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Development and validation of a cholate binding capacity method for DMP 504, a bile acid sequestrant

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Abstract

DMP 504, a highly cross-linked insoluble polymer, is a bile acid sequestrant developed by the DuPont Pharmaceuticals Company for serum cholesterol reduction. Since DMP 504 is insoluble, it was necessary to develop unique specific analytical methods to measure and control the quality of different lots of the drug. Since the mechanism of action of DMP 504 is believed to be by sequestration of bile acids, the in-vitro binding capacity of the polymer for cholic acid was chosen as a surrogate of in-vivo performance and used to assess potency of the compound. In this method, individual aliquots of DMP 504 at three different levels were incubated with a cholate solution of known concentration. The residual cholate solution was filtered and analyzed by a reversed-phase HPLC method using refractive index detection. When the bound cholate was plotted versus the mass of DMP 504, the resulting curve was linear. The slope of this curve is the cholate binding capacity of DMP 504. This method has been shown to be precise and robust. Precision of the method was shown to have an RSD of 2.0% with injection precision of 0.4% and stability of cholate solutions up to 73 h. It is also a unique binding capacity method due to its multi-point determination, and it has been shown to be a suitable quality control method for ensuring lot-to-lot consistency of drug substance.

Keywords: Bile acid sequestrant; DMP 504; Cholate binding capacity; Analytical method validation; HPLC refractive index detection

1. Introduction

Bile acid sequestrants have long been used for serum cholesterol reduction [1–3]. The mechanism of action is believed to involve binding bile acids in the small intestine thereby preventing their reabsorption. The liver must, therefore, produce additional bile salts by enzymatically cleaving cholesterol. Some of this cholesterol is derived from the plasma, resulting in a net reduction in plasma levels of cholesterol [4,5]. Bile acid seques-

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trants are polymers which are not absorbed by the body, and therefore, have minimal systemic side effects.

Two commercially available bile acid sequestrants, cholestyramine and colestipol, are polymers with molecular weights $> 1 \times 10^6$ [6]. Both compounds bind bile acids by a non-specific ion exchange mechanism [7–9]. Due to the non-specific binding of bile acids to these compounds, relatively large doses of each are required for an effective reduction in plasma cholesterol levels [10]. A novel bile acid sequestrant, DMP 504 (Fig. 1), was developed by the DuPont Pharmaceuticals Company, which is also believed to bind bile acids by an ion exchange mechanism. This is done by association of the carboxyl group of the bile acids with the protonated amine on DMP 504. The binding of bile acids by DMP 504 has been determined to be cooperative and the binding constant has been determined [11,12]. This bile acid sequestrant is a highly cross-linked polymer synthesized by reductive amination of 1,10-dibromodecane with hexamethylene diamine [13]. It was developed for the purpose of having a higher binding capacity than the commercially available bile acid sequestrants and therefore a lower dose, maintaining the same efficacy.

The highly cross-linked nature of DMP 504 makes it insoluble, and therefore, unsuitable for direct analysis by HPLC or other traditional analytical techniques (e.g. titration) applied to pharmaceutical or soluble polymeric compounds. An indirect method of analysis using the binding property of DMP 504 to bile acids (cholic acid specifically) was evaluated. The measurement of the polymer's in-vitro binding capacity is a surrogate marker of bile acid sequestration in-vivo, and can be used as a measure of drug potency. A higher in-vitro binding capacity should correspond to a higher in-vivo binding capacity, and therefore, greater cholesterol lowering. Bile acid binding assays are quality control tests applied to cholestyramine and colestipol. The cholestyramine binding is 3.7–4.5 mmol/g and is a single-point HPLC method using sodium glycocholate and is performed versus a USP reference standard. The colestipol binding assay is 1.1–1.6 mmol/g determined by a single-point method in which the binding capacity of cholate is determined titrimetrically. [14,15] The method presented here for DMP 504 is unique in that it uses a multipoint determination, where the slope of the line is the binding capacity. It provides a more accurate and precise assay to ensure lot-to-lot consistency, based on potency of the compound. Binding of cholate and glycocholate by DMP504 were shown to have similar properties [12]. Cholate was selected for this method because of its ready availability.

The binding property measured was the maximum binding or total bile acid bound per unit mass of DMP 504 using an excess of bile acid. This will be referred to as the binding capacity. The method was developed using sodium cholate in a multi-point determination. By performing a linear regression of bound cholate versus mass of DMP 504, any bias associated with a non-zero intercept is removed, because the binding capacity

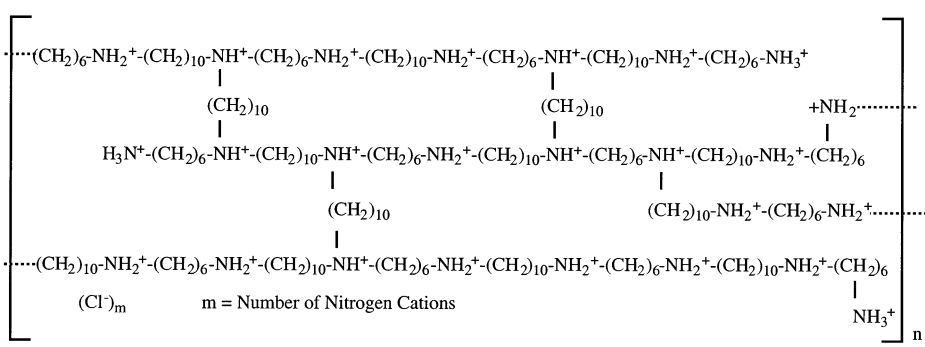

Fig. 1. Representative structure of DMP 504. Chemical Formula: $(C_{18}H_{40}N_2Cl_2)_n$ (approximate). Relative molecular mass: 355.4.

is only a function of the slope and not the intercept. The slope of the line is equal to the binding capacity in mmoles cholate per gram DMP 504. For a discriminating in-vitro binding capacity method for quality control to delineate lot-to-lot differences, the binding conditions were carefully controlled, but there was no attempt to mimic physiological conditions. However, the choice of a pH buffered solution close to physiological pH seemed reasonable. Selection of the binding solution pH was determined based on the solubility of cholic acid in the binding media which determined where the binding capacity had minimal change with respect to pH.

2. Experimental

2.1. Reagents and materials

The cholic acid, sodium salt was obtained from Sigma (St. Louis, MO). The HPLC grade methanol was obtained from Burdick and Jackson (Muskegon, MI), and the HPLC grade water was prepared using a Millipore (Bedford, MA) Milli-Q® system. Glacial acetic acid was purchased from Baxter (Phillipsburg, NJ). The filter units used were Gelman (Ann Arbor, MI) Acrodisk® 0.45 μM nylon. DMP 504 was manufactured by DuPont Pharmaceuticals Chemical Process R&D, Chambers Works, Deepwater, N.J. and Lonza (Visp, Switzerland). Polypropylene labware was used since cholate was found to adsorb to glass.

2.2. Solvent

A 20 mM phosphate buffer using potassium phosphate monobasic was prepared and adjusted to pH 7.0 ± 0.1 with 1.0 N sodium hydroxide.

2.3. Cholate standards

A 20 mM sodium cholate stock solution was prepared using the solvent. Four standards were prepared from this stock solution by diluting with solvent into polypropylene volumetric flasks in the ratios of 5:1, 5:2, 5:3, and 5:4, yielding nominal standard concentrations of 4, 8, 12, and 16 mM.

2.4. Determination of pH for binding capacity

This was determined by visual observation of phosphate buffered solutions of 20 mM cholate for 24 h. Buffered solutions were prepared to be 20 mM in potassium phosphate monobasic (EM Science, Darmstadt, Germany), pH adjusted with sodium hydroxide over a range of 6.0–7.0. The pH was measured after dissolution of the sodium cholate. The relationship of DMP 504 binding capacity to pH was determined in 5 and 20 mM 2-[N-Morpholino]ethanesulfonic acid (MES, J.T. Baker) buffer.

2.5. Binding capacity

Three suspensions of DMP 504 were prepared in 20 mM phosphate buffer at pH 7.0 (± 0.1). Accurate weights of ~ 25, 37.5, and 50 mg of DMP 504 were added to 50-ml polypropylene centrifuge tubes. A 25.0 ml aliquot of the stock cholate standard solution was pipetted into each centrifuge tube and each tube was capped. The centrifuge tubes were laid on their sides and incubated at 37°C in a Lab-Line (Melrose Park, IL) dry air shaker for 16–20 h at a speed of 175–225 rpm. A portion of the supernatant from each vessel was filtered through a 0.45 μm nylon Acrodisk® syringe filter, discarding the first 2 ml through the filter prior to collecting the sample in a polypropylene autosampler vial. The residual cholate solutions were analyzed on a Waters (Milford, MA) HPLC system using a Waters model 410 refractive index detector. The HPLC system conditions are shown in Table 1. From these results, the amount of cholate bound by each sample was calculated as the difference between total cholate and the residual found by HPLC. The binding capacity was then determined by plotting the amount of bound cholate versus the mass of DMP 504 per sample. The binding capacity is the slope of the line in mmol cholate bound/g DMP 504. The amount of incubation time required for the samples to achieve complete binding was determined by studying the binding capacity as a function of time.

Table 1
HPLC conditions[a]

Column	DuPont Zorbax® SB-C8, 15 cm × 4.6 mm i.d.
Column temperature	37°C
Flow rate	0.8 ml/min
Injection volume	25 μl
Detector, refractive index	Sensitivity 32 Scale 20 Internal temperature 37°C

[a] For each unit of mobile phase required, add 730 ml of methanol, 270 ml of water, and 2 ml of glacial acetic acid. Degas and filter through a 0.45-μm filter.

2.6. Linearity of the standards

Linearity was evaluated by preparing four standards over the concentration range of 3.86 mM cholate to 15.42 mM cholate. Linearity for the standards was evaluated by regression analysis of detector response versus standard amount and by residual analysis.

2.7. Precision

The precision of the binding capacity method was determined by three analysts testing the same six lots of DMP 504 independently on different days using different instruments.

2.8. Stability indication

The polymer was subjected to forced degradation by refluxing it in 30% hydrogen peroxide for 4 h. The hydrogen peroxide was then removed by filtration, the DMP 504 was dried and the binding capacity determined. Peroxide was the only means found to degrade the polymer. Acid, base and heat had no effect on the polymer as determined by a lack of change in the binding capacity.

3. Results and discussion

3.1. Determination of optimal pH for binding capacity measurement

The pK_a of cholic acid is 6.4. However, because of the low solubility of cholic acid in aqueous solutions, there is gradual precipitation unless the acid is kept primarily in its dissociated state by maintaining a pH of at least 7.0. Table 2 shows the resulting pH readings and visual observations. When this experiment was repeated using a different lot of sodium cholate from the same supplier, the pH 6.75 solution remained clear for 24 h. In lower pH solutions (6.0–6.5), insufficient buffering capacity exists to maintain a constant pH. In addition, all but the pH 7.0 buffered solution had precipitation of cholic acid in 24 h. The precipitation of cholic acid under slightly acidic conditions, of course, removes cholate from solution and yields an erroneously high binding capacity. The relationship of DMP 504 binding capacity to pH is shown in Fig. 2. The results for 5 and 20 mM buffers were combined since the only difference in the two buffers was the buffering capacity. Although this set of experiments was done using MES buffer rather than phosphate, the general relationship is believed to hold, with the MES buffer yielding a higher binding capacity than does phosphate. There may be some cooperativity

Table 2
pH of 20 mM phosphate buffered 20 mM cholate solutions with visual observations of solubility

Solution	Initial pH	pH of final solution	Observation after 30 min	Observation after 24 h
1	5.99	6.49	Very cloudy	Cloudy
2	6.25	6.41	Cloudy	Crystal clumps
3	6.49	6.57	Suspended fine particles	Crystal clumps
4	6.74	6.75	Clear	Crystal particles
5	6.99	7.00	Clear	Clear

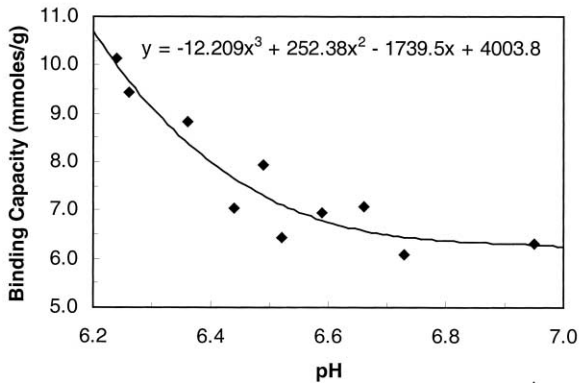

Fig. 2. DMP 504 binding capacity of 20 mM cholate in 5 and 20 mM MES buffer solutions over the pH range of 6.24–6.95.

of binding related to the MES buffer, but this was not investigated. The relationship above pH 7.0 was not determined because of the effects basic solutions have on silica-based HPLC columns. The need to have pH above the point where cholate would precipitate and at a point on the binding/pH curve where the slope is minimized drove the choice of a pH 7.0 phosphate buffer. A pH of 7.0 is above the useful range of MES. The 20 mM phosphate buffer was able to maintain a constant pH.

Table 3
Precision of replicate HPLC injections of a 16 mM cholate standard, the high calibration standard

Injection	Cholate peak area
1	712567.6
2	718977.3
3	717830.5
4	718927.6
5	717187.0
Mean	717098.0
%RSD	0.4

3.2. HPLC system suitability

Fig. 3 shows a typical chromatogram for a cholate standard. The cholate peak is fully resolved from any other peaks and has good peak shape. Analysis of the buffer blank revealed that no peaks co-eluted with the cholate peak. The reproducibility of the HPLC analysis was determined by making replicate injections of a cholate standard solution. The peak area of five injections had a 0.4% RSD (Table 3). The stability of the cholate solution was evaluated by making replicate injections of a cholate standard solution approximately every 12 h for 73 h (Table 4). The

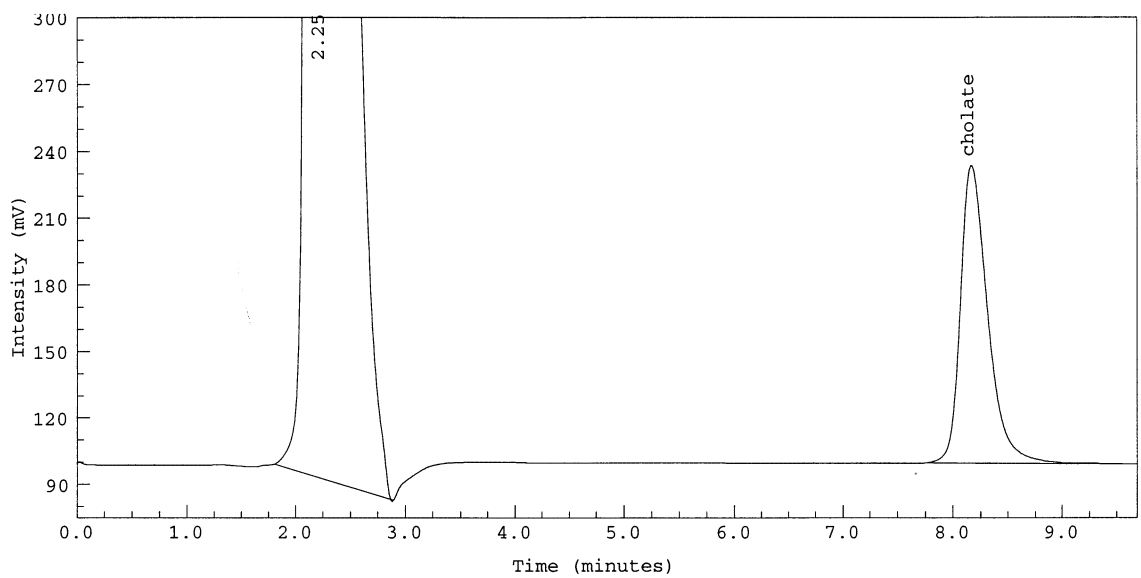

Fig. 3. Typical chromatogram of a residual cholate sample. The HPLC conditions are described in Table 1.

Table 4
Cholate solution stability over 73 h as determined by HPLC

Time (h)	Peak area (μV/s)
0	3733426.5
12.2	3698302.8
24.4	3749235.3
36.6	3744167.3
48.8	3740909.3
61.0	3747099.3
73.2	3741579.3
Mean	3736388.5
%RSD	0.5

precision of these injections was 0.5% RSD with no significant trend, thus demonstrating solution stability over the time period. The binding capacity was measured as a function of incubation time (see Table 5). This study was done in MES buffer yielding a higher binding capacity than in phosphate buffer. Nevertheless, the maximum binding capacity is achieved in 2 h. There is no significant change after that. Therefore, the method is written to incubate the samples between 3 and 24 h to ensure adequate binding and stability.

3.3. Linearity

The linearity of the standards used to determine the concentration of residual cholate was determined over a concentration range of 3.86–15.42 mM cholate using four standards. The linear correlation coefficient for the plot of response versus standard concentration was equal to 1.000 over this range; the slope of the curve was 460.3 ± 0.3 μV-s/mM cholate, and the intercept was -39491 ± 3054 μV/s (see Fig. 4). The standard error of the y-intercept further indicates the precision of the method, which is equivalent to 0.2% of the low standard. The intercept corresponds to -2.2% of the low standard and -0.6% of the high standard. Since all of the samples had a residual cholate concentration that fell within the range of the standards, the concentration of residual cholate could be accurately determined from the standard curve.

Table 5
Effect of incubation time on the binding capacity of DMP 504 using 20 mM sodium cholate in MES buffer

Incubation time (h)	Binding capacity (mmoles cholate/g DMP 504)
Initial	5.76
0.5	5.70
1	5.81
2	6.06
24	6.08

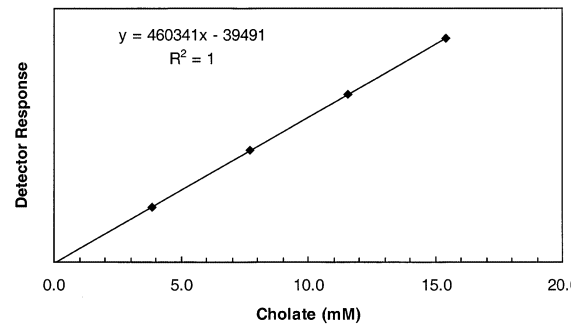

Fig. 4. Cholate standard linearity curve over the range 3.86–15.42 mM cholate.

The binding capacity was determined by a linear regression analysis of mmoles bound cholate versus the amount of DMP 504 in suspension (g). The cholate was present well in excess of that which bound to DMP 504 to ensure that the DMP 504 binding sites were saturated with cholate. A typical binding capacity curve is shown in Fig. 5. Table 6 summarizes the curve data for the binding capacity plots for six different lots of DMP 504 as determined by plotting the amount

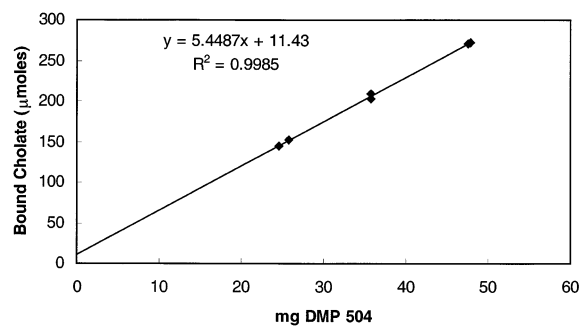

Fig. 5. DMP 504 binding capacity curve for binding of cholate using lot B of DMP 504.

Table 6
Binding capacity data for six lots of DMP 504 by three analysts with two replicates each

	Trial 1			Trial 2		
Lot	Slope (mmol/g)	Intercept (mmol)	Corr. coeff.	Slope (mmol/g)	Intercept (mmol)	Corr. coeff.
Analyst 1						
A	5.5	42.8	1.000	5.4	70.7	1.000
B	5.6	39.5	1.000	5.8	53.8	0.999
C	5.6	38.3	1.000	5.9	51.1	0.998
D	5.3	42.3	1.000	5.4	66.6	0.996
E	5.9	26.9	0.999	5.2	78.3	1.000
F	5.5	40.2	0.994	5.2	80.2	1.000
Analyst 2						
A	5.2	12.5	1.000	5.2	42.5	1.000
B	5.3	8.4	1.000	5.3	38.3	1.000
C	5.1	14	1.000	5.1	44.0	1.000
D	5.0	11.6	1.000	5.0	41.6	1.000
E	5.2	11.4	1.000	5.2	41.3	1.000
F	5.2	10.4	1.000	5.2	40.4	1.000
Analyst 3						
A	5.3	44.2	0.998	5.2	49.8	0.997
B	5.5	44.4	1.000	5.4	43.1	1.000
C	5.3	43.7	0.999	5.3	43.6	1.000
D	5.2	43.7	1.000	5.2	42.2	1.000
E	5.2	52.1	1.000	5.1	57.2	1.000
F	5.4	44.0	0.999	5.3	44.5	0.999

of cholate bound in millimoles versus the mass of DMP 504 in grams. This was done in duplicate by three analysts for six lots of DMP 504. For all six lots the linear correlation coefficient for the binding capacity plot was very close to or equal to 1.000. Residual analysis of these curves supported these curves being linear. Based on these results, the linearity of the binding capacity plots by this method was determined to be acceptable over the range of DMP 504 used. The non-zero intercept is a result of a non-linear binding curve. This curve was generated over a much broader range than that used in the method and is shown in Fig. 6. A cubic equation was fit to these data with an excellent correlation. From the equation in the figure, it can be seen that the quadratic and cubic terms have a negative effect on the bound cholate per unit mass of DMP 504. This is due to the removal of free cholate from solution by the addition of DMP 504 to a fixed amount of cholate. Therefore, a linear fit of the points further from the origin will yield a positive y-intercept. This points out the need to perform a multi-point binding capacity determination, since a single-point determination would yield higher binding capacities than would be obtained from the slope of the line. This also demonstrates that the concentration of cholate binding solution and the mass of DMP 504 used for each point are critical to the determination of binding capacity.

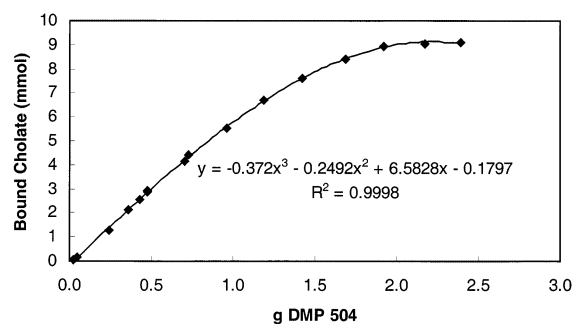

Fig. 6. DMP 504 binding curve over extended range demonstrates non-linearity of binding capacity.

Table 7
Binding capacity precision of duplicate testing by each of three analysts (values in mmol/g)

	Analyst 1		Analyst 2		Analyst 3			
Lot	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Average	%RSD
A	5.5	5.4	5.2	5.2	5.3	5.2	5.3	2.4
B	5.6	5.8	5.3	5.3	5.5	5.4	5.5	3.5
C	5.6	5.9	5.1	5.1	5.3	5.3	5.4	5.8
D	5.3	5.4	5.0	5.0	5.2	5.2	5.2	3.1
E	5.9	5.2	5.2	5.2	5.2	5.1	5.3	5.6
F	5.5	5.2	5.2	5.2	5.4	5.3	5.3	2.4
Average day-to-day RSD	3.6%		0.0%		0.9%		Average RSD	3.8

Some of these minor differences may show up as differences in the y-intercept. However, differences in the intercept are not relevant to the binding capacity.

3.4. Accuracy

Determination of the accuracy for the measurement of cholate is indicated by the low y-intercept of the standard calibration curve. This corresponds to -2.2% of the low standard and -0.6% of the high standard. These values are within the variability of the method and do not represent a bias in the free cholate determination.

3.5. Precision

The standard error of the y-intercept in the cholate linearity results indicates the precision of the method, which is equivalent to 0.2% of the low standard. To evaluate the reproducibility of the binding capacity determination, six different lots of drug substance were analyzed in duplicate on 3 different days by three different analysts. The results from Table 6 have been rearranged and presented in Table 7. The results from Analyst 1 are consistently higher than those of the other analysts with a higher RSD. This was investigated by analyzing each analyst's sample solutions versus each other's standards on the HPLC used by Analyst 1. The sample results for Analysts 2 and 3 analyzed against Analyst 1's standards were higher than their original results. Their samples analyzed against their own standards gave essentially the same results. Analyst 1's samples analyzed versus the other analysts' standards yielded somewhat lower results, but not equivalent. This suggests that Analyst 1's standards were somewhat different. Analyst 1 was less experienced with the method, which may account for these differences and the higher RSD's. If only the results of Analysts 2 and 3 were considered, the standard deviations on all lots would be 0.1, which corresponds to a relative standard deviation of 2.0%. The RSD's for each lot with all three analysts' data considered are shown in Table 7.

3.6. Stability indication

Oxidation of the polymer with 30% hydrogen peroxide decreased the binding capacity of the polymer up to 50% as detected by the binding method. Peroxide was the only means found to degrade the polymer. Acid, base and heat had no effect on the polymer as determined by a lack of change in the binding capacity. Based on the degradation of the polymer by peroxide, this method is stability indicating.

4. Conclusion

It was determined that pH is a critical factor. Therefore, it is controlled by means of a buffered solution where the change in binding capacity with respect to pH is minimized. This binding capacity method has been shown to give repro-

ducible results, and has been established to be rugged under normal laboratory conditions. Therefore, the binding capacity of DMP 504 can be used as a release test for the drug substance and to monitor changes on stability with acceptable precision. This method could also be used for other bile acid sequestrants. However, the experimental conditions for DMP 504 evaluated in this paper would need to be optimized for each specific compound.

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