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Comparison of In Vitro Phosphate-Binding Studies of Sevelamer Carbonate Using Incubator Shaker and USP Dissolution Apparatus II

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ABSTRACT

In the case of most phosphate-binding agents, in vitro phosphate-binding studies are essential for establishing bioequivalence between generic and reference drug formulations. Traditionally, an incubator shaker is used to conduct phosphate-binding studies, but this method is limited by manual sample collection and associated variability. This study aims to evaluate an automatic sampling method using a dissolution tester as an alternative to incubator shakers. Kinetic phosphate-binding studies of sevelamer carbonate tablets (test and reference formulations) were performed using an incubator shaker and USP dissolution apparatus 2. The results from both methods were compared. Though both formulations were equivalent in terms of the phosphate-binding capacity in both methods, the automatic sampling method using the dissolution apparatus had less variability than the manual sampling method using the incubator shaker. Thus, the dissolution apparatus is preferred for bioequivalence and interchangeability studies of sevelamer carbonate tablets.

KEYWORDS: Sevelamer carbonate, phosphate binding, dissolution, ion chromatography

INTRODUCTION

Sevelamer carbonate is a novel cross-link polymer containing allylamine carbonate. This unique drug is indicated for patients suffering from end-stage kidney disease to control and reduce serum phosphate levels (*1*) . The parent molecule, sevelamer hydrochloride, was first developed in 1997 (*2*). Both molecules have a similar mechanism of action, sequestering excess phosphate molecules present in the gastrointestinal tract, thereby hindering the absorption of phosphate and eliminating them via defecation (*2, 3*). Implementation of sevelamer therapy has yielded successful control of phosphate levels in patients with hyperphosphatemia and reduction of vascular calcification in dialysis patients. There are some disadvantages to using sevelamer hydrochloride, mainly because it reduces the concentration of serum bicarbonate, which increases the risk of developing metabolic acidosis in patients undergoing hemodialysis and causes gastrointestinal irritation (*4*). Sevelamer carbonate is preferred because it has a similar mechanism of action but increases serum bicarbonate rather than reducing it.

Due to the inherent characteristics of sevelamer hydrochloride and carbonate molecules as non-absorbable polymers, it is challenging to develop an analytical method to quantify them in blood serum during in vivo studies. Hence, it is difficult to establish bioavailability and bioequivalence (BE) between a reference listed drug (RLD) and generic drug via the conventional in vivo method. The United States Food and Drug Administration (FDA) has provided draft guidance for the pharmaceutical industry on proving BE an in vitro method (*5*). Two in vitro methods are recommended by FDA to evaluate equilibrium phosphate binding and kinetic phosphate binding. In the equilibrium phosphate-binding study, the drug product is exposed to eight different concentrations of phosphate for a predetermined duration to determine the phosphate-binding capacity at various concentrations. In the kinetic phosphate-binding study, the drug product is tested at the lowest and highest concentrations of phosphate used in the equilibrium phosphate-binding study to investigate the binding of phosphate over time. The time that shows maximum phosphate binding in the kinetic study is used

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for the equilibrium study. For both studies, the phosphate solutions should contain 80 mM of sodium chloride and 100 mM of N, N-Bis (hydroxyethyl)-2-aminoethanesulfonic acid) (BES), prepared at pH 4 and pH 7. Whole tablets of the drug product are used directly (as is) or pre-treated with acid (*6*). Phosphate-binding studies are conducted by placing sample solutions onto an orbital shaker (referred as "incubator shaker" in this study) at 37 °C for 2 hours and manually sampled and filtered. The concentration of phosphate ranges from 1–38.7 mM (*6*).

Many other related works have used a similar sample incubation method where samples were shaken or stirred and sampled manually at predetermined intervals. In each work, sevelamer carbonate tablets were tested in 300 mL of phosphate solution at pH 3.0–7.0, and phosphate concentrations of 1–40 mM (*7*–*9*). This method poses a few constraints for sevelamer carbonate BE studies. When kinetic phosphate-binding studies are performed using multiple manual sampling time points, it is difficult to ensure the accuracy of sampling time and volume. When multiple samples are tested, more time is required for sample collection at each time point.

To address these issues, this study evaluates an alternative method for conducting BE studies with sevelamer hydrochloride and carbonate using USP dissolution apparatus 2 (*10*). To our knowledge, there is currently no published work on the concept of using a dissolution apparatus for phosphate-binding studies. This work aims to demonstrate the suitability of using a dissolution tester as compared to an incubator shaker in kinetic phosphate-binding studies of sevelamer carbonate conducted at pH 4 and pH 7 with 1–40 mM phosphate concentration and without acid pre-treatment of the sample. This work does not include equilibrium phosphate-binding studies.

MATERIALS AND METHODS

Materials

Sevelamer carbonate tablets (800 mg, Renvela) were sourced from Sanofi-Aventis Canada Inc and used as the RLD in this work. In-house formulated sevelamer carbonate tablets (800 mg) were used as the test formulation.

BES used was procured from Fisher Scientific. Sodium hydroxide 50% w/w was obtained from Fischer Chemical. Other chemicals such as sodium chloride, sodium hydroxide, 37% hydrochloric acid (HCl) and potassium dihydrogen phosphate were sourced from Merck. Milli-Q water was supplied in-house via the Millipore Milli-Q System. All chemicals used are of reagent grade or higher. Whatman UNIFLO syringe filters with a pore size of 0.45 µm were used.

Equipment

The Dionex Aquion Ion (Thermo Scientific) chromatography system coupled to a Dionex AS DV Autosampler (Thermo Scientific) was used to quantify the amount of phosphate in each sample. The system was set up with a Dionex-Ion Pac AS-11 analytical column (250 × 4 mm) and an AG-11 guard column (50 × 4 mm) (Thermo Scientific). Suppression was achieved with Dionex ADRS 600 Suppressor (Thermo Scientific). The Electrolab 14 Station (EDT-14Lx Offline) dissolution tester fitted with USP apparatus 2 was used for testing samples. Sample collection was performed by Dx Sample Collector (Electrolab). Samples were also shaken with New Brunswick Innova 40/40R Shaker (Eppendorf).

Sample Preparation

An aqueous solution containing 100 mM BES and 80 mM sodium chloride was prepared and pH was adjusted to pH 4 and 7 using 10 N sodium hydroxide, which was prepared by dissolving 40 g of sodium hydroxide pellets in 100 mL of Milli-Q water. Phosphate solutions at concentrations of 1 and 40 mM were prepared by dissolving a known amount of potassium dihydrogen phosphate in the prepared aqueous solution. HCl solution (1 N) was prepared for pH adjustment.

For the experiment conducted in the incubator shaker, sevelamer carbonate tablets (RLD and test formulations) were added to 300 mL of 1 and 40 mM phosphate solutions. For the pH 4 samples, an appropriate amount of 1 N HCl solution was added to the phosphate solution to maintain pH of samples at pH 4. For the pH 7 samples, 300 mL of phosphate solutions were used without any pH adjustment needed. All samples were prepared in triplicates. The samples were shaken with the incubator shaker at 37 °C, 100 rpm for 4 hours. At each time point (0.5, 1, 2, 3, and 4 hours), samples were removed manually using a micropipette and the same volume was replenished using the respective pH 4 and 7 aqueous solution.

For the experiment conducted in the dissolution tester, sevelamer carbonate tablets (RLD and test formulations) were added into 500 mL of 1 and 40 mM phosphate solutions preheated to 37 °C and run at 100 rpm. Similary to incubator shaker, pH adjustment of pH 4 samples with 1 N HCl solution was required. All samples were prepared in triplicates. The dissolution tester was set to collect the sample from each vessel at 0.5, 1, 2, 3, and 4 hours, and each vessel was replenished with respective pH 4 and pH 7 aqueous solution.

All collected samples from the incubator shaker and dissolution tester were filtered through a 0.45-µm syringe filter then diluted with deionized water. Samples at 40 mM phosphate concentration were diluted 100 times and 1-mM phosphate concentration samples were undiluted.

An eight-point calibration curve for both pH 4 and pH 7 was generated by diluting 40-mM phosphate solution to various concentrations ranging from 1–30 mM. Eight concentrations of phosphate was further diluted 100 times. A graph of the area of phosphate peak versus concentration of phosphate was plotted, and the correlation coefficient was targeted to be more than 0.99.

Chromatographic Conditions

Phosphate content in standards and samples were analyzed by ion chromatography with columns as mentioned above. The eluent used was 30-mM sodium hydroxide, with a flow rate of 1.5 mL/min. Suppression was achieved through recycled eluent mode. The injection volume is 25 µL.

Calculations

As ion chromatography measures the amount of phosphate present in the sample, the unbound phosphate concentration present in each collected sample was calculated using the intercept and slope generated from the calibration curve equation:

$$\textit{Unbound phosphate concentration (mM)} = \frac{\textit{peak area of phosphate - intercept}}{\textit{slope}} \quad (1)$$

The concentration in millimoles of unbound phosphate can be determined by multiplying unbound phosphate concentration (mM) with the initial volume used in milliliters. Similarly, the initial concentration of phosphate used (mM) is converted to millimoles by multiplying by the volume used (mL) in the experiment. Out moles refer to the concentration of phosphate sampled at each time point, as shown in the equation:

$$\textit{Out moles (milli moles)} = \frac{\textit{Sample volume removed at each time point (mL)} \times \textit{unbound phosphate concentration (mM)}}{1000} \quad (2)$$

The amount of unbound phosphate remaining in the sample solution can be determined by subtracting out mols from the amount of unbound phosphate. To calculate the amount of phosphate bound at each time point, deduct the amount of unbound phosphate (mmol) at the respective time point from the amount of unbound phosphate remaining from the previous time point. For the first time point, the amount of phosphate bound can be calculated by subtracting the amount of unbound phosphate (mmol) from the initial phosphate concentration. Finally, the phosphate-binding capacity of each time point can be determined, as shown in the equation below:

$$\textit{Phosphate binding capacity} \left(\frac{mmol}{g}\right) = \frac{\textit{cumulative amount of phosphate bound(mmol) at respective time point}}{\textit{label claim (g)}} \quad (3)$$

Analytical Method Validation

The analytical method was validated as per ICH Q2(R1).

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities, degradants, matric, component, etc. To demonstrate specificity, interference from blank and sevelamer Carbonate API at the retention time of phosphate peak were determined by preparing blank solutions, placebo solutions, and sevelamer carbonate tablet solution in pH 4 and pH 7 aqueous solution, without the presence of phosphate. Results showed that area from blank, placebo, and sevelamer carbonate solutions at the retention time of phosphate peak were not more than 1% of area under the calibration curve for the 40-mM standard solution.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample. Linearity was established at pH 4 and pH 7 and 1–40 mM phosphate concentration. The correlation co-efficient (r) and regression coefficient (r^2) for both linearity plots were 1.00 (Fig. 1).

Accuracy and Precision

The accuracy of an analytical method expresses the closeness of agreement between the value that is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed as trueness. Three concentrations of phosphate were prepared, each level in triplicates, at 0.7, 40, and 50 mM, at pH 4 and pH 7. The recoveries of phosphate were 92.8–101.3% at pH 4 and 96.3–100.6% at pH 7. The precision of accuracy at pH 4 and pH 7 are determined by the percent relative standard deviation (RSD), which was 3.5% and 1.7%, respectively.

Filter Validation

Filter validation is to prove the compatibility of filters that will be used for filtration of solutions of test preparations. The study was conducted at pH 4 by comparing unfiltered

sample with samples filtered with 0.45-µm nylon and PVDF filters, respectively. The results show that there was negligible difference between the unfiltered samples versus filtered samples. The percent difference for was 0.1% for 0.45-µm nylon filtered samples and 0.7% for 0.45-µm PVDF filtered samples. Therefore, both filters are suitable for use.

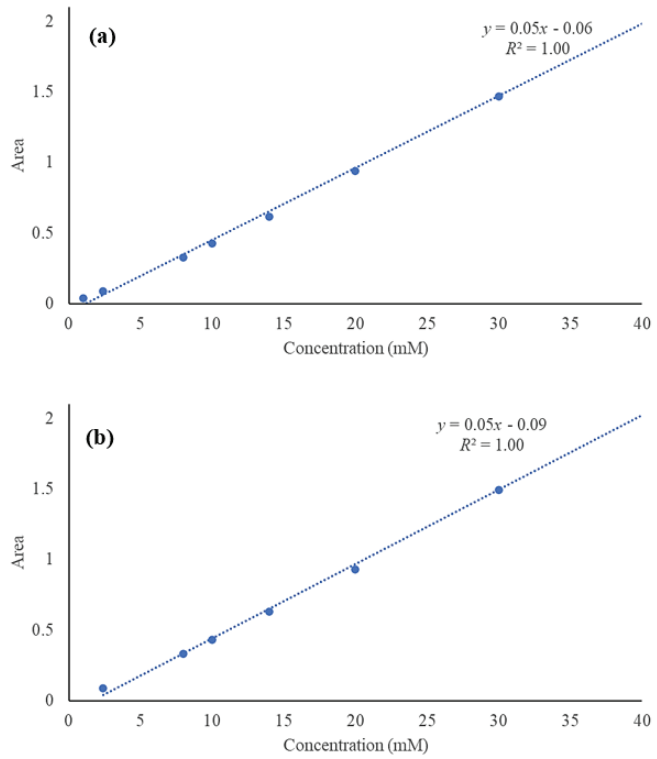

*Figure 1. Linearity plot generated at pH 4 (**a**) and pH 7 (**b**) for 1–40 mM phosphate solution.*

RESULTS

The results are presented in Table 1 (pH 7), Table 2 (pH 4), Figure 2 (40 mM), and Figure 3 (1 mM).

The phosphate-binding capacity values obtained using the incubator shaker demonstrated high RSD, especially at the 30-min time point, and the effect was more prominent with 1 mM and the phosphate-binding capacity is a minuscule value. Fluctuations of phosphate-binding capacity between the test formulation and RLD were observed, more so at the first 30-min time point. The resulting test/reference ratio (T/R) at the first time point had higher fluctuation (range 82–118) as compared to other time points of the same experiment.

On the other hand, phosphate-binding capacity values generated using the dissolution tester were better in terms of RSD and T/R for both conditions. RSD was smaller compared to the incubator shaker, especially in the 1-mM concentration, and the T/R for each timepoint was within 97–104 (Tables 1 and 2). Moreover, the overall profiles of the test formulation and RLD in the dissolution tester for all conditions were similar, with lesser fluctuations, especially in the first few time points.

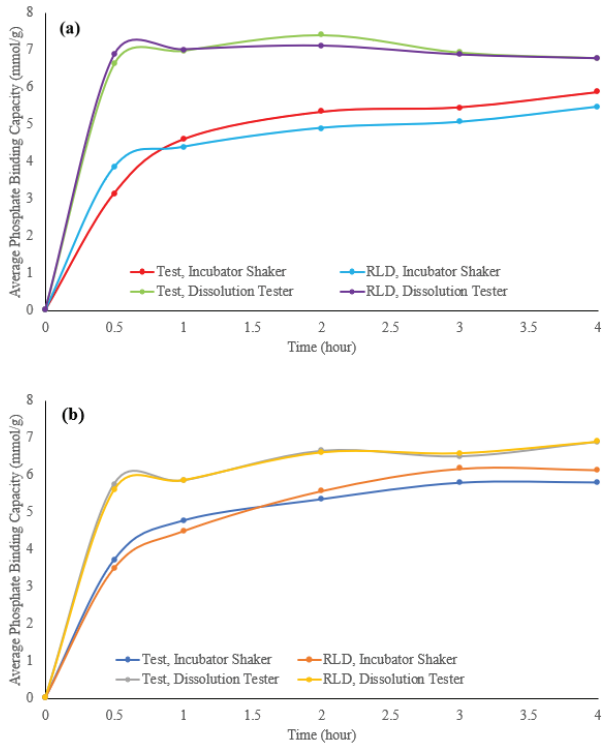

*Figure 2. Phosphate-binding capacity of test versus reference (RLD) in 40-mM phosphate solution at pH 7 (**a**) and pH 4 (**b**).*

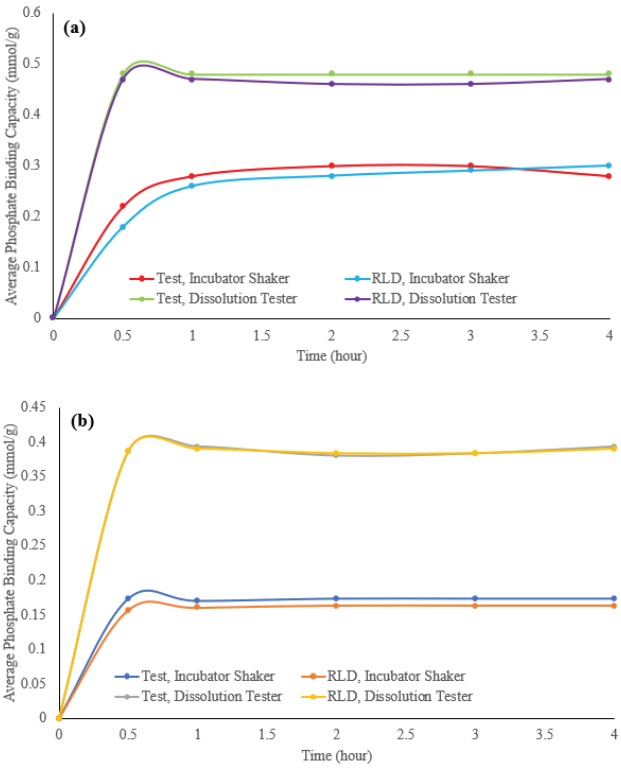

*Figure 3. Phosphate-binding capacity of test versus reference (RLD) in 1-mM phosphate solution at pH 7 (**a**) and pH 4 (**b**).*

Table 1. Results of Phosphate-Binding Capacity Studies at pH 7

Time (h)	Test Formulation			RLD			T/R
	Mean phosphate-binding capacity (mmol/g)	SD	RSD	Mean phosphate-binding capacity (mmol/g)	SD	RSD	
40 mM using incubator shaker							
0.5	3.15	0.20601	6.54	3.86	0.09148	2.37	82
1	4.60	0.14122	3.07	4.40	0.22308	5.07	105
2	5.34	0.29637	5.55	4.91	0.11980	2.44	109
3	5.46	0.26426	4.84	5.07	0.11103	2.19	108
4	5.87	0.28939	4.93	5.48	0.05918	1.08	107
1 mM using incubator shaker							
0.5	0.22	0.03055	13.89	0.18	0.01732	9.62	118
1	0.28	0.00577	2.06	0.26	0.02309	8.88	106
2	0.30	0.00000	0.00	0.28	0.00577	2.06	106
3	0.30	0.00000	0.00	0.29	0.00000	0.00	103
4	0.28	0.00577	2.06	0.30	0.00000	0.00	93
40 mM using dissolution tester							
0.5	6.63	0.13923	2.10	6.87	0.23770	3.46	97
1	6.99	0.14177	2.01	7.01	0.10866	1.55	100
2	7.41	00.13454	1.85	7.11	0.18984	2.67	104
3	6.94	0.10017	1.43	6.87	0.15251	2.22	101
4	6.79	0.12124	1.85	6.77	0.42109	6.22	100
1 mM using dissolution tester							
0.5	0.48	0.00197	0.41	0.47	0.00244	0.52	102
1	0.48	0.00278	0.58	0.47	0.00202	0.43	102
2	0.48	0.00216	0.45	0.46	0.00207	0.45	103
3	0.48	0.00557	1.16	0.46	0.00124	0.27	103
4	0.48	0.00173	0.36	0.47	0.00024	0.05	102

RLD: reference listed drug; SD: standard deviation, RSD: relative SD, T/R: test/RLD ratio.

DISCUSSION

The data generated using the dissolution tester were overall more consistent as compared to incubator shaker. Even though the volume used in both experiments may be different, the data can be discussed objectively by looking at the T/R of each experiment and the variation between samples via RSD. There is better control of T/R with the dissolution tester because the T/R values did not fluctuate drastically (range 97–104) as compared to T/R values of incubator shaker (range 82–118).

One attribute of using a dissolution tester that contributes to improved RSD is automated sampling. A calibrated dissolution tester ensures that each sample volume collected is uniform and the collection time of each sample is simultaneous for all samples, unlike the incubator shaker where manual sampling is required, which carries a greater risk of human error. There is no guarantee that each sampling time is uniform for all the samples when doing manual sampling, especially when multiple samples are taken simultaneously. The volume that is sampled at each time point may also vary. Although the instrument used during manual sampling may be calibrated, it heavily relies on the competency of the user to follow exact procedures to have accurate and precise sample collection.

Another factor to consider when using an incubator shaker is the run must stop to collect the sample at every time point. This not only leads to non-continuous phosphate binding but eventually leads to a delay of subsequent time points. Thus, the T/R data are at risk because the cumulative time delay in sampling the test formulation may not be the same for the RLD, and the extent of phosphate binding will be varied. In the present study, this effect was most prominent at the first time point, as the binding of phosphate occurred rapidly due to the concentration of free phosphate being highest.

Table 2. Results of Phosphate-Binding Capacity Studies at pH 4

Time (h)	Test Formulation			RLD			T/R
	Mean phosphate-binding capacity (mmol/g)	SD	RSD	Mean phosphate-binding capacity (mmol/g)	SD	RSD	
40 mM using incubator shaker							
0.5	3.73	0.20308	5.45	3.50	0.19228	5.49	106
1	4.78	0.11136	2.33	4.50	0.19915	4.43	106
2	5.36	0.21475	4.01	5.58	0.30420	5.46	96
3	5.80	0.17583	3.03	6.18	0.13628	2.21	94
4	5.81	0.11288	1.94	6.14	0.08325	1.36	95
1 mM using incubator shaker							
0.5	0.17	0.01528	8.81	0.16	0.01528	9.75	111
1	0.17	0.01000	5.88	0.16	0.00000	0.00	106
2	0.17	0.00577	3.33	0.16	0.00577	3.53	106
3	0.17	0.00577	3.33	0.16	0.00577	3.53	106
4	0.17	0.00577	3.33	0.16	0.00577	3.53	106
40 mM using dissolution tester							
0.5	5.76	0.11557	2.01	5.61	0.13710	2.44	103
1	5.85	0.11095	1.90	5.86	0.10019	1.71	100
2	6.64	0.13364	2.01	6.61	0.05326	0.81	101
3	6.49	0.03270	0.50	6.59	0.06194	0.94	99
4	6.89	0.08586	1.25	6.90	0.07046	1.02	100
1 mM using dissolution tester							
0.5	0.39	0.00577	1.49	0.39	0.00577	1.49	100
1	0.39	0.00577	1.47	0.39	0.00000	0.00	101
2	0.38	0.00000	0.00	0.38	0.00577	1.51	99
3	0.38	0.00577	1.51	0.38	0.00577	1.51	100
4	0.39	0.00577	1.47	0.39	0.00000	0.00	101

RLD: reference listed drug; SD: standard deviation, RSD: relative SD, T/R: test/RLD ratio.

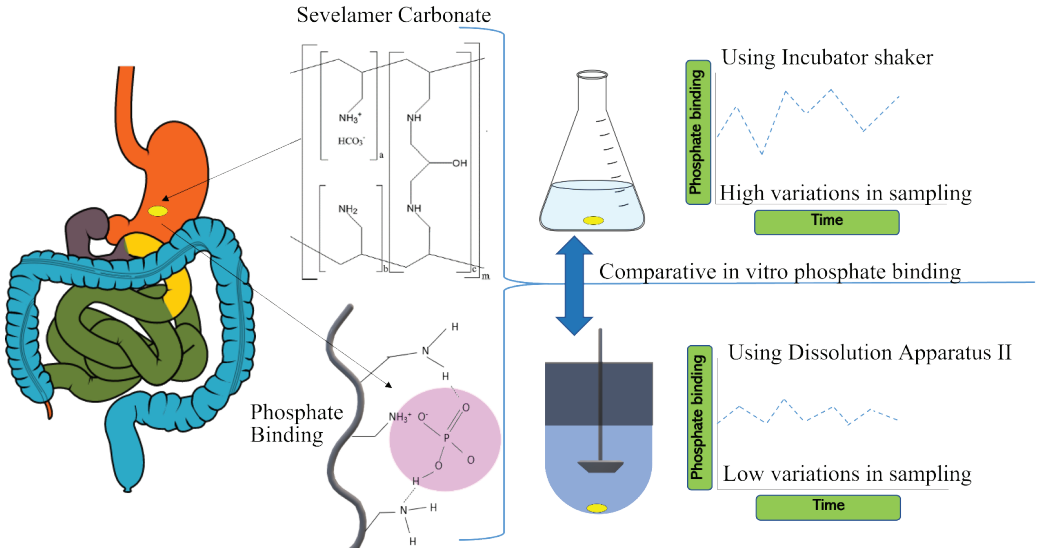

Figure 4. Illustration depicting phosphate binding of sevelamer carbonate (left) and the comparative in vitro phosphate-binding study (right) using an incubator shaker and dissolution apparatus II. "GI System" image (left) by Olek Remesz (https://commons.m.wikimedia.org/wiki/File:GISystem.svg), reused and adapted under the Creative Commons attribution-share alike license 2.5 (https://creativecommons.org/licenses/by-sa/2.5/deed.en).

Especially in the 1 mM solution where the amount of free phosphate is limited, the binding rate of sevelamer to phosphate is rapid and any minor delay in sample collection time greatly affects the phosphate=binding capacity results. This issue can be avoided by utilizing an automated sampling method such as a dissolution tester. Figure 4 illustrates the phosphate-binding process of sevelamer carbonate and compares the in vitro phosphate-binding methods (incubator shaker versus dissolution) investigated in this study.

CONCLUSIONS

The bioequivalence of generic and reference phosphate-binding drug products is established via in vitro methods, so it is important to use a method that is appropriate, reliable, and robust. Implementation of a dissolution tester (automatic sampling) was advantageous compared to an incubator shaker (manual sampling) for establishing the bioequivalence of sevelamer carbonate. Investigation of the suitability for a dissolution tester in vitro studies of other phosphate-binding agents could be beneficial, and if implemented, all in vitro phosphate-binding studies could be harmonized.

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CONFLICT OF INTEREST

The authors disclosed no conflicts of interest related to this article.

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