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Determination of phosphate binding capacity in sevelamer carbonate by HPLC using refractive index (RI) detector

Kumarswamy Ummiti*, Kalyan Reddy Chennuru, Sudhakar Vakkala, Venugopal Dama, Malleswara Reddy Annarapu
Generics- Analytical Research and Development, IPDO-Innovation Plaza, Dr. Reddy's Laboratories Limited, Hyderabad-500090, (INDIA)
E-mail : kumar.ummiti@gmail.com

ABSTRACT

Sevelamer carbonate is the active ingredient of Renvela® tablets; a cross linked polymeric amine that binds phosphate. It was developed as a pharmaceutical alternative to sevelamer hydrochloride (Renagel®). Renvela® is indicated for the control of hyperphosphatemia in patients with end-stage renal disease. An in vitro phosphate-binding assay is required to measure the drug's efficiency. Several attempts have been made in the prior art to develop analytical methods like ion chromatography, high performance capillary electrophoresis and ultraviolet spectroscopy for determining phosphate binding for sevelamer. A significant disadvantage of high performance capillary electrophoresis are the high concentration limit of detection and high expensive. The reported ultraviolet spectroscopy method involves derivitisation. Unfortunately difficulty of carry out and time consumption are one of the disadvantages of ultraviolet spectroscopy method. A novel isocratic reverse phase HPLC method has been developed in order to detect and quantify the bound phosphate. This method involves mixing the drug with a solution of known phosphate concentration, filtering off the polymer-phosphate complex, and quantitating the unbound phosphate concentration by HPLC using refractive index detector. The method was validated according to ICH guidelines. The presented method provides equal sensitivity with ion chromatography, high sensitive, specific, and inexpensive analytical procedure and therefore is highly suitable for in-process and stability analysis of sevelamer carbonate API.

KEYWORDS

Sevelamer carbonate;
HPLC;
Phosphate binding;
Validation.

INTRODUCTION

Sevelamer carbonate (Figure 1) cross-linked poly (allylamine carbonate), is a potent phosphate binder used for the reduction of serum phosphate levels in patients with end-stage renal disease (ESRD)[1-4]. It contains multiple amines separated by one carbon from the polymer backbone. These amines become partially protonated in the intestine and interact with phosphate ions through ionic and hydrogen binding.

Sevelamer carbonate is the active ingredient of Renvela® tablets. It was developed as a pharmaceutical alternative to sevelamer hydrochloride (Renagal®). The advantage of Renvela® for end-stage renal dis-

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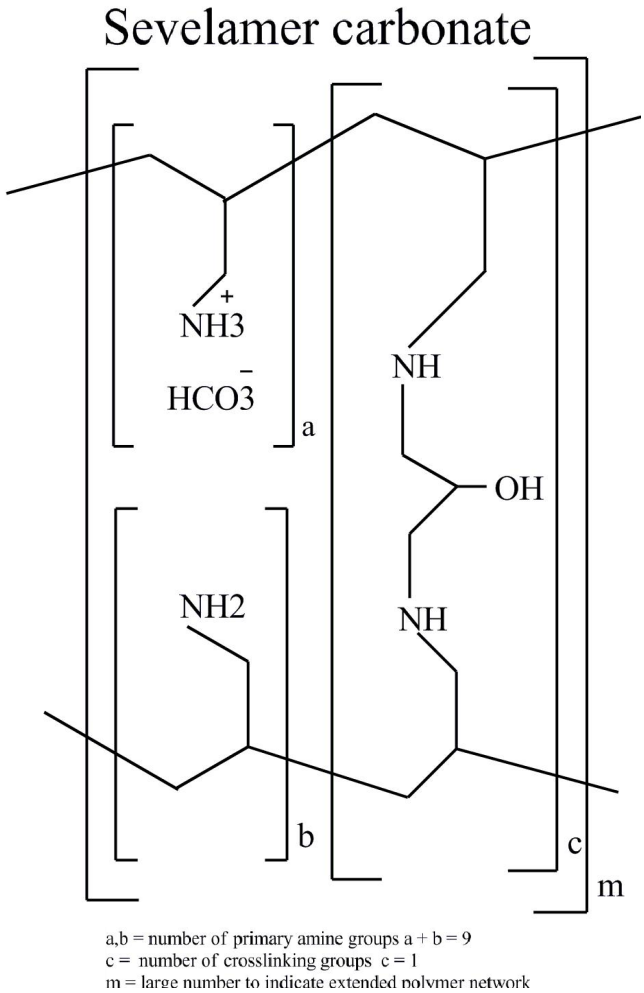

Figure 1:Structure of sevelamer carbonate

ease (ESRD) over existing therapies, calcium or aluminium supplementation, is that it is non-absorbed, leading to an improved safety profile. An in vitro phosphate-binding assay study is the important aspect of the analytical characterization of sevelamer carbonate. Several attempts have been made in the prior art to develop analytical methods like ion chromatography, high performance capillary electrophoresis and ultraviolet spectroscopy for determining phosphate binding for sevelamer[5-7]. A significant disadvantage of high performance capillary electrophoresis are the high concentration limit of detection and high expensive. The reported ultraviolet spectroscopy method involves derivitisation. Unfortunately difficulty of carry out and time consumption are one of the disadvantages of ultraviolet spectroscopy method. But till now there are no reports on in vitro phosphate binding assay for sevelamer carbonate by HPLC.

This paper describes in vitro phosphate binding assay for sevelamer carbonate using isocratic HPLC method with refractive index (RI) detector. The proposed HPLC method was validated as per ICH guidelines[8] and its updated international convention. The presented method provides equal sensitivity with ion chromatography, specific, and inexpensive analytical procedure and therefore is highly suitable for detect and quantify the bound phosphate by sevelamer carbonate API.

EXPERIMENTAL

Materials and reagents

Sevelamer carbonate API was purchased from Formosa laboratories, Inc. (Taiwan, China). N, N-Bis(hydroxyethyl)-2-aminoethanesulfonic acid (BES) was purchased from Sigma Aldrich (St. Louis, MO). Formic acid, ammonia, sodium chloride, sodium hydroxide and potassium phosphate monobasic (KH_2PO_4) were purchased from Merck (Mumbai, India). All chemicals were of analytical grade.

Apparatus

All chromatographic experiments were carried out on HPLC system with a refractive index detector (Waters, USA). The system was controlled and acquired data processed by the Empower software (Waters, USA). Shaking water bath (Lab companion, model: BS-11, Global medical instrumentation, Inc., Ramsey, Minnesota, USA) was used for sample preparation.

Chromatographic conditions

Chromatographic separations were performed on an Allsep anion column (150 mm, 4.6 mm i.d., 7µm particle size) (Grace Davison Discovery Sciences, Deerfield, USA). Mobile phase contains 0.05% (*v/v*) formic acid, the pH of the solution was adjusted to 3.8 with ammonia. The flow rate was 1.0 mL min^{-1}. The column temperature was maintained at 60 °C and optical unit temperature (detector temperature) was maintained at 35 °C. The injection volume was 10µL. Attenuation (detector sensitivity) was 64 and polarity was positive. The chromatographic run time was 20 min. A typical phosphate standard chromatogram is shown in Figure 2.

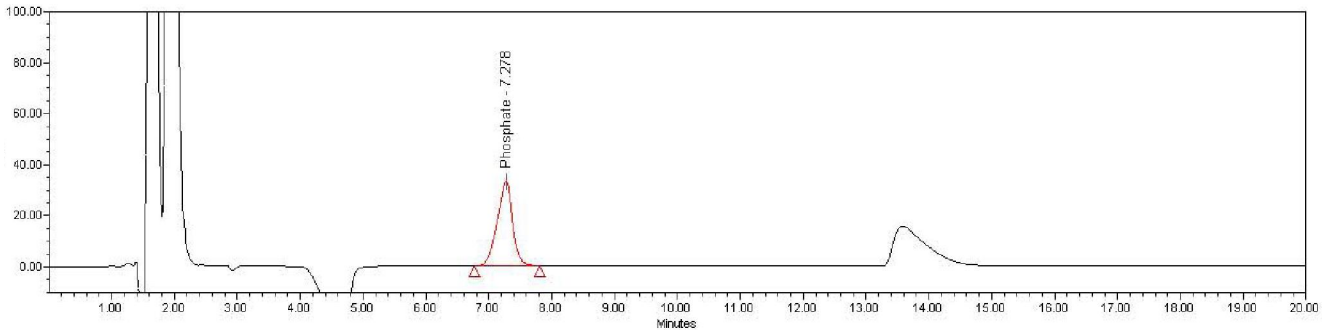

Figure 2: Representative HPLC chromatogram of phosphate standard solution.

Sample preparation

A stock phosphate solution containing 20 mM KH_2PO_4, 100 mM BES and 80 mM sodium chloride was prepared. The pH of the solution was adjusted to 6.95 (± 0.05) with 1N sodium hydroxide. System suitability solutions were prepared by diluting the phosphate stock to get the concentration of phosphate 10 mM. 12 mM. 14 mM. 16 mM and 18 mM. These solutions were filtered through 0.22 µm membrane filter (Millipore).

To perform the binding step, duplicate samples of sevelamer carbonate about 100 mg were weighed into a 250 mL conical flask, to which 100 mL of 20 mM phosphate stock solution was added. The flasks were then capped and kept in shaking water bath with 100 RPM at 37 ± 2 °C for 120 min. Approximately 7 mL of the resultant slurry was filtered through membrane filter (Millipore). Binding capacities for the duplicate samples were required to agree within 2%.

System suitability

The system suitability test was performed to ensure that the chromatographic assay was suitable to the analysis intended. System suitability solutions containing 10 mM. 12 mM. 14 mM. 16 mM and 18 mM of phosphate concentration were injected into HPLC. A linearity graph was plotted by taking concentration on X-axis and peak response of phosphate on Y-axis. From this, coefficient of correlation (R^2) value was calculated. The coefficient of correlation (R^2) value should be not less than 0.990.

Calculations

The unbound phosphate concentration remaining in a sample was calculated with the following equation:

$$y = a\,x + b;\quad x = (y - b)/a$$

where 'x' is unbound phosphate concentration, 'y' is peak area of phosphate solution in the sample preparation, 'a' is slope of the linearity graph and 'b' is intercept of the linearity graph.

The phosphate binding capacity. in mmol of phosphate g of Renvela®, was calculated as follows:

$$\frac{\text{(20 mM – unbound phosphate concentration) x 0.1 lit x100}}{\text{g of polymer x (100 - LOD)}}$$

where 20 mM is the phosphate concentration applied, *unbound phosphate concentration* is the unbound phosphate concentration, in mM, determined by HPLC, 0.1 lit is the volume of 20 mM phosphate solution applied, *g of polymer* is the weight of sevelamer carbonate used, and LOD is the loss on drying of the sample obtained by thermo gravimetric analysis.

Validation procedure

The chromatographic method was validated by evolution of the analytical parameters including specificity, linearity, precision, accuracy and robustness.

Specificity

A stock solution containing 100 mM BES and 80 mM sodium chloride was prepared. The pH of the solution was adjusted to 6.95 (± 0.05) with 1N sodium hydroxide. Duplicate samples of sevelamer carbonate about 100 mg were weighed into a 250 mL conical flask, to which 100 mL of stock solution was added. The flasks were then capped and kept in shaking water bath with 100 RPM at 37 ± 2 °C for 120 min. Approximately 7 mL of the resultant slurry was filtered through membrane filter (Millipore). This solution was used as placebo for identifying any interference at the retention time of phosphate peak.

Linearity

A stock phosphate solution containing 40 mM

KH_2PO_4, 100 mM BES and 80 mM sodium chloride was prepared. The pH of the solution was adjusted to 6.95 (± 0.05) with 1N sodium hydroxide. Linearity solutions were prepared by diluting the phosphate stock to get the concentration of phosphate 4.8 mM. 8.0 mM. 11.2 mM. 14.0 mM, 20.0 mM, 24.0 mM and 28.0 mM. These solutions were filtered through 0.22 µm membrane filter (Millipore). Standard plots were constructed and linearity was evaluated by statistically by linear regression analysis that was calculated by least-squares regression.

Precision

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Six sample solutions of sevelamer carbonate were prepared on two different days. The results were expressed as an RSD of the analytical measurements.

Accuracy

The accuracy was determined by adding known amounts of the sevelamer carbonate API to the sample. The added levels were 75%, 100% and 125% of the sample preparation. The results were expressed as the percentage of bound phosphate in the sample. All solutions were prepared in six times and assayed.

Robustness

To determine the robustness of the method the experimental conditions were deliberately changed and the R^2 value of the linearity solutions was evaluated. To study the effect of flow rate on R^2 value it was changed to 0.8 and 1.2 mL min^{-1}. The effect of column temperature was studied at 55 °C to 65 °C. The effect of pH was studied at pH 3.6 and pH 4.0 for mobile phase. In all these experiments the mobile phase components were not changed.

RESULTS AND DISCUSSION

Method development and optimization

The main objective of the chromatographic method was to separate critical closely eluting phosphate and blank peaks and to elute phosphate as a symmetrical peak. Attempts were made with isocratic elution with mobile phase using different pH buffers (pH 3.4 - pH 4.5), but separations between phosphate and blank peaks was not satisfactory if the buffer pH is beyond 4.0 and run time of the chromatogram increasing if the buffer pH is below 3.6. The buffer pH was fixed as pH 3.8. Normal C18 and C8 columns are not useful for phosphate binding analysis. A different type of column like Allsep Anion Column (150 mm, 4.6 mm i.d., 7µm particle size) has been used for method development and good resolution was achieved. Attempts were made with different column temperatures (35-65 °C), but phosphate peak symmetry was not satisfactory if the column temperature is below 55 °C. The column temperature was fixed as 60 °C.

System suitability

In pharmaceutical analysis (quality control), the suitability tests were focused on the analytical instrument and method. The analytical results are only valid if the defined system suitability criteria are fulfilled. In this work, the experimental results (TABLE 1) indicated that the chromatographic system was suitable for the intended analysis. The retention time for phosphate peak was 7.28 min. The coefficient of correlation (R^2) value calculated from the system suitability solutions was 1.000.

Validation procedure

Specificity

There was no peak found at the retention time of phosphate in blank and placebo chromatograms prove no interference from blank and placebo (Figure 3a and Figure 3b).

Linearity

The correlation coefficient, slope and y-intercept of the calibration plot are reported in TABLE 2. The calibration plot was linear over the ranges tested. The correlation coefficient was >0.998. This result shows there was an excellent correlation between the peak area and concentration for the phosphate.

Precision

The precision of the method verified by injecting six individual preparations of sample solutions. The results are shown in TABLE 3. In intra-assay precision, the % RSD of phosphate binding was s found within 2%. The intermediate precision was deter-

TABLE 1 : Results from system suitability determination

Concentration (mM)	Area	Average	%RSD	Regression equation(y)		
				Slope (b)	Intercept (a)	Correlation coefficient
10.0	542414					
10.0	543835	543021	0.1			
10.0	542814					
12.0	654420					
12.0	653623	653622	0.1			
12.0	652822					
14.0	763962					
14.0	761510	761958	0.2	55643.44	-14318.2	1.000
14.0	760402					
16.0	875858					
16.0	877500	876920	0.1			
16.0	877402					
18.0	988898					
18.0	985870	987786	0.2			
18.0	988590					

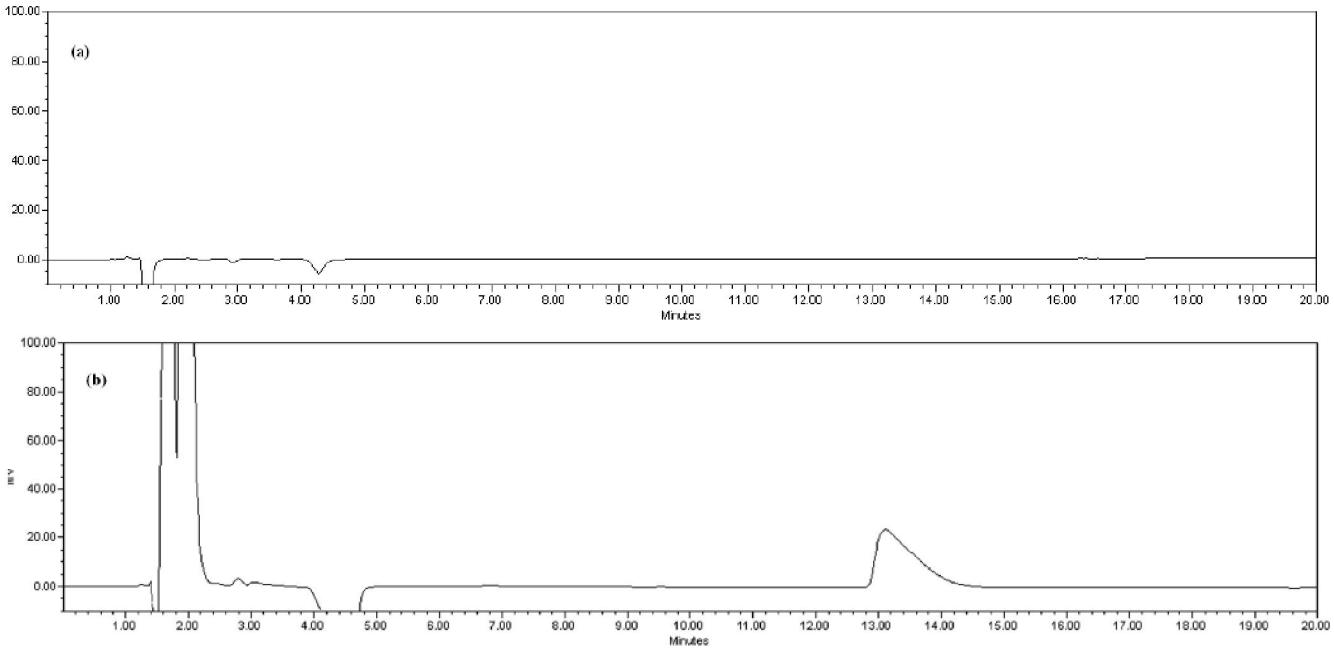

Figure 3 : Representative HPLC chromatogram of (a) blank (b) placebo.

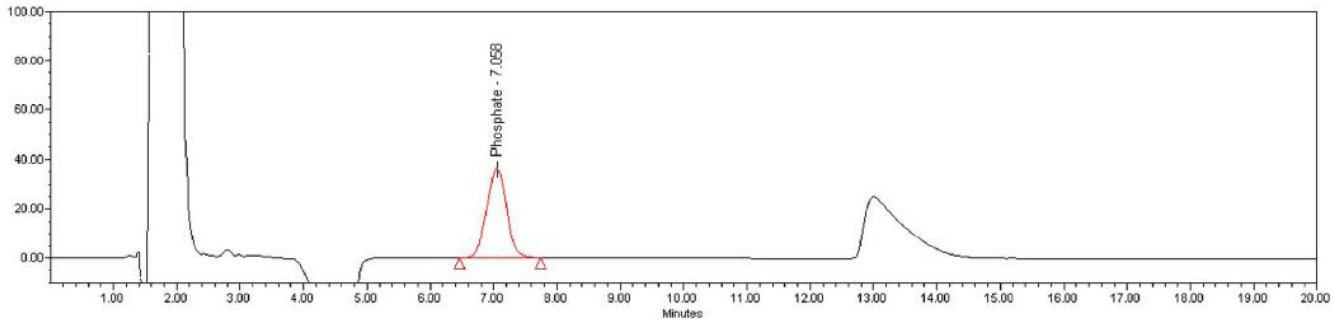

Figure 4 : Representative HPLC chromatogram of test.

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TABLE 2 : Linearity data

Concentration (mM)	Area	Average	%RSD	Regression equation (y)		
				Slope (b)	Intercept (a)	Correlation coefficient
4.8	254484					
4.8	254395	253860	0.4			
4.8	252700					
8.0	424188					
8.0	422405	424672	0.6			
8.0	427423					
11.2	595488					
11.2	600034	599980	0.7			
11.2	604417					
14.0	755803					
14.0	757174	754848	0.4	54302.379	-8085.685	1.000
14.0	751567					
20.0	1079048					
20.0	1069986	1073579	0.4			
20.0	1071704					
24.0	1292500					
24.0	1294794	1294887	0.2			
24.0	1297368					
28.0	1515603					
28.0	1513094	1514397	0.1			
28.0	1514494					

TABLE 3 : Precision data

Analyst	Phosphate Binding (mmol/g) (n=6)						Average (mmol/g) (n=6)	% RSD[a] (n=6)
Intra-day Precision	5.57	5.56	5.54	5.59	5.58	5.61	5.56	0.46
Inter-day Precision	5.60	5.67	5.67	5.64	5.62	5.62	5.63	0.49

[a]**Relative Standard deviation of six results**

mined by analysis in different day with different analyst using different HPLC system. The low values of RSD for both the repeatability and intermediate precision demonstrate the good precision of the method proposed. A HPLC chromatogram obtained from a sample is shown in Figure 4.

Accuracy

The average value of phosphate binding in repeatability i.e., 5.56 mmol/g was taken as 100%. By comparing this value, the accuracy of the method was evaluated for three concentration levels, i.e. 75%, 100% and 125%. The percent of recovery was calculated at each level and found in the range of 95.0-105.0 % (TABLE 4).

Robustness

Robustness is the ability to reproduce the analytical method under different circumstances without the occurrence of unexpected differences in the obtained results. To determine the robustness of the method the experimental conditions were deliberately changed and the correlation coefficient (R^2) value of the system suitability solutions was evaluated (TABLE 5). The coefficient of correlation (R^2) value should be not less than 0.990. In all the deliberate varied chromatographic conditions the selectivity as well as the performance of the method were unchanged proves the robustness of the

TABLE 4 : Accuracy data

Accuracy level	Weight of sevelamer carbonate (mg)	Phosphate binding (mmol/g)	% Recovery	Average % recovery (n=6)	%RSD [a] (n=6)
75%	75.2	5.57	100.2		
75%	75.8	5.60	100.8		
75%	75.1	5.58	100.3	100.5	0.7
75%	75.0	5.64	101.5		
75%	75.0	5.53	99.5		
75%	74.8	5.59	100.6		
100%	101.9	5.57	100.2		
100%	102.1	5.56	100.0		
100%	102.8	5.54	99.6	100.3	0.4
100%	101.8	5.59	100.5		
100%	101.4	5.58	100.4		
100%	102.4	5.61	100.9		
125%	125.0	5.51	99.0		
125%	124.7	5.51	99.0		
125%	124.9	5.49	98.8	98.8	0.2
125%	125.4	5.48	98.5		
125%	125.4	5.48	98.6		
125%	125.4	5.49	98.7		

[a] **Relative Standard deviation of six results**

TABLE 5 : Robustness data.

Robustness parameter	Correlation coefficient (R^2 value)
pH 3.6 buffer	1.000
pH 4.0 buffer	1.000
Column Temperature-55°C	0.998
Column Temperature-65°C	1.000
Flow rate-0.8 mL/min	0.997
Flow rate-1.2 mL/min	0.998

method.

CONCLUSION

A validated isocratic RP-HPLC method was developed for in vitro phosphate binding assay of sevelamer carbonate using refractive index detector. The method is simple, specific, linear, precise, accuarate, robust and can estimate phosphate binding capacity of the drug as per ICH requirements. Hence the method can be used for routine analysis of phosphate binding assay in bulk samples and for assay of pharmaceutical formulations. The method has obvious advantages over those previously reported such economical, easy to carry out and high separation efficiency.

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