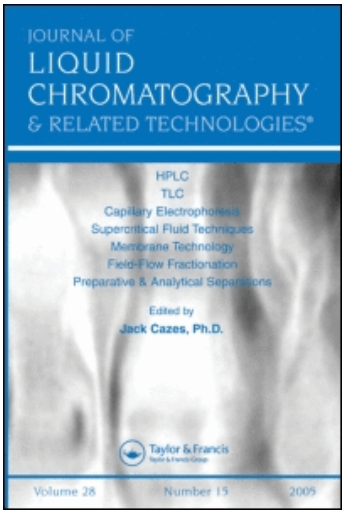

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Development and Validation of a High Performance Liquid Chromatographic (HPLC) Method for the Determination of Phenytoin Prodrug (Fosphenytoin) in Solutions, Parenteral Formulations, and Active Drug Substance

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DEVELOPMENT AND VALIDATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC (HPLC) METHOD FOR THE DETERMINATION OF PHENYTOIN PRODRUG (FOSPHENYTOIN) IN SOLUTIONS, PARENTERAL FORMULATIONS, AND ACTIVE DRUG SUBSTANCE

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ABSTRACT

A rapid, stability indicating high performance liquid chromatographic (HPLC) procedure for the determination of phenytoin prodrug (fosphenytoin) content in solutions, parenteral formulations and the active drug substance was developed.

Reverse-phase liquid chromatography was performed using a 15 cm microparticulate (5 µm) NOVA-PAK phenyl packed column, a variable wavelength detector (214 nm), and a mobile phase that consisted of a buffer that was 0.025 M in potassium phosphate monobasic and 0.05 M in 1-heptanesulfonic acid sodium salt (pH = 4.1 with phosphoric acid) and methanol (65:35) pumped at 1.0 mL/minute. The method is precise and accurate, with three separate analysts achieving an accuracy greater than 99.0% and a relative standard deviation of less than 0.8%. A limit of detection determined by visual examination was found to be approximately 0.1 µg/mL. Accuracy of this method decreases at concentrations of less than 10 µg/mL fosphenytoin. Either peak height or peak area ratios at 214 nm can be used for quantitation.

INTRODUCTION

Fosphenytoin (phenytoin prodrug) is a licensed anticonvulsant compound[1], derived from phenytoin and is under development for the treatment of *status epilepticus* of the *grand mal* type and prevention and treatment of seizures occurring during neurosurgery. A formulation was developed[2] which afforded enhanced stability over the proposed shelf-life of the product and would also be safe for use as an injectable. The primary advantage fosphenytoin has over phenytoin is that it can be administered as either an IM or an IV solution and it has a much higher maximum rate of administration, thereby increasing its safety and usefulness in a critical care setting. It is synthesized as a simple prodrug of phenytoin through a proprietary six step process to yield the final product ($C_{16}H_{13}N_2O_6PNa_2 \cdot xH_2O$).

The goal of this study was to develop an analytical method that is stability-indicating and specific for fosphenytoin. It was to also be accurate, precise, rugged and specifically applicable to parenteral formulations and the active drug substance. The method was to also have a simple dilution scheme prior to injection and the analytical run time should be less than 15 minutes.

The following data and discussion details the development and validation of an assay meeting all the pre-established criteria.

MATERIALS

Samples used in this investigation include fosphenytoin synthesized by a contract manufacturer and its degradation product 5,5-diphenylhydantoin[a] (phenytoin). Monobasic potassium phosphate (HPLC grade)[b], 1-heptanesulfonic acid sodium salt[c], glass-distilled methanol[d], Lactated Ringer's Solution[e], 5% Dextrose[e] and 0.9% Sodium Chloride Solution[e], Normal Saline Solution[f], and 5% Sodium Bicarbonate[f] were used as received. Milli-Q[g] purified waster was used throughout the studies. 0.1 M Tromethamine Solution was prepared using tromethamine[h] and Milli-Q purified water.

METHODS

Apparatus

The liquid chromatographic system consisted of a solvent delivery system[i], an automatic sampler[j], a selectable wavelength detector[k], a 10 mV recorder[l], and an on-line data system[m]. A 15 cm x 3.9 mm column packed with NOVA-PAK phenyl (5 µm)[n], a detector wavelength of 214 nm, and a chart speed of 10 cm/hour were employed. A 50 microliter injection volume was used throughout the study.

Mobile Phase

Approximately one liter of mobile phase was prepared as needed by dissolving 2.21 grams of potassium phosphate (monobasic) and 7.16 grams of 1-heptanesulfonic acid sodium salt in 650 mL of water. The pH was adjusted to 4.1 ± 0.2 with phosphoric acid (85%). This was followed by addition of 350 mL of methanol. The mobile phase was thoroughly mixed and degassed by filtration through a 0.5 micron filter[e] prior to use. A constant flow rate of 1.0 mL/minute resulted in a pressure of approximately 2000 psi.

Diluent

Approximately one liter of diluent was prepared by dissolving 2.21 gm of potassium phosphate (monobasic) in 650 mL of water. The pH was adjusted to 4.1 ± 0.2 with phosphoric acid (85%). This was followed by addition of 350 mL of methanol. The diluent was thoroughly mixed prior to use.

Calibration

Linearity was tested using a series of standard curves. An accurately weighed sample of approximately 100 mg of fosphenytoin reference standard was placed into a 100 mL volumetric flask and brought to volume with diluent (1.0 mg/mL). Lower concentrations were obtained by appropriate dilutions with diluent. Standard concentrations of 50, 40, 30, 20, 10 and 5 µg/mL were prepared for analysis by HPLC. Sequential dilutions were prepared for determination of the limit of

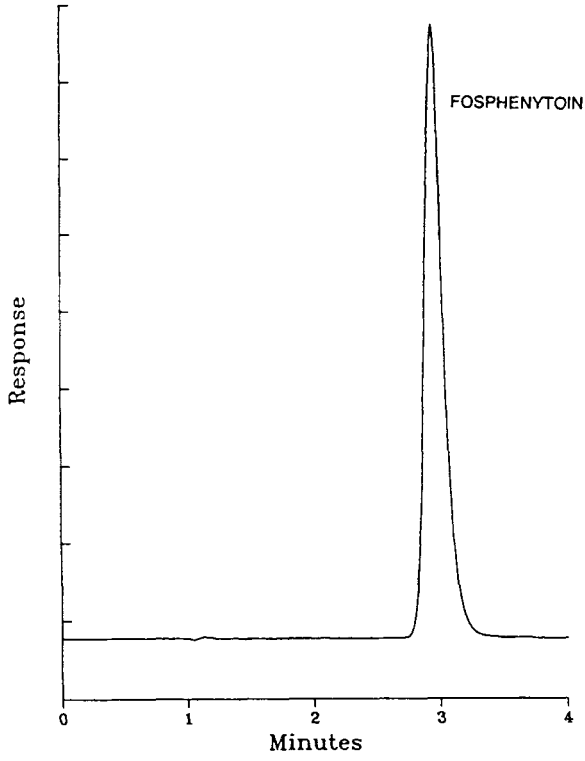

FIGURE 1. Typical HPLC chromatogram for the fosphenytoin assay method.

quantitation and the limit of detection. Each standard was chromatographed and the fosphenytoin peak heights were measured manually and peak areas were measured by the data system (Figure 1 and 2, Table 1). Data from standard curve injections before and after a set of sample injections were collected to determine the suitability and effectiveness of the HPLC system.

Experiments to determine if the method was suitable for use with a single point standard were performed. Three injections of the 40 μg/mL fosphenytoin standard were made before and after a set of samples that were previously analyzed using the standard curve (Table 2).

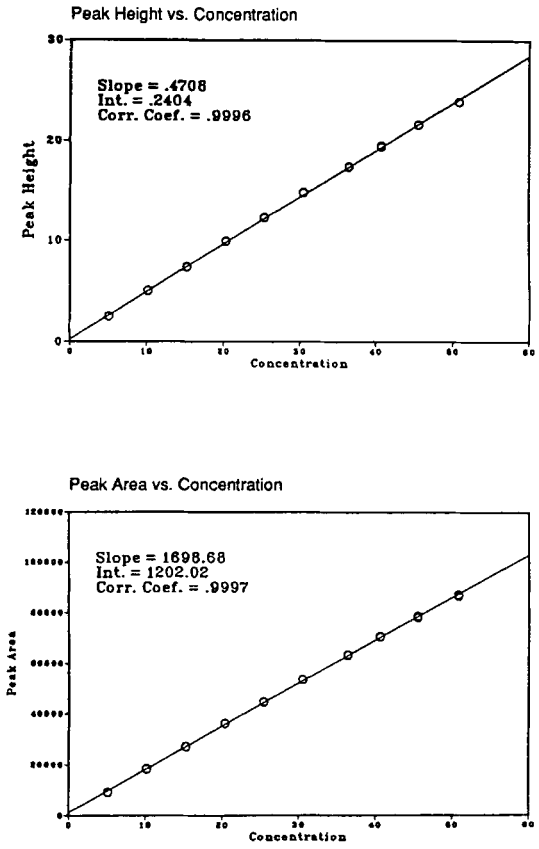

FIGURE 2. Typical calibration curves for the fosphenytoin assay method.

Reproducibility of replicate injections of fosphenytoin standard solution were used to provide suitability and effectiveness of the HPLC system. Data from three injections before and after a set of sample injections were collected.

To determine method accuracy, triplicate samples of three lots were analyzed by three analysts, on three separate days (Table 3). All samples were initially diluted in methanol and the final dilution was performed with diluent to yield a theoretical concentration of 45 μg/mL.

TABLE 1

Typical calibration curve of fosphenytoin.
Comparision of peak height to peak area values.

Fosphenytoin Concentration (µg/mL)	% of Theoretical Using Peak Height	% of Theoretical Using Peak Area
50.861	98.93	99.21
45.550	99.82	99.69
40.689	100.59	100.50
36.440	100.25	100.38
30.517	101.5	100.85
25.431	100.61	100.54
20.344	100.89	101.08
15.259	98.97	100.01
10.172	99.87	99.67
5.086	92.97	92.52
Slope	0.4708	1698.68
Y-Intercept	0.2404	1202.02
Correlation Coefficient	0.9996	0.9997

Replicate injections of a standard solution were chromatographed to determine system precision. Replicate sample preparations were made of a single log to fosphenytoin formulated product and chromatographed to determine method precision.

Three lots of fosphenytoin drug substance were analyzed to confirm precision (Table 5) of the method for the drug substance.

Specificity

The method, as developed, is intended for use with fosphenytoin solutions and formulations. Therefore, the excipient present in the formulated product (tromethamine) was examined for any interferences in the HPLC analysis. Several large volume parenterals (LVP) were tested to check for possible interferences which might occur during analysis of the diluted product. Lactated Ringer's Solution, 5% Sodium Bicarbonate, Normal Saline Solution, Dextrose 5%, and 5% Dextrose and 0.9% Sodium Chloride were chromatographed undiluted using the analytical method described.

Table 2
Comparison of results using a single point standard and a standard curve (linear regression).

Sample	(A) Single Point Standard Peak Height	(B) Single Point Standard Peak Area	(C) Linear Regression Peak Height	(D) Linear Regression Peak Area	A-B	A-C	C-D
1	98.758	98.750	98.695	99.148	0.008	0.063	-0.453
1	99.210	99.370	99.141	99.769	-0.160	0.069	-0.628
1	98.814	98.580	98.751	98.977	0.234	0.063	-0.226
1	99.267	99.632	99.197	100.032	-0.365	0.070	-0.835
1	98.819	99.046	98.769	99.446	-0.227	0.050	-0.677
1	99.048	99.963	98.994	100.365	-0.915	0.054	-1.371
1	99.561	99.313	99.500	99.714	0.248	0.061	-0.214
1	99.276	99.499	99.219	99.900	-0.223	0.057	-0.681
1	98.517	98.496	98.477	98.896	0.021	0.040	-0.419
1	99.033	99.117	98.985	99.518	-0.084	0.048	-0.533
1	98.918	99.243	98.872	99.645	-0.325	0.046	-0.773
1	99.090	98.519	99.042	98.919	0.571	0.048	0.123
2	99.210	99.313	99.380	99.747	-0.103	-0.170	-0.367
2	98.944	99.031	99.118	99.464	-0.087	-0.174	-0.346
2	98.878	98.898	99.052	99.332	-0.020	-0.174	-0.280
2	99.011	99.129	99.183	99.563	-0.118	-0.172	-0.380
2	98.639	98.782	98.483	99.219	-0.143	-0.204	-0.376
2	98.977	99.076	99.176	99.514	-0.099	-0.199	-0.338
2	99.044	99.353	99.242	99.791	-0.309	-0.198	-0.549
2	99.247	99.900	99.442	100.340	-0.653	-0.195	-0.898
2	98.958	99.466	99.151	99.904	-0.508	-0.193	-0.753
2	99.429	99.934	99.616	100.000	-0.505	-0.187	-0.384
2	99.765	99.540	99.947	99.978	0.255	-0.182	-0.031
2	99.563	99.902	99.748	100.340	-0.339	-0.185	-0.592
3	100.689	100.440	100.465	100.821	0.249	0.224	-0.356
3	100.740	100.494	100.515	100.875	0.246	0.225	-0.360
3	100.383	100.460	100.163	100.841	-0.077	0.220	-0.678
3	100.587	100.115	100.364	100.495	0.472	0.223	-0.131
3	100.611	100.674	100.377	101.054	-0.063	0.234	-0.677
3	100.560	100.651	100.327	101.031	-0.091	0.233	-0.704
3	100.813	101.346	100.576	101.728	-0.533	0.237	-1.152
3	100.611	100.511	100.377	100.931	0.100	0.234	-0.554
3	100.002	100.346	99.780	100.727	-0.344	0.222	-0.947
3	101.776	101.681	101.529	102.064	0.095	0.247	-0.535
3	100.776	101.303	100.680	101.685	-0.389	0.234	-1.005
3	101.776	101.785	101.529	102.168	-0.009	0.247	-0.639

Variable	N	Mean	Standard Deviation	STD Error of Mean	Pair-t
sample 1					
A-C	12	0.0558	0.0096	0.0028	0.0001
A-B	12	-0.1014	-0.3729	0.1076	0.3663
C-D	12	-0.5572	0.3743	0.1081	0.0003
sample 2					
A-C	12	-0.1861	0.0118	0.0034	0.0001
A-B	12	-0.2211	0.2475	0.0715	0.0101
C-D	12	-0.4412	0.2278	0.0658	0.0001
sample 3					
A-C	12	0.2317	0.0091	0.0027	0.0001
A-B	12	-0.0287	0.2902	0.0838	0.7387
C-D	12	-0.6448	0.2912	0.0841	0.0001

Table 3. Results from three analysts over three days.

Sample Name	Anal. 1 Day 1	Anal. 1 Day 2	Anal. 1 Day 3	Anal. 2 Day 1	Anal. 2 Day 2	Anal. 2 Day 3	Anal. 3 Day 1	Anal. 3 Day 2	Anal. 3 Day 3
1-1	101.07	101.46	100.84	101.67	101.35	98.92	100.95	99.38	100.14
1-1	101.17	101.36	100.59	101.80	99.72	98.86	100.82	99.71	100.37
1-2	101.26	101.16	100.51	100.88	100.89	102.01	100.72	100.92	101.23
1-2	101.23	101.72	101.76	100.92	99.09	100.11	100.72	101.06	101.08
1-3	100.46	101.42	100.67	101.13	101.48	99.46	100.82	101.04	100.32
1-3	<103.05>	102.03	100.94	100.91	101.33	99.41	100.85	100.82	100.58

Analyst	N	Mean
1	17	101.156
2	18	100.552
3	18	100.629

Day	N	Mean
1	17	101.011
2	18	100.886
3	18	100.433

2-1	102.12	101.70	100.58	99.88	99.59	100.06	100.37	101.02	<94.31>
2-1	102.64	100.41	100.55	99.84	99.54	100.16	100.68	100.74	<94.49>
2-2	101.56	99.93	99.90	99.91	100.04	99.98	101.39	100.76	100.31
2-2	101.33	100.96	99.94	100.02	100.31	99.78	101.54	100.38	101.27
2-3	101.20	101.37	100.08	99.94	100.32	99.96	100.80	100.86	100.64
2-3	101.16	101.72	100.47	100.01	100.47	99.74	100.82	100.94	100.68

Analyst	N	Mean
1	18	100.979
2	18	99.975
3	16	100.888

Day	N	Mean
1	18	100.845
2	18	100.614
3	16	100.319

3-1	100.13	<101.19>	100.28	101.14	100.75	101.44	100.10	99.71	99.35
3-1	99.86	99.92	101.11	100.79	100.83	101.03	100.19	99.86	99.38
3-2	99.71	98.17	100.52	101.26	101.37	101.00	100.00	98.90	99.60
3-2	99.81	98.75	100.09	101.40	101.39	100.98	100.03	99.42	99.69
3-3	99.74	99.29	100.95	101.28	100.75	100.53	100.45	99.69	99.63
3-3	99.68	99.18	<102.89>	101.28	100.71	100.55	100.38	99.71	99.89

Analyst	N	Mean
1	16	99.824
2	18	101.027
3	18	99.777

Day	N	Mean
1	18	100.402
2	17	99.906
3	17	100.354

Note: Results in brackets were removed using the Q-test.

Table 3-A

Average Results of Reproducibility Assay

Sample 1	Mean	=	100.772
	Standard Deviation	=	0.759
	Relative Standard Deviation	=	0.753%
Sample 2	Mean	=	100.603
	Standard Deviation	=	0.694
	Relative Standard Deviation	=	0.690%
Sample 3	Mean	=	100.224
	Standard Deviation	=	0.771
	Relative Standard Deviation	=	0.769%

Table 4

Degradation of Fosphenytoin Formulation at 75°C for Four Months

Time at 75°C	Percent of Initial Potency
1 Week	84.31%
2 Weeks	71.86%
3 Weeks	59.41%
4 Weeks	45.16%
6 Weeks	25.02%
8 Weeks	15.55%
3 Months	4.42%
4 Months	2.05%

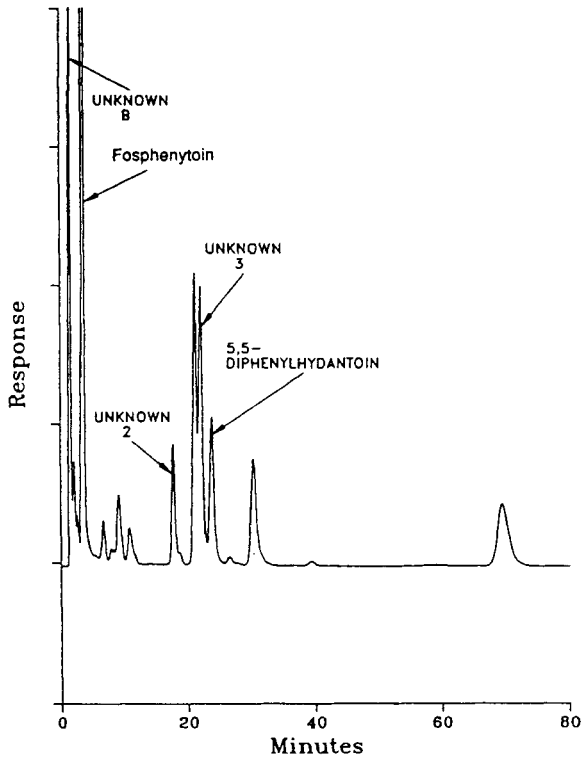

FIGURE 3. Chromatogram demonstrating the degradation of formulated fosphenytoin after exposure to 75 °C for four months.

Table 5

Precision of Active Drug Substance Results

Sample	Potency 1	Potency 2	Potency 3	Average Potency	Relative Standard Deviation
A	99.525	99.808	100.072	99.802	0.275
B	100.025	99.969	100.914	100.303	0.528
C	99.828	99.957	100.587	100.124	0.406

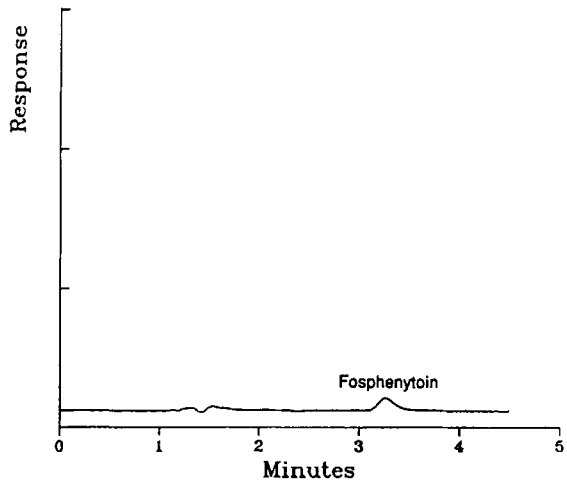

FIGURE 4. Chromatogram showing the remaining fosphenytoin after exposure to 75 °C for four months. Degradation products are not observed in this chromatogram.

A thermal degradation experiment was performed to challenge this HPLC procedure for specificity, and simultaneously, to determine whether the procedure was stability-indicating. Several 5 mL ampuls of fosphenytoin (75 mg/mL) were stored at 75°C up to four months (Table 4). Samples were periodically removed at the following intervals: 1, 2, 3, 4, 6, and 8 weeks, and at 3 and 4 months. These samples were monitored for degradation (Figure 3) and potency (Figure 4).

RESULTS AND DISCUSSION

Method Development and Ruggedness

Once an apparently suitable set of chromatographic conditions had been identified, the effects of small changes in the mobile phase were explored. Ruggedness is not a mathematically quantifiable term and therefore, in this study, it is defined as a qualitative look at the method's ability to resist small changes in the mobile phase. Thus, the

retention time of fosphenytoin was monitored while each component in the mobile phase was varied over a moderate range.

Effect of Buffer Concentration

The concentration of potassium dihydrogen phosphate in the aqueous portion of the mobile phase was varied between zero and 0.05 M buffer while holding the 1- heptanesulfonic acid concentration at 0.05 M. When no potassium dihydrogen phosphate buffer was present, the fosphenytoin peak tailed unacceptably. Stepwise increases in the concentration of potassium dihydrogen phosphate resulted in a slight increase in the retention time and a concomitant improvement in the peak shape. A mobile phase containing 0.025 M potassium dihydrogen phosphate was chosen because an increase or decrease in molarity of 0.005 does not alter the retention time nor peak shape of the fosphenytoin peak.

These results indicate that the potassium dihydrogen phosphate buffer concentration is not a significant factor in retention, but must be present for proper chromatographic elution to occur.

Similarly, the concentration of 1-heptanesulfonic acid sodium salt in the aqueous portion of the mobile phase was varied between zero and 0.1 M (Figure 6) while holding the phosphate concentration at 0.025 M. When no 1-heptanesulfonic acid is present, the fosphenytoin peak is retained twice as long as when 0.05 M 1-heptanesulfonic acid is present. A mobile phase containing 0.05 M 1-heptanesulfonic acid was chosen because an increase or decrease of 0.01 M does not alter the retention time nor peak shape of fosphenytoin. The presence of 1-heptanesulfonic acid sodium salt is necessary to elute fosphenytoin within a reasonable time, with adequate separation from the solvent front, as well as separation of degradation peaks.

Effect of pH

The pH effect of the aqueous portion of the mobile phase was varied from 3.7 to 4.6 in order to observe the effect of small changes in pH on the retention time of fosphenytoin (Figure 7). The buffer and organic concentrations were held constant. The retention time of

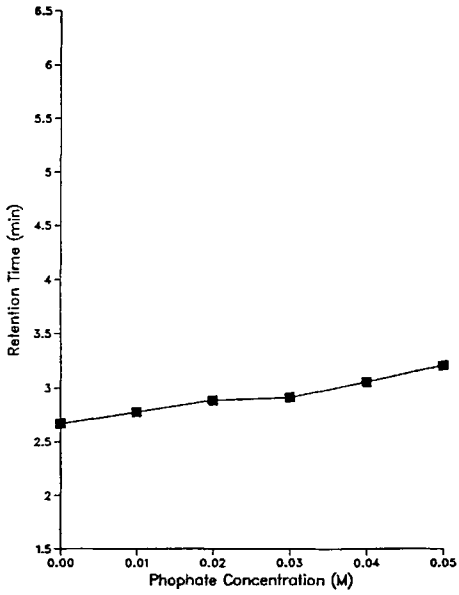

FIGURE 5. Effect of potassium dihydrogen phosphate concentration on fosphenytoin retention time.

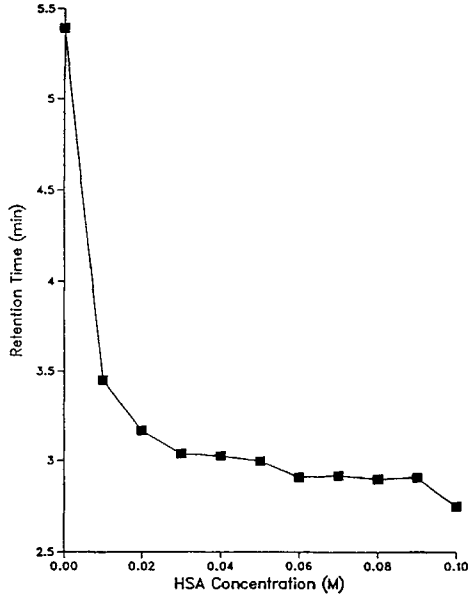

FIGURE 6. Effect of 1-heptanesulfonic acid concentration on fosphenytoin retention time.

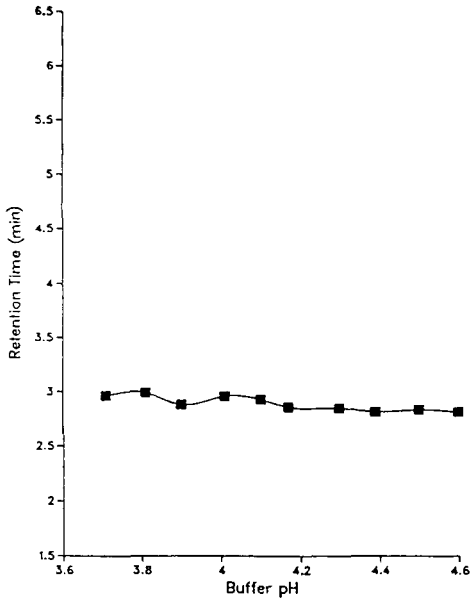

FIGURE 7. Effect of buffer pH on fosphenytoin retention time.

fosphenytoin remained constant while the pH of the buffer was altered. Therefore, the method is rugged with regard to small changes in pH.

Effect of Aqueous/Organic Ratio

The effect of the change in ratios of aqueous to organic composition of the mobile phase was also studied (Figure 8). Initially, using 50% buffer and 50% methanol, the fosphenytoin peak was very sharp but the retention time was less than 2 minutes which was too close to the void volume of the column. Increasing the buffer in 5% increments consistently increased the retention time. Buffer concentrations of 70% or greater yielded extensive peak broadening. A buffer/methanol ratio of 65:35 yielded a sharp fosphenytoin peak at an acceptable retention time. Five percent variations in the mobile phase composition will affect the retention time to a small degree (± 0.5 min.). Therefore, careful

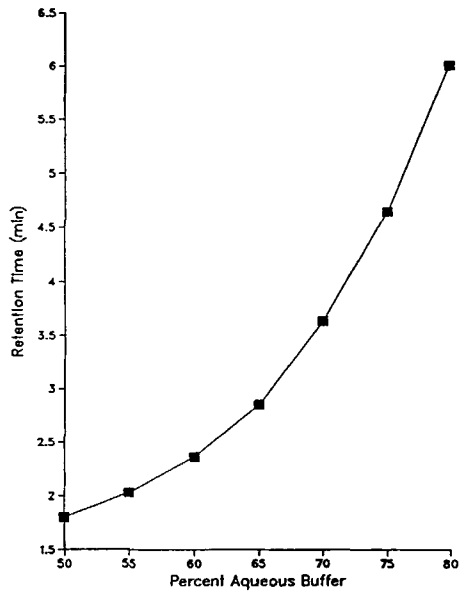

FIGURE 8. Effect of aqueous buffer concentration on fosphenytoin retention time.

preparation of the mobile phase is taken to ensure that the proper organic to buffer ratio is obtained.

Degradation Studies/Stability Indicating Nature of the Method

The extreme conditions of the thermal challenge were used to demonstrate method specificity. Other conditions could not be used due to the inherent instability of the prodrug to acid and base conditions. A log linear correlation between degradation of fosphenytoin and the storage time was observed (Table 5) indicating a first order (or pseudo first order) degradation pathway. Several unidentified degradation peaks (Figure 3) were noted in the HPLC chromatograms of the thermally treated samples. The samples at 75°C degraded to 2% of the

original concentration of fosphenytoin (Figure 4) after 4 months. Work has been completed on the complete degradation pathway and will be published in a separate report. Separation conditions were optimized throughout this report using fosphenytoin as well as separation from the peaks observed in Figure 3. Therefore, good confidence in the method's stability indicating nature is expected as the potency has dropped to a level nearing the experimental variance of the method.

Selectivity

No interferences were observed in the area of interest when 0.1 M tromethamine, Lactated Ringer's Solution, 5% Sodium Bicarbonate, Normal Saline Solution, Dextrose 5%, and 0.9% Sodium Chloride were chromatographed using the described method. Therefore, the current method clearly is capable of resolving all anticipated and potential degradation products and excipients from fosphenytoin.

Method Statistics and Comparison

Fosphenytoin was quantitated by direct measurement using the newly developed HPLC procedure. Three separate analysts using three separate HPLC systems on three separate days achieved an accuracy greater than 99.0%, with a relative standard deviation of less than 0.8% (Table 3).

Several standard curves were chromatographed for determination of method linearity. The peak areas and heights for fosphenytoin standards were then plotted versus the fosphenytoin concentration to yield calibration curves (Table 1 and Figure 2). All standard curves typically had a correlation coefficient of greater than 0.999. All standard peak heights or areas were shown to be within 3% of the linear regression line and the fosphenytoin peak retention time was always within a range of 3 to 4 minutes, thereby passing preset system suitability requirements.

The response for fosphenytoin was shown to be linear with a near zero intercept, therefore, the use of a single point standard was investigated. The 40 µg/mL standard was used to analyze a set of samples previously analyzed using the standard curve approach (Table 2). Comparison of the results yielded a standard deviation of less than 0.4% and a standard error of the mean not more than 0.1%. These statistical results along with Pair-t results confirm that there are no differences in results, therefore a single point (or level) standard may be used in the analysis.

Replicate injections of a standard solution were chromatographed to determine the inherent system precision. Relative standard deviations of less than 0.5% using peak heights or areas were obtained. Six sample preparations of one lot of formulated product were chromatographed to determine the method precision. Relative standard deviations were less than 0.8% using either peak heights or areas. These results indicate that the described method is precise.

The limit of detection was determined to be 0.1 µg/mL and the limit of quantitation was determined to be 5 µg/mL, both experimentally by serial dilution.

Drug Substance

Validation of the method for determination of fosphenytoin in Drug Substance was performed in parallel with the formulated product method. The analysis is identical with the exception of sample preparation. Results from the analysis of three lots of fosphenytoin drug substance confirm the precision of the described method (Table 5). The results do not differ by more than ± 0.5%, standard deviations are less than 0.6, and relative standard deviations are not more than 0.6%.

CONCLUSION

The described method was found to be suitable for the determination of fosphenytoin content in fosphenytoin solutions, parenteral formulations, and the active drug substance. The described method met all of the following criteria: it yields accurate and precise data, when performed by 3 analysts, accuracy is greater than 99.0% with a relative standard deviation of less than 0.8%; an analysis time of ten minutes per injection; analysis can be performed using a variable or fixed wavelength detector set at 214 nm; the results can be calculated using integrated peak area or peak height. The response for fosphenytoin is linear within a range of 5 to 50 µg/mL, therefore, the method as described is suitable for use with a single level standard. Using the method, many degradation products can be detected and the results of long term degradation studies will be described in a separate report.

FOOTNOTES

a. Sigma Chemical Company, St. Louis, MO
b. Fisher Scientific Company, Fair Lawn, NJ
c. Eastman Kodak Company, Rochester, NY
d. Burdick and Jackson, Muskegon, MI
e. McGaw Laboratories, Irvine, CA
f. Baxter, Deerfield, IL
g. Millipore Corporation, Bedfore, MA
h. Helco Chemicals Division, Whittaker Corporation, Delaware Water Gap, PA
i. Series 10 Pump, Perkin-Elmer Corporation, Norwalk, CT
j. LC600 Autosampler, Perkin-Elmer Corporation, Norwalk, CT
k. Model 160 Selectable Wavelenght Detector, Beckman Instruments Inc., Berkley, CA
l. Model 555, Linear Instruments Corporation, Fair Lawn, NJ
m. Multichrom, Fisons VG Laboratory Systems, Altrincham, Cheshire, England
n. Nova-pak Phenyl, Waters Associates, Bedford, MA

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