Figure 3: 3 Dimensional spectra of Nebivolol and Hydrochlorthiazide (HPTLC)

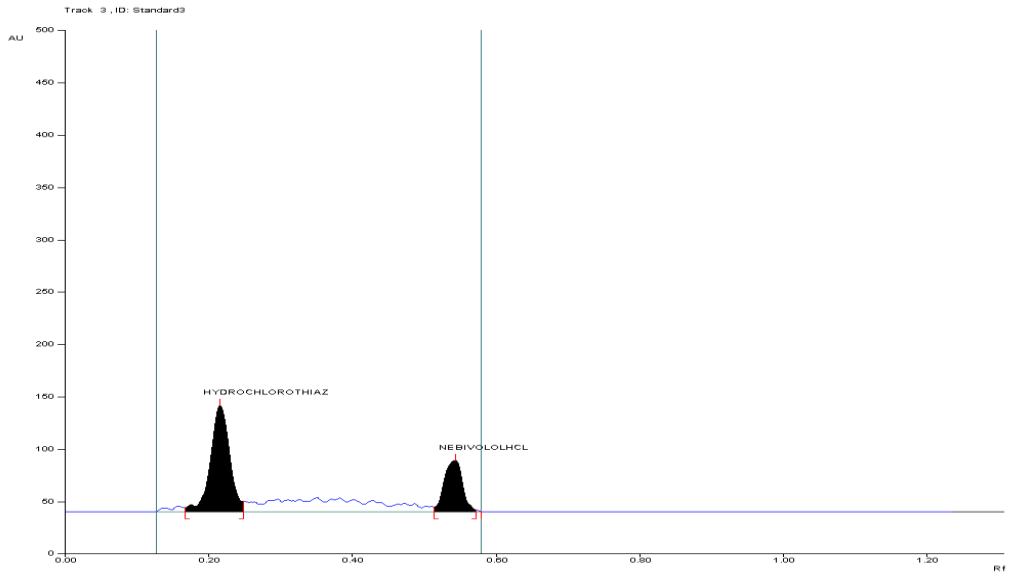

Figure 4: Densitogram of Nebivolol hydrochloride and Hydrochlorothiazide (HPTLC)

CONCLUSION

The UV Spectrophotometry, RP-HPLC and HPTLC methods developed in the present study are thus shown to be simple, rapid, accurate, precise, specific, linear and rugged. They are thus suitable for the estimation of raw materials and formulations the newly developed analytical methods may be used in research institutions, quality control department in industries, approved testing laboratories and clinical pharmacokinetic studies.

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