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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this research project is to better understand the interaction of dietary supplements with cancer chemotherapeutic drugs. This information may be useful to decrease the toxicity and increase the effectiveness of chemotherapy. The scope of the research involves *in vivo* assessments in rats of nutritional supplement-chemotherapeutic drug interactions and *in vitro* studies of the mechanisms of nutraceutical-chemotherapeutic drug interactions. High performance liquid chromatography (HPLC) methodology has been adapted/refined for the detection and determination of doxorubicin and vitamin E. Under the conditions used by us, we found that there was no significant effect of vitamin E supplementation on hematologic toxicity or survival in rats treated with a range of doxorubicin doses. There were no important perturbations of the pharmacokinetics of doxorubicin associated with changes in the vitamin E content of rat diets. Preliminary results indicated that treatment with St. John's wort alters the plasma pharmacokinetics of doxorubicin. Our studies suggest that even relatively high doses of vitamin E do not adversely affect the toxicity of doxorubicin. On the other hand, St. Johns wort may decrease peak levels of doxorubicin. Further studies will help to determine whether important interactions occur between these nutrients and cancer chemotherapeutic agents.

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INTRODUCTION

The general subject of this research project is the effects of dietary supplements on the pharmacokinetics and pharmacodynamics of chemotherapeutic drugs used to treat women with breast cancer. More specifically, the research focuses on the effects of St. John's wort as an example of a nutraceutical and Vitamin E as an example of a nutritional supplement on doxorubicin, docetaxel, and cyclophosphamide. The hypothesis to be tested is that nutritional supplements have important effects on the pharmacokinetics and pharmacodynamics of cancer chemotherapeutic agents. The purpose of the research is to better understand the interaction of dietary supplements with cancer chemotherapeutic drugs and then utilize this knowledge to alert patients and their physicians to these interactions. This information also may be useful to decrease the toxicity and increase the effectiveness of chemotherapeutic drugs. The scope of the research involves *in vivo* assessments in rats of nutritional supplement-chemotherapeutic drug interactions and *in vitro* studies of the mechanisms of nutraceutical-chemotherapeutic drug interactions.

BODY

Task 1. Evaluate the effects of supplementation with St. John's wort and vitamin E on the pharmacokinetics of cyclophosphamide, docetaxel and doxorubicin.

a. Establish and refine, as necessary, methodology for analysis of plasma concentrations of 4-hydroxycyclophosphamide, docetaxel, doxorubicin, vitamin E and hypericin.
b. Maintain 3 groups of rats on diet alone, or diet plus St. John's wort or vitamin E.
c. Inject rats with chemotherapeutic agents and collect plasma samples.
d. Measure drug levels in plasma
e. Analyze pharmacokinetic data.
f. Repeat phamacokinetic studies with different doses.

The work is proceeding as outlined in Task 1. We have chosen to begin with studies of the effects of St. John's wort and vitamin E on doxorubicin.

α-Tocopherol (vitamin E) concentrations in plasma were measured with a reverse-phase high-performance liquid chromatography method using UV detection as previously reported by Julianto *et.al.* (1). Briefly plasma samples were deproteinized by treating 100μL plasma with 200μL acetonitrile-tetrahydrofuran (3:2 [vol/vol]). The mobile phase consisted of 6% tetrahydrofuran in methanol with a flow rate of 1mL/min through an Econosphere C18 5μ column (Alltech, Deerfield, IL) and an Econosphere C18 5μ guard column (Alltech). The detector was operated at a wavelength of 292nm and the samples were quantified using peak area. A good concentration-peak area relationship was obtained with a correlation coefficient of 0.96.

Hypericin (the active ingredient in St. John's wort) concentrations in plasma will be measured with a reverse-phase high-performance liquid chromatography method using

fluorescence detection as previously reported by Kerb *et al.* (2). Briefly, plasma samples will be prepared by treating 250μL plasma with 200μL dimethyl sulfoxide and then with 75μL acetonitrile-2-butoxyethanol (75:25 [vol/vol]). Samples will then be extracted with 2mL ethyl acetate and the supernatant dried under nitrogen. The dried extract is redissolved in 100μL acetonitrile. The mobile phase consists of methanol-tetrahydrofuran-0.1M Na_2PO_4 (pH 4.0) (45:30:25 [vol/vol]) with a flow rate of 0.8mL/min through a LiChrospher RP Select B 5μ column (Alltech, Deerfield, IL) with a LiChrospher RP Select B 5μ guard column (Alltech). An excitation wavelength of 315nm and emission wavelength of 590nm is used for fluorescence detection and the samples are quantified using peak area. Our original plan was to focus on analysis of hypericin as an indicator molecule for our pharmacological studies of St. John's wort. Because some recent studies have identified hyperforin as an important contributor to the pharmacological actions of this herbal product we plan, in analytical studies about to be initiated, to select methodology (HPLC probably with fluorimetric detection) that accurately detects and determines both of these agents.

Doxorubicin concentrations in plasma were measured with a reverse-phase high-performance liquid chromatography method using fluorescence detection as previously reported by Warren *et al.* (3) and as modified by us. Briefly, plasma samples were deproteinized by treating with 4 volumes of ethanol and the supernatant evaporated under nitrogen and redissolved in the mobile phase. The mobile phase consisted of 0.4M ammonium formate pH 4.0-acetonitrile (79:21 [vol/vol]) with a flow rate of 2mL/min through a μ-Bondapak phenyl column (Waters Corporation, Milford, MA) with a PS-GU phenyl 5μ guard column (Thomson Instrument Company, Springfield, VA). An excitation wavelength of 480nm and emission wavelength of 595nm was used for fluorescence detection and the samples were quantified using peak area. A standard calibration curve is shown in Figure 1. Excellent concentration-peak area relationships (correlation coefficients > 0.99) were obtained for samples of pooled rat plasma containing doxorubicin (Figure 1).

Weanling female Fisher 344 rats, 4 or 5 rats per group, were fed either a cereal-based, standard rat diet that supports growth and maintenance (Harlan-Teklad Global, Product # TD 00217) that contains 16% protein, 3.5% fat and 102 I.U./kg Vitamin E (α-tocopherol), or the same diet supplemented with either a high level of vitamin E, 750 I.U./kg (Harlan-Teklad Product # T.D. 01375) or a low level of vitamin E, 50 I.U./kg (Harlan-Teklad Product # T.D. 01374).

In our initial experiments rats were injected intravenously with doxorubicin at a dose of 5 mg/kg, but we observed plasma concentrations at later time points that were at or below the limit of quantitation and thus increased the dose modestly to 7.5 mg./kg. Plasma samples were collected at 0, 10, 20, 30, 45, 60, 180, 300, 420, 1440 and 2880 minutes. Figure 2 illustrates HPLC analyses prior to and at 10 and 180 minutes after intravenous administration of doxorubicin. For all conditions we observed a biexponential elimination pattern, with rapid distribution and slow elimination typically described for doxorubicin in rodents doses comparable to the dose that we used. Estimated pharmacokinetic parameters were comparable for rats on control, low vitamin

E and high vitamin E diets. For example, in animals receiving the low vitamin E diet the elimination half-life was in the 24 hour range and volume of distribution and clearance were 35 liters/kg and clearance was 17 ml/min/kg respectively; these parameters were closely similar to those estimated recently for doxorubicin in rats in a study of the effects of a P-glycoprotein inhibitor on the pharmacokinetics of doxorubicin (4). Estimated "peak" plasma concentrations of doxorubicin 10 minutes after intravenous administration were in the 500- 600 nanomolar range for rats in each study group. Estimated plasma concentrations of doxorubicin 24 hours after intravenous adminstration were also comparable in rats on the high and low vitamin E diets. For example the mean doxorubicin concentrations at 24 hours were 35 and 39 nanomolar respectively for the low and high vitamin E diets. (Figures 3 and 4). We have retained selected tissue samples for future analysis to assess whether the comparable plasma pharmacokinetics observed for the 3 groups studied extends to tissues.

Pharmacokinetic studies are being conducted with extracts of St. John's wort. Initial exploratory studies were performed with a standardized preparation of the herb (HBC St. Johns wort) that was used in clinical trials and pharmacokinetic studies supported by the National Institutes of Health. This same preparation will be used in a human study of the effects of St. John's wort on the pharmacokinetics of docetaxel to be conducted by Cancer and Leukemia Group B (CALGB). The extract is standardized to 0.3% Hypericin and 4% Hyperforin. Therefore it seemed like a logical preparation to use for our studies. The herb was suspended in water at a dose level of 100 mg/kg. This converts to approximately the same concentration that is available to humans ingesting these tablets. The suspension was administered daily to rats by gavage. A 14 day course was planned prior to injecting doxorubicin for pharmacokinetic studies. Unfortunately, all of the animals lost weight, and most died before the completion of 14 days. Rats in the control group that were given water by gavage survived, indicating that the deaths were due to the St. John's wort preparation rather than to faulty gavage technique. Post-mortem examination by the veterinarian identified esophageal irritation with abscess formation in some animals, and intestinal obstruction in most of the rats. As is the case for other commercially available herbal preparations, the St. John's wort preparation contains a variety of components other than hyperforin and hypericin, the putative active agents. A review of the ingredients of this St. John's wort preparation indicated that the excipients included silicon dioxide. We believe that this ingredient may be causing the intestinal complications. We currently are evaluating other preparations of St. John's wort in exploratory toxicological studies to aid us in selecting an optimal preparation for our extended studies. One such preparation is stated to contain only rice powder as an exipient. Although the St. John's wort preparation initially selected is not suitable for our purposes we did make the provocative obesrvation in the course of our preliminary studies with this preparation that treatment with the St. John's wort preparation for 14 days prior to administration of doxorubicin results in a substantial (at least 2-fold) reduction in plasma concentrations of doxorubicin, suggesting significant modification of the disposition of this important antineoplastic agent (Figure 5).

Task 2. Measure the effects of supplementation with St. John's wort and vitamin E on the toxicity of cyclophosphamide, docetaxel and doxorubicin.

a. Maintain 3 groups of rats on diet alone, or diet plus St John's wort or vitamin E.
b. Administer chemotherapeutic drugs in LD50 doses.
c. Observe for toxicity and collect blood samples.
d. Analyze blood samples for evidence of hematologic, renal, hepatic and cardiac toxicity.
e. Analyze toxicity data.
f. Repeat toxicological studies with different doses.

The work is proceeding as outlined in Task 2. Weanling female Fisher 344 rats were maintained on the same diets described in Task 1; that is, cereal-based or supplemented with a low dose (50 I.U./kg) or a high dose of Vitamin E (750 I.U./kg). The rats grew at the same rate on all 3 diets (Figure 6). After 8 weeks the rats were injected with increasing doses of doxorubicin (5.0, 7.5, 9.8, 12.8 and 17.9 mg/kg). Figure 7 shows that the LD50 of doxorubicin was approximately the same for all 3 diets, and that there was no dose-response relationship for Vitamin E. Measurements of hematocrit and white blood cell counts at Days 4, 9 and 14 following injection of doxorubicin showed no important differences among the dietary groups.

Task 3. Study the mechanisms of nutraceutical-chemotherapeutic drug interactions.

a. Measure hepatic p450 activity where appropriate.
b. Measure hepatic P-glycoprotein expression where appropriate.
c. Measure glutathione S-transferase activity in liver samples where appropriate.
d. Collate and analyze data.

These studies have not started because the nutraceutical-chemotherapeutic drug interactions have not been defined yet.

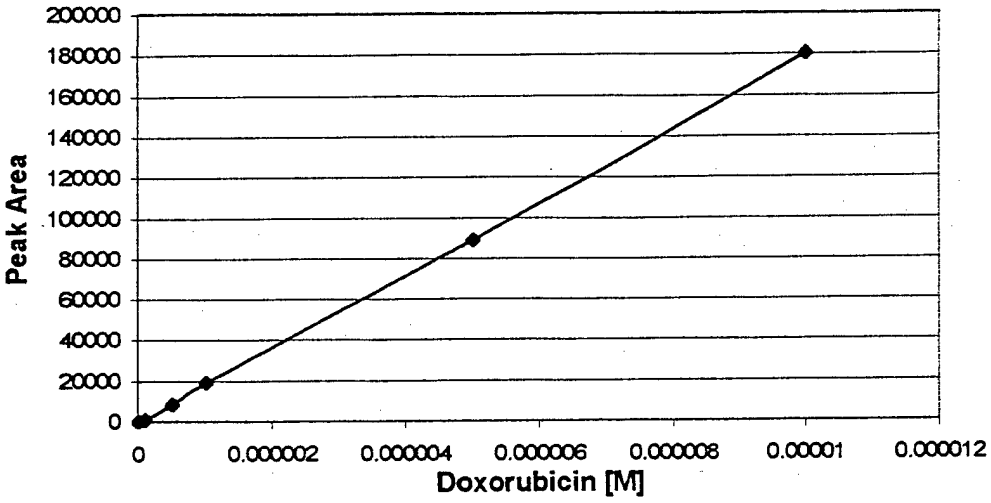


Figure 1

Standard calibration curve obtained by spikeing blank pooled rat plasma with doxorubicin at a concentration range of $1.0x10^{-7}$ to $1.0x10^{-5}$ M.

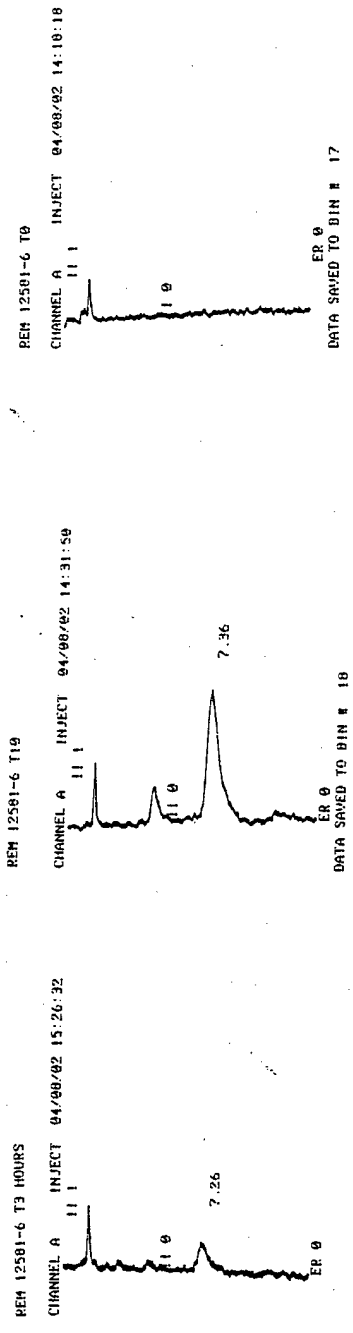


Figure 2

HPLC analysis of rat plasma samples collected before (upper), 10 minutes after (middle) and 180 minutes after (lower) intravenous administration of doxorubicin (7.5mg/kg).

Figure 3

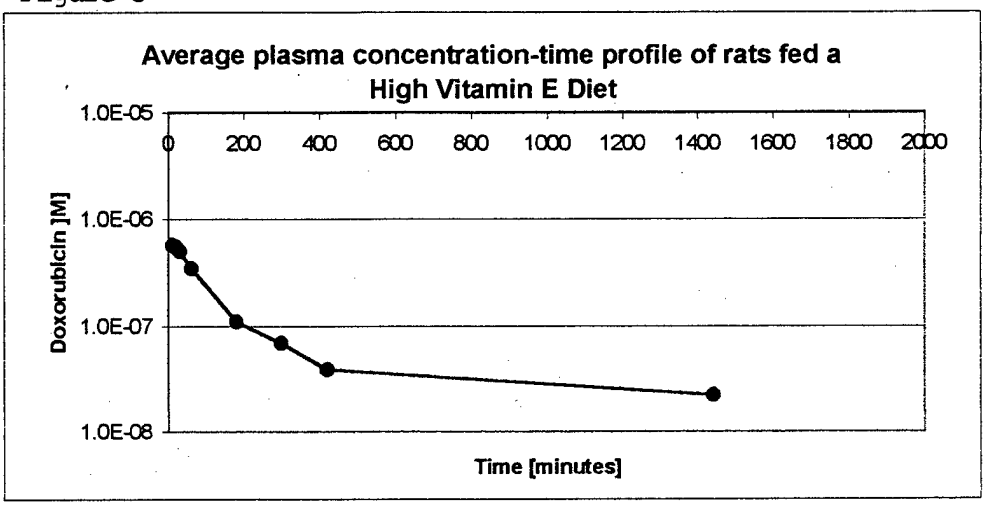
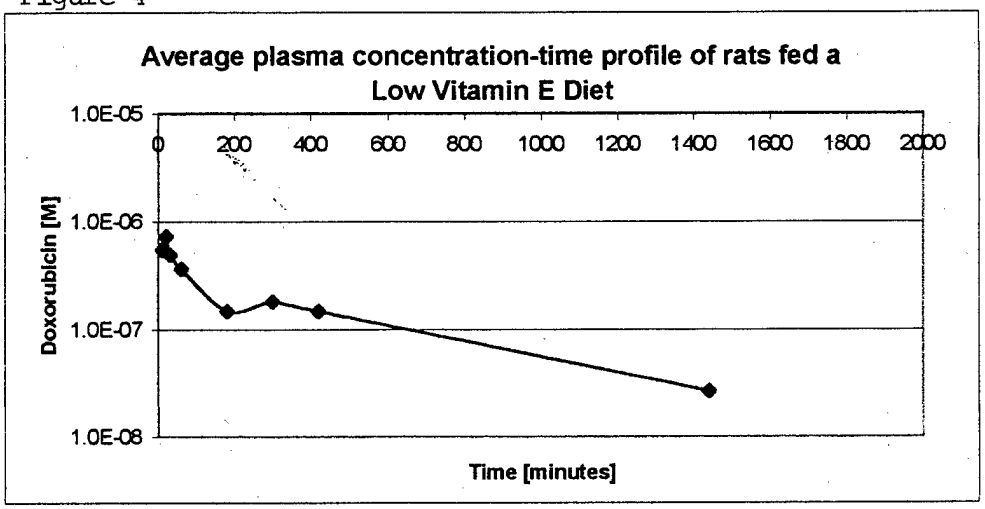


Figure 4



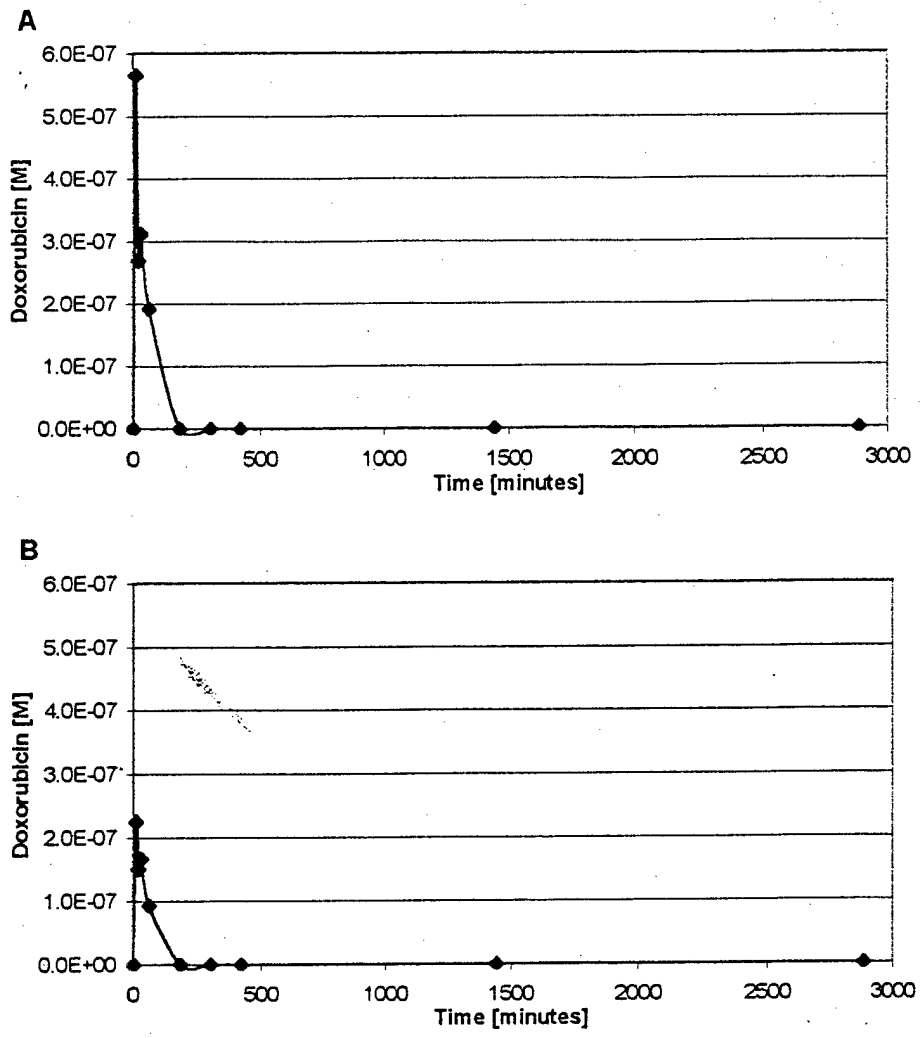


Figure 5

Plasma concentration-time profile of rats treated with a single dose of 7.5mg/kg doxorubicin and fed a cereal-based diet (A) without St. John's Wort supplementation and (B) with supplementation. St. John's Wort was administered by gavage daily for 14 days at 100mg/kg before dosing with doxorubicin.

Figure 6. **Comparison of Average Weekly Weights of Each Dietary Group For Animals Receiving 12.8 mg/kg of Doxorubicin**

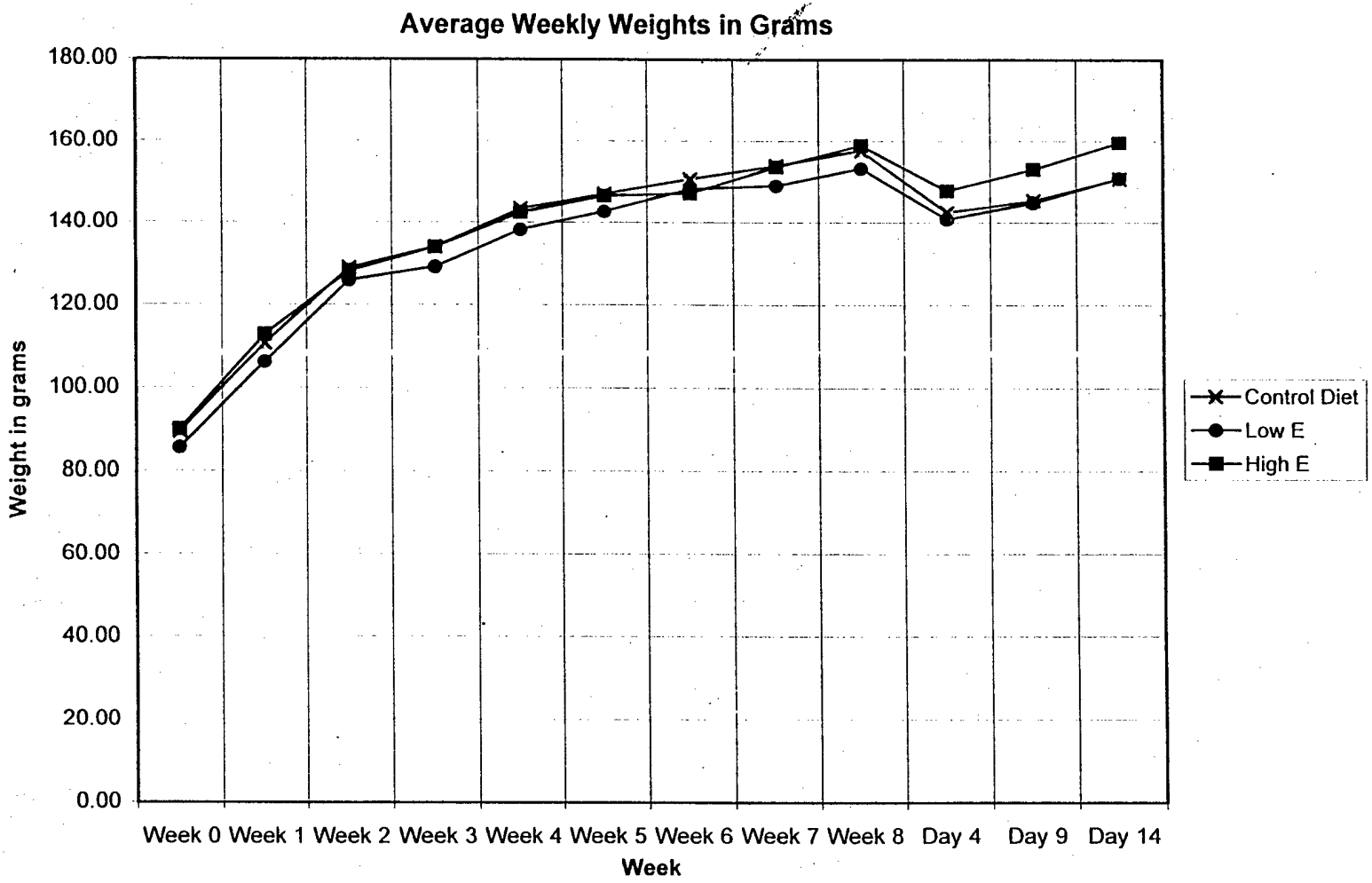
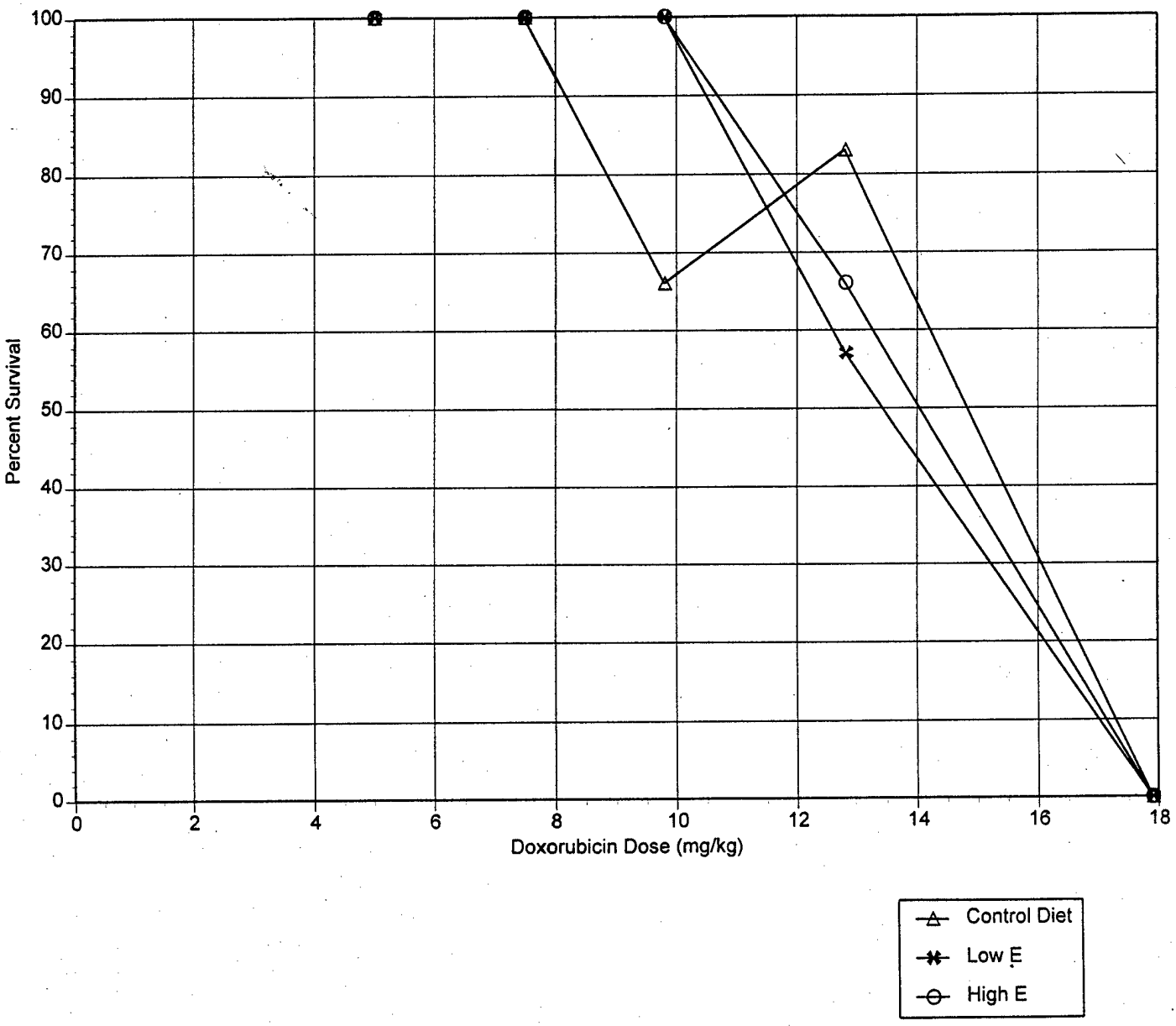


Figure 7. **Comparison of Three Levels Of Dietary Vitamin E On Doxorubicin Toxicity**



KEY RESEARCH ACCOMPLISHMENTS

- High performance liquid chromatography (HPLC) methodology has been adapted/refined for the detection and determination of doxorubicin and vitamin E.
- Under the conditions used by us, we found that there was no significant effect of vitamin E supplementation on hematologic toxicity or survival in rats treated with a range of doxorubicin doses.
- Studies have been completed and await detailed analysis to ascertain whether effects of vitamin E on the pharmacokinetics of doxorubicin parallel the effects on toxicity. Preliminary analysis indicates that there were no important perturbations of the pharmacokinetics of doxorubicin associated with changes in the vitamin E contents of rat diets.
- Preliminary results indicated that treatment with St. John's wort alters the plasma pharmacokinetics of doxorubicin.

REPORTABLE OUTCOMES

Funding applied for based on work supported by this award:

- American Society of Clinical Oncology (ASCO) Young Investigator Award to Zafer Yildirim, MD, PhD, for a post-doctoral fellowship to work on this project.

CONCLUSIONS

The experiments described in this Annual Report analyze the relationship between dietary supplements and chemotherapeutic drugs used to treat patients with breast cancer. Herbal medicines and dietary supplements are frequently used by patients with cancer, but there is a paucity of information on their interactions with prescribed drugs. Since a majority of women with breast cancer are taking dietary supplements, there is a pressing need to better understand the effects of these supplements on cancer chemotherapy. Our studies suggest that even relatively high doses of vitamin E do not adversely affect the toxicity of doxorubicin. On the other hand, preliminary results suggest that St. Johns wort may decrease peak levels of doxorubicin. Further studies will help to determine whether important interactions occur between these nutrients and cancer chemotherapeutic agents.

REFERENCES

1. Julianto, T., K.H. Yuen, A.M. Noor. Simple high-performance liquid chromatographic method for detemination of α-tocopherol in human plasma. J. Chromat. B 732:227-321, 1999.
2. Kerb, R., J. Brockmoller, B. Staffeldt, M. Ploch, I. Roots. Single-dose and steady-state pharmacokinetics of hypericin and pseudohypericin. Antimicrobial Agents and Chemotherapy 40:2087-2093, 1996.
3. Warren, K.E., C.M. McCully, T.J. Walsh, F.M. Balis. Effects of fluconazole on the pharmacokinetics of doxorubicin in nonhuman primates. Antimicrobial Agents and Chemotherapy 44:1100-1101, 2000.
4. Mellows, G., Mistry, P., Norris, D., Waterfall, J. The effect of XR9576 (tariquidar), a specific P-glycoprotein inhibitor, on the pharmacokinetics of doxorubicin, paclitaxel and vincristine in the rat. Proc. AACR 43:275, 2002.