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PREPARATION OF GELATIN:PHENYTOIN SODIUM MICROSPHERES: AN *IN VITRO* AND *IN VIVO* EVALUATION

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ABSTRACT

In this study phenytoin sodium microspheres were formulated with biodegradable acid-treated gelatin. The microspheres were subjected to *in vitro* and *in vivo* testing. The percent drug retained in the microspheres, as well as its release from the microspheres, was tested. *In vitro* data revealed a decrease in percent drug retained in the microspheres with an increase in addition of glutaraldehyde to the microsphere formulations. The statistically most consistent drug release was observed from formulations containing 10 g of gelatin and 2 g of phenytoin sodium. From this formulation the slowest release was observed when 5 ml

of glutaraldehyde were added to the various formulations, whereas the fastest release was observed when no glutaraldehyde was added. In vivo studies consisted of administering phenytoin sodium in microsphere form and an aqueous solution via various routes of administration and determining phenytoin plasma concentration vs. time profiles in female Sprague Dawley Rats. Computer fitting of the in vivo data and subsequent statistical testing enabled comparison of the effect of microsphere formation and the effect of microsphere dose on selected pharmacokinetic parameters.

INTRODUCTION

Microencapsulation of either phenytoin or phenytoin sodium has been explored very little in the literature. Therefore, it was decided that gelatin:phenytoin sodium microspheres should be formulated and evaluated on an in vitro and in vivo basis. In vitro evaluations of percent phenytoin sodium retained in the microspheres and dissolution studies of percent released as a function of time were performed. In vivo evaluations consisted of administering phenytoin sodium microspheres and phenytoin sodium solution to rats and the determination of select pharmacokinetic parameters.

METHODS

Preparation of Microspheres

Gelatin microspheres containing phenytoin sodium were prepared by modifying procedures reported

earlier.[1,2]. The microspheres were prepared by dissolving gelatin[1] in 50 ml of distilled water at 65°C with stirring, then phenytoin sodium[2] was dispersed in the gelatin solution. The gelatin:drug suspension was then added to 250 ml of light white mineral oil[3], previously equilibrated at 65°C +/- 0.02 °C in a water bath[4]. The mixture was stirred with a variable speed stirrer[5] with the controller set at 3 for three minutes to disperse the gelatin:drug suspension as tiny droplets in the mineral oil. The entire system was then transferred from the 65°C water bath to a refrigerated water bath[6] maintained at 5°C +/- 0.05°C. After stirring the mixture for an additional three hours to ensure gelation of the individual microspheres, the beaker was then covered and placed in a freezer at minus 10°C for 24 hours. After 24 hours, 250 ml of chilled, 5°C, isopropanol[7] (70% V/V) was added to the slurry. After filtering the mixture under vacuum, the microspheres were washed twice with 250 ml of chilled isopropanol. At the end of the second washing, the microspheres were allowed to air dry.

The microspheres to be treated with glutaraldehyde were prepared in the same manner, except for the addition of glutaraldehyde before removal of the microspheres from the 5°C water bath. Five, ten, or twenty mls of glutaraldehyde[8] (25% W/W) were added in one aliquot and the mixture stirred for 30 minutes. The

beaker was then covered and placed in a freezer maintained at minus 10°C for 24 hours.

Triplicate batches of microspheres were prepared using each procedure. The individual batches were designated as 10:2, 15:2, or 20:2 to indicate either 10, 15, or 20 g of gelatin and 2 g of phenytoin sodium in the formulation.

Total Assay of Microspheres for Drug Content

Triplicate samples of microspheres from each batch were weighed and placed in a homogenizing flask containing 50 ml of 0.05 M borate buffer having a pH of 9.0. The microspheres were homogenized[9] for one hour. After one hour, the flask assembly was washed with an additional 50 ml of borate buffer. A portion of the homogenized solution was then filtered through a 0.45 um membrane filter.[10] Absorbance of the filtered solution was then determined at 254 nm with a spectrophotometer[11], using gelatin microspheres prepared and treated in the same manner as a blank. Comparison to a Beer's-Lambert plot was made, and the percent drug contained in the microspheres was determined.

Dissolution Studies

Dissolution[12] was followed by examining triplicate samples containing 50 mg of phenytoin sodium equivalents, using Method II as presented in the United States Pharmacopeia.[3] Microspheres were placed on the surface of 900 ml of 0.05 M borate buffer having a pH

of 9.0 previously equilibrated at 37°C and contained in a 1000 ml round-bottom dissolution vessel. The mixture was stirred at 50 rpm, and 5 ml aliquots were withdrawn at specified time intervals, using a 5 ml glass pipette fitted with a piece of polyvinyl chloride tubing plugged with glass wool. A constant volume of dissolution medium was maintained throughout, and the polyvinyl chloride tubing plugged with glass wool was added to the dissolution media after each aliquot was withdrawn.

Each aliquot was filtered using a 0.45 um membrane filter. Comparison to a Beer's-Lambert plot was made and the amount of drug retained at each time interval was determined at an ultraviolet absorbance of 245 nm.

In Vivo Formulation Selection Criteria

The selection of gelatin:phenytoin sodium microsphere formulations for further in vivo testing was made as the result of statistical analysis. The objective of the statistical testing procedure was to select an in vitro slow-release and fast-release formulation for administration to the test animals. A repeated measures analysis of variance 3-factor factorial model was used. The three factors used were: 1) gelatin:phenytoin sodium ratio, 2) quantity of glutaraldehyde, and 3) in vitro dissolution sampling time interval. The analysis was conducted using SPSS Release 9^{13} on an IBM Model 370 Computer System.

Percents of drug released from dissolution data were transformed, using the expression $X = 2\arcsin(X^{o})^{1/2}$, where X = the transformed percent and X^{o} = percent drug released. The transformed values were further analyzed by simple main effects, simple-simple main effects, and Newman-Keuls means tests.

Plasma Calibration Curve

A calibration curve was prepared by mixing equal volumes of spiked phenytoin free acid[14] plasma samples, acetonitrile[15], and internal standard, cyheptamide[16], dissolved in acetonitrile. The mixtures were vortexed for 30 seconds and centrifuged at 4500 rpm for 10 minutes. The supernatant was transferred to a separate test tube, and 25 ul was injected into a high pressure liquid chromatograph[17] for analysis. A wavelength of 198 nm was employed for analysis using a variable wavelength detector.[18] The chromatograph was equipped with a 10% carbon load column.[19] The mobile phase consisted of 40% acetonitrile and 60% of 0.01 M phosphate buffer having a pH of 6.0. Chromatograms were run at a chart speed of 0.25 cm/min. Utilizing a flow rate of 1.0 ml/min, retention times for phenytoin and cyheptamide were determined to be 10 and 14 minutes, respectively. Peak-height ratios of phenytoin to cyheptamide were determined and a calibration curve prepared by plotting peak-height ratios versus known phenytoin concentrations.

In Vivo Studies

In vivo studies were conducted by dosing female Sprague-Dawley rats, weight range 210-260 grams, with phenytoin sodium microspheres and phenytoin sodium alkaline solution intramuscularly, intraperitoneally, and orally. Two microsphere formulations of phenytoin sodium were statistically selected for in vivo administration. The two formulations were selected based upon the percent phenytoin sodium released throughout the sampling periods in the dissolution studies.

All gelatin:phenytoin sodium microspheres were resuspended in 1.0% polyoxyethylene sorbitan monooleate[20] in normal saline prior to administration. Intraperitoneal injections were made using an 18 gauge needle and intramuscular injections were made using a 25 gauge needle. All oral dosing was administered via a 16 gauge gastric intubation needle.

Blood samples of approximately 300 ul were collected into a heparinized test tube at selected time intervals via the tail vein. The method of collection, utilized a modified Liebig condenser jacket connected to a vacuum line.[4] As in preparation of the calibration curve, the amount of plasma used for analysis was 100 ul, and each sample was treated as described earlier. Plasma concentrations were then determined using the calibration curve prepared earlier.

The individual *in vivo* plasma concentration data from each formulation administered were fitted to an open one-compartment extravascular model:[5]

$$C = (FXo/V)(Ka/(Ka-K)(EXP(-Kt)-EXP(-Kat)).$$

The fraction of dose absorbed, or absolute bioavailability, was calculated by determining the quotient of the AUC divided by the dose of the respective formulation and the AUC divided by the dose of an intravenous solution.

$$F = (AUC_f/Dose_f)/(AUC_{I.V.}/Dose_{I.V.})$$

where f = respective formulation and I.V. = intravenously administered solution of phenytoin sodium. The intravenous data were obtained from the literature using the same species and gender of rat.[6] The volume of distribution, V, was calculated by multiplying the dose administered, Xo, by the absolute bioavailibility, F, and dividing this product by the extrapolated plasma concentration at time zero, Cpo. The absorption rate constant, Ka, was estimated by way of the feathering or back-projection technique of the ascending portion of the plasma concentration vs. time profiles. The elimination rate constant, K, was estimated from the elimination or descending phase of the plasma concentration vs. time profiles.

From these initial parameter estimates, a range of values were then inserted into the regression model. The non-linear regression model iterations were

performed using a non-linear regression program.[21] Information obtained for further use from the computer printout included K, Ka, and Cpt. Pharmacokinetic parameters calculated from these computer-generated values were AUC, tmax, and Cmax.

The trapezoidal method was used to determine the AUC from the computer-generated plasma concentrations at the various time points. The time to reach the maximum plasma concentration was calculated using the equation:

$$tmax = (1/Ka-K))\ln(Ka/K).$$

The maximum plasma concentration was calculated from the equation,

$$Cmax = ((KaFXo)/(V(Ka-K)))(EXP(-Ktmax)-EXP(-Katmax)).$$

These pharmacokinetic parameters were then further statistically tested by a t-test.

RESULTS AND DISCUSSION

In Vitro

The percents of drug retained in the various microsphere formulations are shown in Table 1. An analysis of variance performed on the transformed percent phenytoin sodium retained in the microspheres determined that there were significant differences between the ratios of gelatin and phenytoin sodium and the milliliters of glutaraldehyde added upon the transformed percent drug retained in the microspheres.

TABLE 1

Percent Phenytoin Sodium Retained In Microspheres*

Ratio of Gelatin: Phenytoin	Volume of Glutaraldehyde 0 ml	5 ml	10 ml	20 ml
10:2	11.60	9.89	9.04	8.05
15:2	10.68	8.00	8.10	6.28
20:2	8.70	5.16	4.85	4.08

*Each value represents the average of three determinations on three batches of microspheres prepared at different times.

A Newman-Keuls Procedure showed that a significant difference exists in transformed percent drug retained in the microspheres between all three ratios of gelatin to phenytoin sodium. These differences may be attributed to a dilution effect produced by increasing the amount of gelatin in the formulations while holding the amount of phenytoin sodium constant.

A Newman-Keuls Procedure showed that no significant difference in transformed percent phenytoin sodium retained in the microspheres occurred when 5 or 10 ml of glutaraldehyde was added to the microsphere formulations. However, a significant difference did

occur when zero or 20 ml of glutaraldehyde was added to the formulations. Table 1 shows that for each ratio of gelatin to phenytoin sodium the percent drug retained in the microspheres tends to decrease as the volume of glutaraldehyde added is increased. This inverse relationship between the percent drug retained in the microspheres and volume of glutaraldehyde may be due to cracking or fissuring of the microspheres enabling drug to be extracted during the washing. This was pointed out in a study of gelatin microcapsules containing clofibrate where microscopic examination of microcapsules hardened for more than 8 hours in a 10% solution of formaldehyde in 2-propanol revealed numerous fractured and broken microcapsules.[7] It was felt that this prolonged hardening probably caused the microcapsule walls to become brittle and subject to fracture, resulting in subsequent loss of drug during washing.

Dissolution profiles for the release of phenytoin sodium from the various microsphere formulations are shown in Figures 1-4. Figure 1 shows when glutaraldehyde was not utilized in the microsphere formulations, the 20:2 ratio of gelatin to phenytoin sodium appears to produce microspheres having a faster dissolution. No discernible differences were noted for the 10:2 ratio of gelatin to phenytoin sodium or the

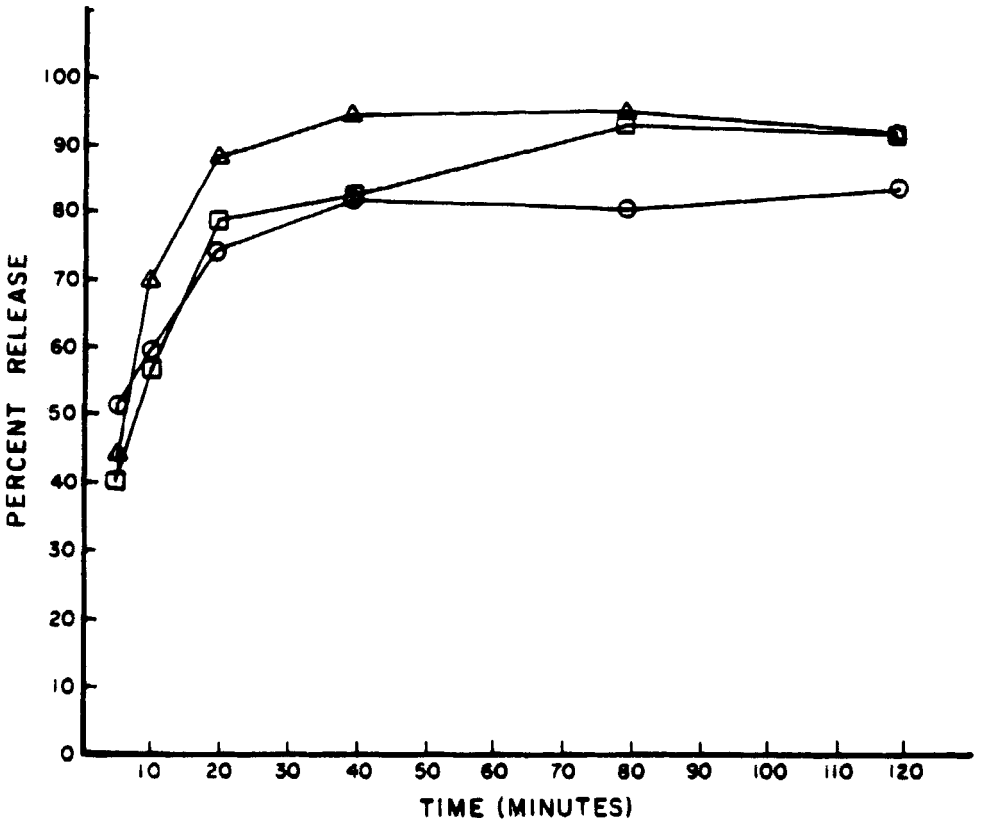
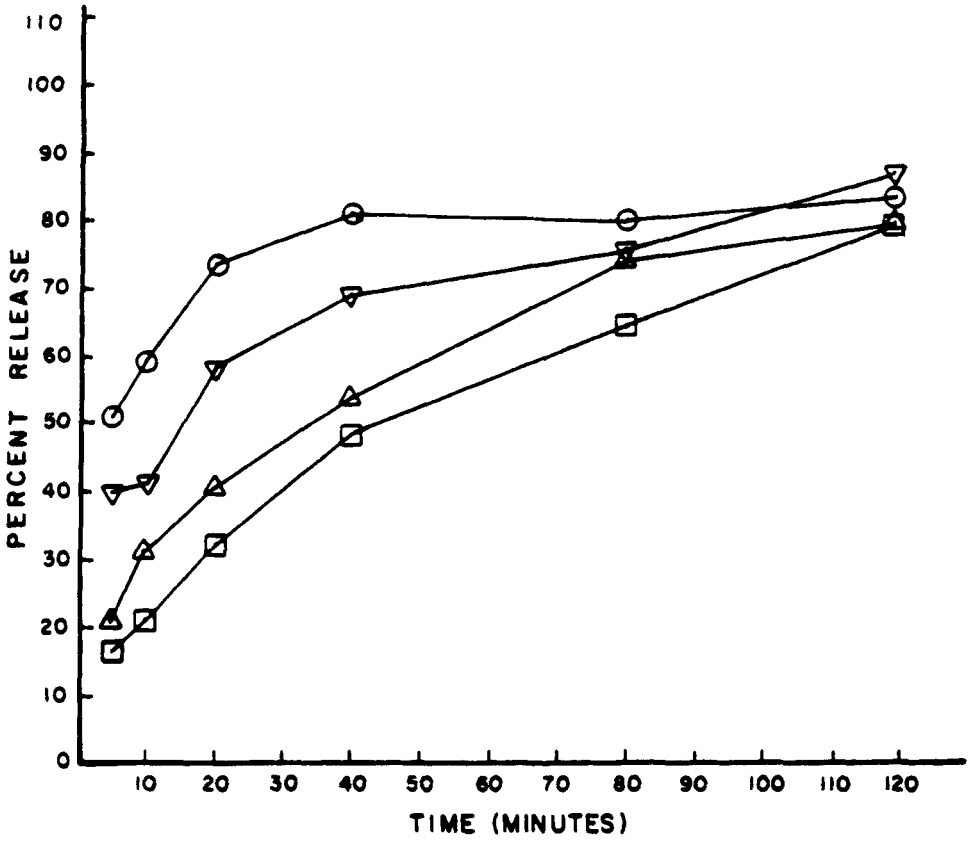


FIGURE 1

Dissolution Profile of Phenytoin Sodium From Gelatin: Phenytoin Sodium Microspheres Treated With 0 ml of Glutaraldehyde. Ratio in Grams of Gelatin:Phenytoin Sodium in Formulation.

15:2 ratio of gelatin to phenytoin sodium during the initial stages of the dissolution. During the latter stages of the dissolution, the 15:2 ratio appears to release phenytoin sodium faster than the 10:2 ratio. These results are similar to those reported for



○= 0 ml, □= 5 ml, △= 10 ml, and ▽= 20 ml.

FIGURE 2

Dissolution Profile of Phenytoin Sodium From Gelatin: Phenytoin Sodium (10:2) Microspheres Treated With Various Volumes of Glutaraldehyde. Volume of Glutaraldehyde in ml Added to Formulation.

phenytoin release from phenytoin-PVP coprecipitates.[8] It was shown that the greater the ratio of PVP to phenytoin, the faster the phenytoin dissolution.

The release profiles for the glutaraldehyde:phenytoin sodium formulations are shown

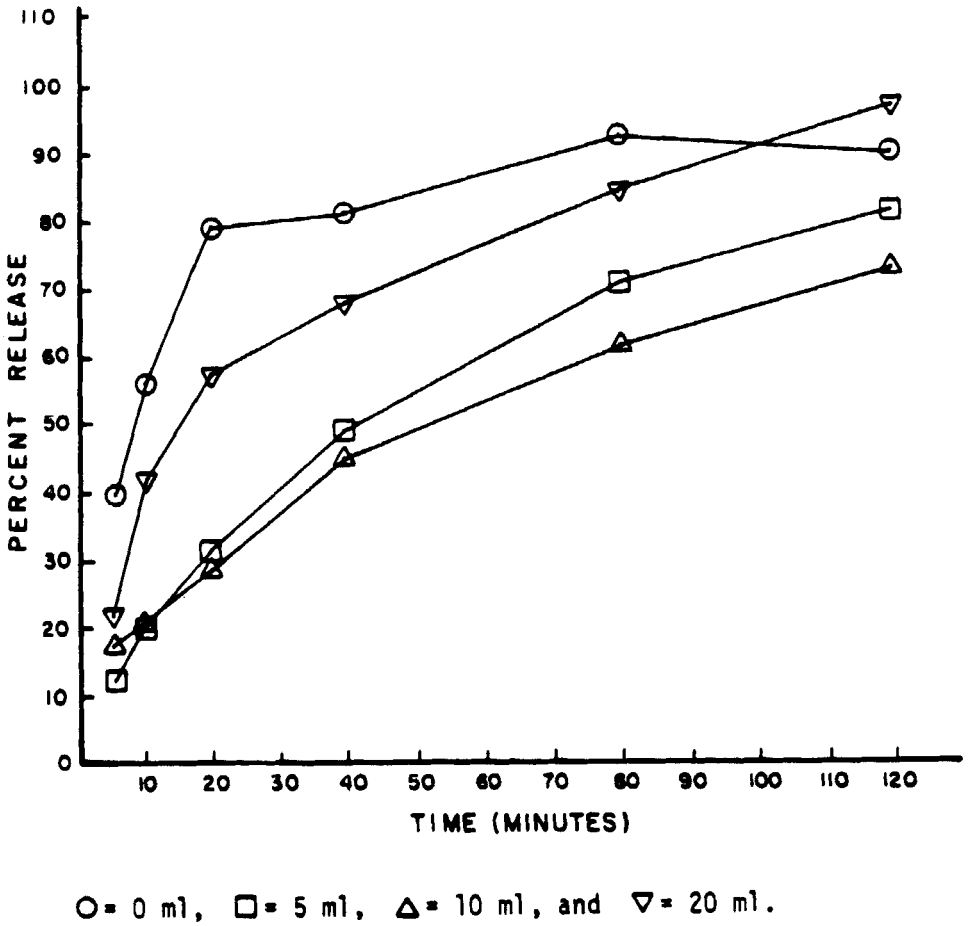
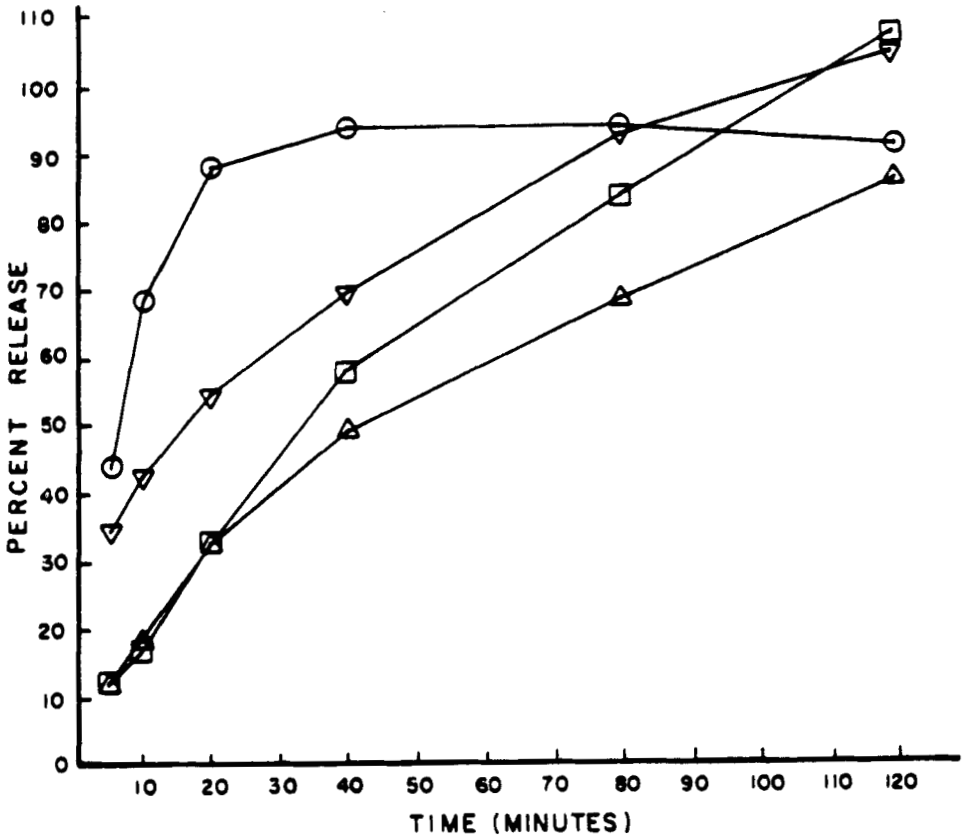


FIGURE 3

Dissolution Profile of Phenytoin Sodium From Gelatin: Phenytoin Sodium (15:2) Microspheres Treated With Various Volumes of Glutaraldehyde. Volume of Glutaraldehyde in ml Added to Formulation.

in Figures 2-4. Phenytoin sodium appears to have a faster dissolution from the microspheres, regardless of the gelatin:phenytoin sodium ratio, when no glutaraldehyde was used in the formulations. When glutaraldehyde was added to the formulations, the



○= 0 ml, □= 5 ml, △= 10 ml, and ▽= 20 ml.

FIGURE 4

Dissolution Profile of Phenytoin Sodium From Gelatin: Phenytoin Sodium (20:2) Microspheres Treated With Various Volumes of Glutaraldehyde. Volume of Glutaraldehyde in ml Added to Formulation.

release of phenytoin sodium from the microspheres was delayed. Figure 2 indicates that, for the 10:2 ratio of gelatin to phenytoin sodium, the effect of glutaraldehyde upon delaying the release of phenytoin sodium is in the order of 5 ml > 10 ml > 20 ml. Figure

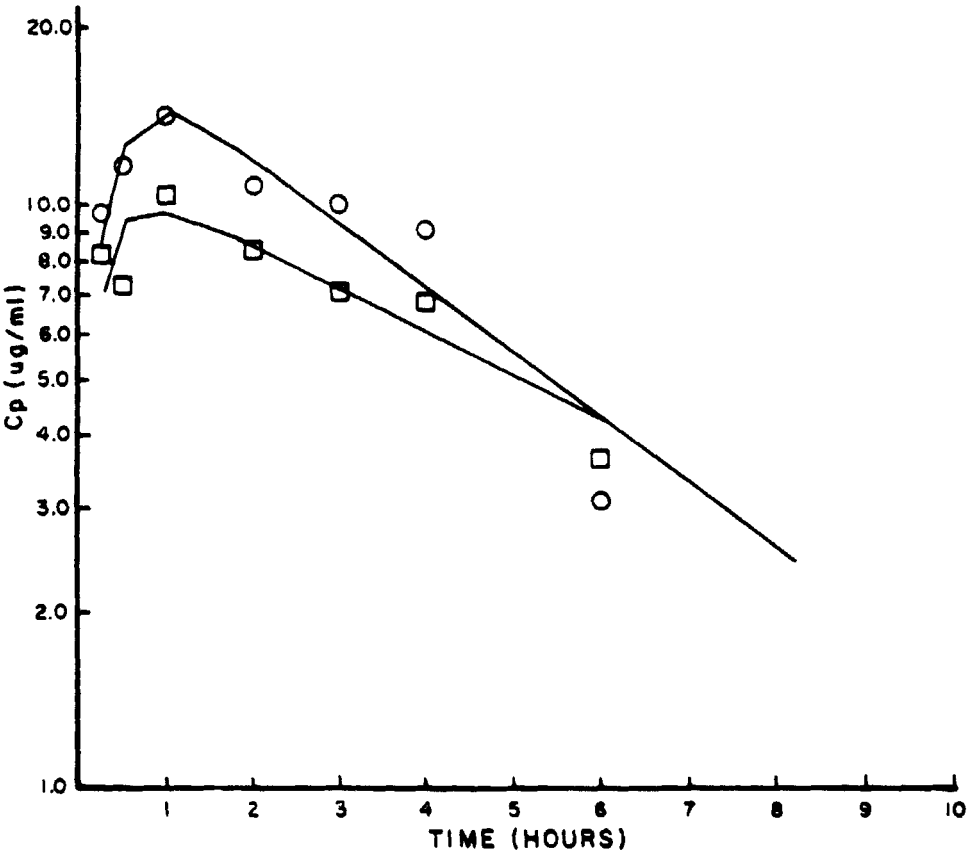
3 indicates that, for the 15:2 ratio, the effect of glutaraldehyde is in the order of 10 ml > 5ml > 20 ml. Figure 4 indicates that, for the 20:2 ratio, the effect of glutaraldehyde is also in the order of 10 ml > 5 ml > 20 ml. Glutaraldehyde in the formulations would be expected to produce cross-linking between gelatin molecules and thereby hinder the dissolution of phenytoin sodium from the microspheres. However, an earlier observation of the effect of formaldehyde upon the amount of pentobarbituric acid extracted from gelatin-acacia microcapsules revealed that microcapsules exposed to larger volumes of formaldehyde released greater percents of their contents when exposed to gastric fluids.[9] It was hypothesized that this may have been due to cracking, resulting from excessive denaturization, of the microcapsule shell upon drying when greater amounts of formaldehyde were used during the microcapsule preparation.

In Vivo

Microsphere formulations which consistently exhibited the fastest and slowest dissolution of phenytoin sodium were selected for in vivo testing as a result of statistical analysis of the data. The transformed in vitro percent drug-released data were analyzed using a three-factor factorial repeated measures model, with all factors considered as fixed.

As a result of the statistically significantly interaction terms, an analysis of simple simple main effects was performed according to the procedure outlined by Kirk.[10] The statistically significant simple simple main effects were further analyzed using a Newman-Keuls Procedure. From these results microspheres prepared using 10 g of gelatin and 2 g of phenytoin sodium in their formulations were chosen for in vivo testing. For microspheres prepared using this ratio of gelatin to phenytoin sodium, the dissolution of phenytoin sodium was fastest when no glutaraldehyde was added to the formulation and slowest when 5 ml of glutaraldehyde was added to the formulation.

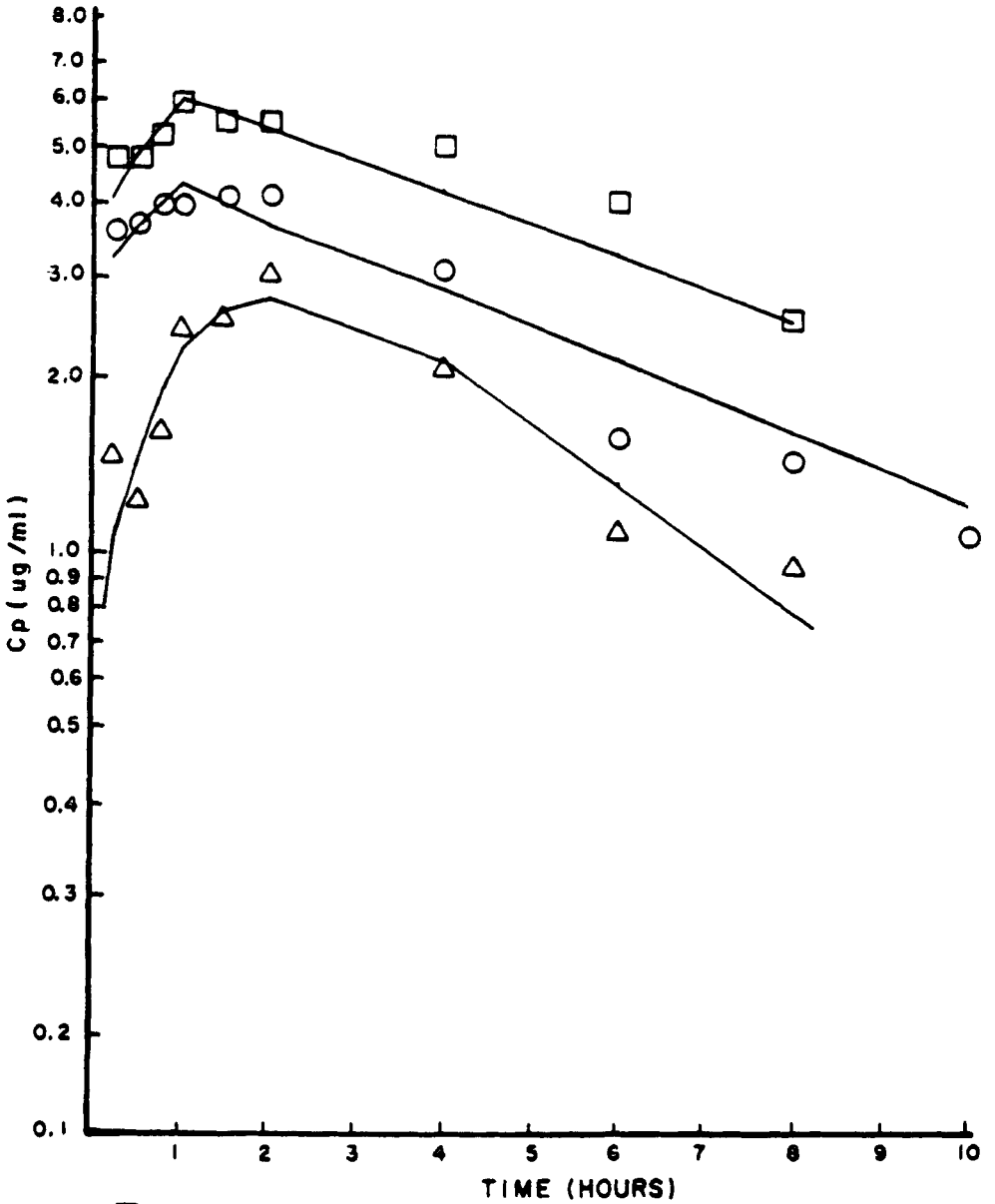
The plasma concentration profiles of phenytoin for the two microsphere formulations and phenytoin sodium alkaline solution are displayed in Figures 5-7. Averaged plasma concentration data obtained from the various formulations converged or fit the regression model with the exception of data from the formulation of 10 g of gelatin and 2 g of phenytoin sodium with 5 ml of glutaraldehyde administered intraperitoneally at a dose of 20 mg/kg phenytoin sodium equivalents. An attempt was made to fit this set of data to a two-input extravascular model.[11] However, convergence was not met indicating the experimental data did not fit this model. Because of the difficulty in fitting this data to a



□ = phenytoin sodium solution. Each value represents the average of five animals.
○ = gelatin:phenytoin sodium microspheres (10:2 treated with 0 ml of glutaraldehyde). Each value represents the average of four animals.

FIGURE 5

Plasma Concentration Profile of Phenytoin Following Intraperitoneal Administration of Phenytoin Sodium Solution and Gelatin:Phenytoin Sodium Microspheres at 20 mg/kg Phenytoin Sodium Equivalents.



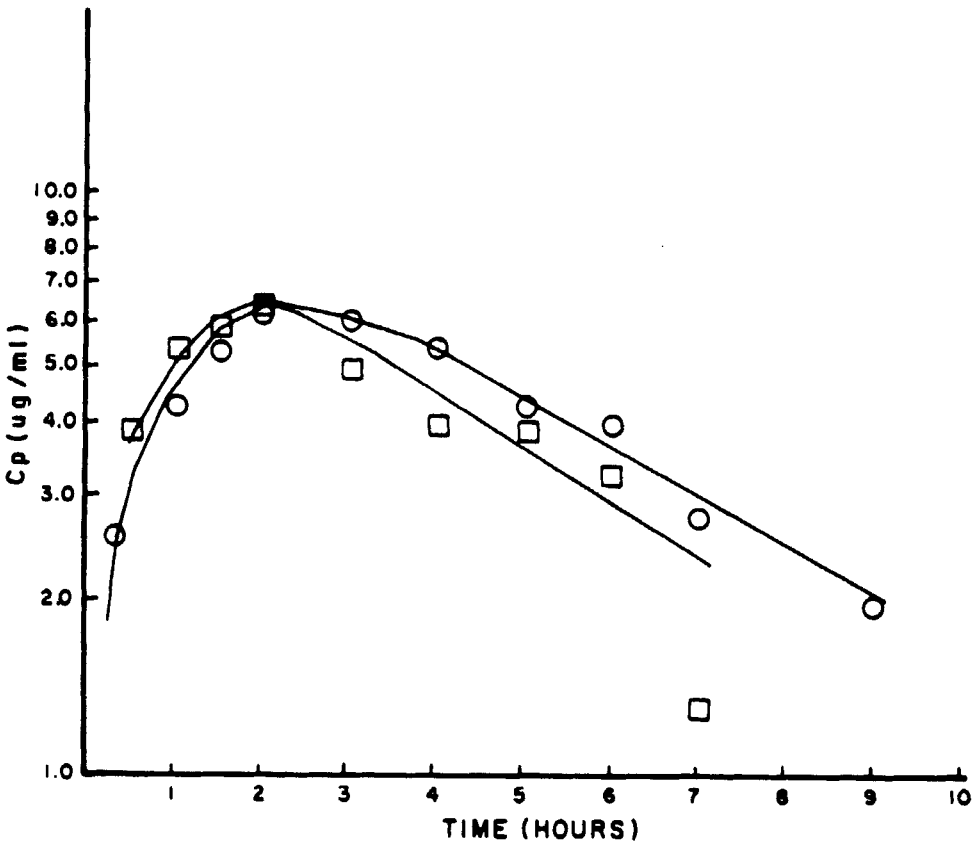
□ = phenytoin sodium solution. Each value represents the average of three animals.

○ = gelatin:phenytoin sodium microspheres (10:2 treated with 0 ml of glutaraldehyde). Each value represents the average of seven animals.

△ = gelatin:phenytoin sodium microspheres (10:2 treated with 5 ml of glutaraldehyde). Each value represents the average of two animals.

FIGURE 6

Plasma Concentration Profile of Phenytoin Following Oral Administration of Phenytoin Sodium Solution and Gelatin:Phenytoin Sodium Microspheres at 30 mg/kg Phenytoin Sodium Equivalents.



□ = phenytoin sodium solution. Each value represents the average of three animals.
○ = gelatin:phenytoin sodium microspheres (10:2 treated with 0 ml of glutaraldehyde). Each value represents the average of four animals.

FIGURE 7

Plasma Concentration Profile of Phenytoin Following Oral Administration of Phenytoin Sodium Solution and Gelatin: Phenytoin Sodium Microspheres at 50 mg/kg Phenytoin Sodium Equivalents.

suitable model, it was eliminated from any further testing.

In order to determine variation between animals, the individual animal data were subjected to the same procedures as the group data. If individual data for a majority of the animals within a group converged, the group was retained for further testing. For the groups retained, only the within-group individual animal data which converged were statistically tested. The following groups met convergence in a majority of the animals tested: 1) phenytoin sodium alkaline solution administered intraperitoneally at 20 mg/kg phenytoin sodium equivalents; 2) microspheres prepared using 10 g of gelatin and 2 g of phenytoin sodium with 0 ml of glutaraldehyde administered intraperionteally at 20 mg/kg phenytoin sodium equivalents; 3) microspheres prepared using 10 g of gelatin and 2 g of phenytoin sodium with 0 ml of glutaraldehyde administered orally at 20 mg/kg phenytoin sodium equivalents; and 4) microspheres prepared using 10 g of gelatin and 2 g of phenytoin sodium with 0 ml of glutaraldehyde administered orally at 50 mg/kg phenytoin sodium equivalents.

The pharmacokinetic parameters selected for testing were the AUC, Cmax, and tmax. The intraperitoneal data allowed the effect of microsphere formulation to be

tested. The oral data allowed testing of the effect of microsphere dose. Statistical analysis of the means of each of the select pharmacokinetic parameters was performed using a t-test.

The microsphere formulation had no effect upon the AUC and tmax of phenytoin when given intraperitoneally since both t-tests were not statistically significant. There was a significant difference in the Cmax of phenytoin obtained from the phenytoin sodium alkaline solution and the microsphere formulation, with the microspheres producing the higher Cmax. It has been reported that prompt and extended release phenytoin sodium capsules produce a higher Cmax than phenytoin sodium injection.[12] Absorption following oral administration of the phenytoin sodium was found to be erratic and highly variable. The authors postulated that this may have been due to precipitation of poorly soluble phenytoin acid in the stomach. A similar mechanism has been proposed for the poor absorption of phenytoin following intramuscular administration.[13] It is possible that the lower Cmax observed with the intraperitoneal solution administered in this study may also have been due to precipitation of phenytoin. If precipitation of phenytoin occurs from the intraperitoneal administration of phenytoin alkaline solution, redissolution would be the rate-limiting step

in the absorption process. Slow and/or incomplete redissolution may possibly result in lower plasma levels. On the other hand, the release of phenytoin from the microspheres may be the rate-limiting step in the absorption process when phenytoin sodium microspheres were administered intraperionteally.

Altering the dose of phenytoin sodium in microsphere form had no effect upon the Cmax of phenytoin when administered orally. There was a significant difference was noted in the AUC and tmax of phenytoin when given in microsphere form, with the higher dose producing the greater AUC and tmax.

CONCLUSIONS

The purpose of this study was to formulate and evaluate by in vitro and in vivo methods gelatin:phenytoin sodium microspheres. After completion of the experimentation and statistical analyses of the data, the following conclusions were made:

1. Gelatin:phenytoin sodium microspheres can be formulated using the process of emulsification.
2. The transformed percent phenytoin sodium retained in the microspheres was significantly affected by altering both the gelatin:phenytoin sodium ratio and the volume of glutaraldehyde in the microsphere formulations.

3. It appears that, when glutaraldehyde was not utilized in the microsphere formulations the 20:2 ratio of gelatin to phenytoin sodium produced microspheres having the fastest dissolution.
4. The dissolution of phenytoin sodium from the microspheres appears to be delayed by the addition of glutaraldehyde to the microsphere formulations.
5. The transformed percent phenytoin sodium retained in the dissolution studies revealed that microspheres prepared using the 10:2 ratio of gelatin to phenytoin sodium had the most consistent release. It was also found, for microspheres prepared from this ratio of gelatin to phenytoin sodium, that those microspheres treated with 0 ml of glutaraldehyde had the fastest release of phenytoin sodium while those microspheres treated with 5 ml of glutaraldehyde had the slowest release of phenytoin sodium.
6. When given intraperitoneally, phenytoin sodium microspheres produced a significantly higher Cmax than phenytoin sodium alkaline solution in female Sprague-Dawley rats.
7. When phenytoin sodium microspheres were given orally to female Sprague-Dawley rats, a significant difference in AUC and tmax was observed when the dose of phenytoin sodium was increased.

In general, it may be concluded that some formulation variables influenced the dissolution of phenytoin sodium from gelatin:phenytoin sodium microspheres as well as selected pharmacokinetic parameters.

FOOTNOTES

1. Type I from swine skin, acid-treated, 300 Bloom; Sigma Chemical Co.; St. Louis, Missouri.
2. Grade I, crystalline; Sigma Chemical Co.; St. Louis, Missouri.
3. Amend Drug and Chemical Co.; Irvington, New Jersey.
4. Blue M; Magni Whirl Constant Temperature Bath; Blue M Electric Company; Blue Island, Illinois.
5. GT21-18 Variable Speed Stirrer fitted with a GT-21 Motor Controller; G. K. Heller Corp.; Floral Park, New York.
6. Model B-2; Lauda Corp.; West Germany.
7. Fisher Scientific Co.; Fair Lawn, New Jersey.
8. Fisher Scientific Co.; Fair Lawn, New Jersey.
9. Virtis 45; The Virtis Company; Gardiner, New York.
10. HATF 02500; Millipore Corporation; Bedford, Massachusetts.
11. Model 139 UV-Vis; Perkin Elmer; Norwalk, Connecticut.
12. Vanderkamp 600; Van-Kel Industries Inc.; Chatham, New Jersey.

13. Statistical Package for the Social Sciences; SPSS, Inc.; Chicago, Illinois.
14. Grade I, crystalline; Sigma Chemical Co.; St. Louis, Missouri.
15. HPLC-Grade; Fisher Scientific Co.; Fair Lawn, New Jersey.
16. Pierce Chemical Co.; Rockford, Illinois.
17. Model 6000A Solvent Delivery System; Waters Associates; Milford, Massachusetts.
18. Beckman Model 155; Beckman Instruments, Inc.; Berkeley, California.
19. Radial-PAK A Liquid Chromatography Cartridge; Waters Associates; Milford, Massachusetts.
20. Sigma Chemical Co.; St. Louis, Missouri.
21. NLIN; SAS Institute, Inc.; Cary, North Carolina.

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