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Synthesis, Properties, and Intratumoral Evaluation of Mitoxantrone-loaded Casein Microspheres in Lewis Lung Carcinoma

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Abstract—Smooth, round, uniform bovine casein microspheres of 1–5 and 10–20 μm size were readily prepared by a steric stabilization technique previously developed in this laboratory for synthesis of albumin microspheres. The avid phagocytic uptake of casein and albumin microspheres was demonstrated with fluorescein-labelled microspheres using a macrophage-like mouse myelomonocytic leukaemia cell line. Post-synthesis loading of 25% mitoxantrone was achieved for casein microspheres containing 20% polyglutamic acid. Preliminary intratumoural chemotherapy experiments with a mouse Lewis lung carcinoma indicated that mitoxantrone and mitoxantrone-loaded casein-polyglutamic acid microspheres exhibited lower toxicity when administered intratumorally.

A major goal for improved cancer chemotherapy is the selective, efficient and targeted delivery of cytotoxic drugs to desired sites of action (McLaughlin & Goldberg 1983). To enhance therapeutic efficacy of anticancer drugs and at the same time reduce their toxicity to normal, healthy tissues in the body, various targeting concepts have been investigated. Microsphere carrier systems based on the natural circulatory protein carrier, albumin, have received increasing attention (Morimoto & Fujimoto 1985; Gupta & Hung 1990). Such microspheres are particularly promising for clinical use because of their inherent long term stability and the relatively high drug payloads which can be achieved.

Surface characteristics of microspheres play a significant role in their in-vivo fate. In general, hydrophobic particles are usually removed more rapidly from the circulation than are hydrophilic particles (Illum et al 1986). Particle hydrophilicity is also important for easy wetting and rapid reconstitution of injectable dispersions in aqueous vehicles (Longo et al 1982). Recently, we developed a synthetic method for the preparation of microspheres based on the amphiphilic protein, casein, which afforded even more hydrophilic microspheres than those based on albumin (unpublished work). The casein microspheres, which are cross-linked with glutaraldehyde via the organic phase with glutaraldehyde-toluene, also appear to be smoother, rounder and more uniform in structure than casein microspheres prepared by conventional aqueous glutaraldehyde cross-linking (Chen et al 1987). The drug-loading efficiency and in-vitro release of the drug methotrexate from our casein spheres was very similar to albumin. We therefore believe that it may be advantageous to use relatively inexpensive casein in place of albumin as a matrix for microsphere drug carriers prepared by our method. Our protein microsphere synthesis (Longo et al 1982) is different from other reported methods in that the glutaraldehyde cross-linking agent is added via the organic phase in an organic solvent (toluene) which results in an initial surface cross-linking. Greater hydrophilicity and improved dispersability in aqueous vehicles is achieved, as well as a uniform, spherical structure. Furthermore, microspheres made by our method are readily post-loaded with many drugs by absorption from aqueous drug solutions.

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This report is concerned with the preparation of mitoxantrone-loaded casein microspheres and evaluation of antitumour activity in a mouse Lewis lung carcinoma model via intratumoural chemotherapy. Mitoxantrone (Fig. 1), an anthracenedione, was investigated because it is a potentially important new anticancer drug with a wide spectrum of antitumour activity, comparable in many ways with doxorubicin, but with reduced toxicity (Batra et al 1986).

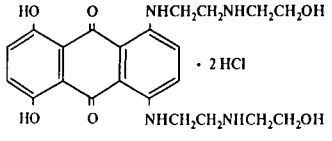

FIG. 1. Structure of mitoxantrone.

Materials and Methods

Materials

Bovine casein, bovine serum albumin (BSA), fluorescein isothiocyanate casein (FITC–CAS) and albumin (FITC-BSA), poly-L-glutamic acid (PGA), protease (Type VIII, 7·8 units mg^{-1}), Iscove's modified Dulbecco's medium, Hanks' buffered salt solution, foetal bovine serum, L-glutamine, antibiotic cocktail, non-essential amino acids and light mineral oil were obtained from Sigma Chemical Company, USA. Mitoxantrone hydrochloride (Novantrone), 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino] ethyl] amino]-9,10-anthracenedione dihydrochloride, was a generous gift from Lederle Laboratories, USA. Sorbitan sesquioleate (Arlacel C) was from ICI Americas Inc., USA. All other reagents were of analytical or equivalent grade from Fisher, USA.

Preparation of microspheres

Casein microspheres (CAS/MS) of 1–2 μm size were prepared by a reverse emulsion method. Typically, 1 mL of a 20% solution of casein in 0·5 M NaOH was added to 50 mL of a mixture of light mineral oil and *n*-hexane (1:4) containing 1 g of the surfactant. The mixture was sonicated using the Q-horn of a sonicator (Heat Systems-Ultrasonics Inc., Model W-375, USA) at a power setting of 9 for 5 min. Cross-linking of the protein was accomplished via the organic phase by the addition of 10 mL glutaraldehyde saturated toluene (Longo et al 1982). The contents were stirred in a 100 mL round bottomed flask at 2000 rev min^{-1} for 1 h at room temperature (25°C) using a Teflon paddle stirrer. Microspheres were washed several times with petroleum ether followed by several acetone washes, and dried under vacuum at room temperature.

Bovine serum albumin microspheres (BSA/MS) were prepared in a similar fashion except that the solution of albumin was prepared in distilled water, and the dispersion was sonicated for only 1 min at a power setting of 5.

Microspheres containing poly-L-glutamic acid (PGA) were prepared by dissolving 20% PGA with casein in the standard syntheses. FITC-labelled BSA/MS and CAS/MS were prepared by incorporating 4% FITC-BSA or FITC-CAS with the unlabelled protein in the standard syntheses.

Incorporation of mitoxantrone in microspheres

Microspheres containing mitoxantrone were prepared either by incorporation of the drug during synthesis or by post-synthesis loading. Mitoxantrone (50 mg) was mixed with a casein or albumin solution and dispersed and cross-linked as before to incorporate the drug during synthesis. The loading obtained by this technique for 1–2 μm microspheres was 8–10%. Microspheres of 10–20 μm size were also readily prepared with BSA and casein except that dispersion was achieved with a Vortex mixer (Fisher Scientific) at a setting of 8. Loading of ca. 14% of mitoxantrone during microsphere synthesis was obtained for these larger microspheres. Post-synthesis loading was conducted with CAS/MS containing 20% PGA. Typically, microspheres (100 mg, 1–2 μm) were added to 5 mL of a 10% solution of mitoxantrone in a screw-cap test tube and rotated in a test tube rotator (Labquake Inc., USA) overnight at room temperature. After washing twice with water and twice with acetone, microspheres were vacuum dried at room temperature. Enhanced mitoxantrone loading (25%) was obtained for 1–2 μm microspheres by this post-loading method. However, post-loading of 10–20 μm BSA–PGA and CAS–PGA/MS with mitoxantrone was about 12%.

Analysis of the drug content

The amount of drug incorporated was determined by digesting the spheres with protease (Kim & Oh 1988). Microspheres (5 mg) were suspended in 20 mL phosphate buffer (pH 7·4, 0·1 M) containing 5 mg protease in a 50 mL screw-cap test tube. The suspension was incubated at 37°C until all the spheres were digested (4–5 h). An aliquot was diluted with an equal volume of trichloroacetic acid to precipitate the proteins. After centrifugation at 1500 rev min^{-1} for 10 min, the supernatant was filtered through a 0·45 μm filter and the filtrate analysed spectrophotometrically at 610 nm (Perkin Elmer UV-Vis spectrophotometer 552). Controls were performed with pure drug incubated with protease and pure drug plus unloaded spheres incubated with protease. No degradation of the drug due to the enzyme nor association of the drug with precipitated protein from the digested microspheres was observed.

In-vitro drug release

Approximately 10 mg of the mitoxantrone-loaded spheres was suspended in 300 mL phosphate buffer in stoppered Erlenmeyer flasks. The flasks were placed in a shaker bath (Blue M, USA) at 37°C and tumbled at a speed setting of 10. Aliquots of 2 mL were withdrawn at various time intervals, filtered and analysed spectrophotometrically for drug. Fresh buffer (2 mL) was added after the withdrawal of each aliquot to maintain a constant volume.

Phagocytic uptake of microspheres

Mouse myelomonocyte leukaemia cells (ATCC TIB68 WEHI 3), a macrophage-like line derived from BALB/c mice, were cultured in Iscove's modified Dulbecco's medium containing foetal bovine serum, L-glutamine, antibiotic cocktail and non-essential amino acids in a 5% CO_2 atmosphere at 37°C. Immediately before each experiment, the supernatant was removed from each tissue culture flask, leaving a confluent layer of cells adhered to the bottom of the flask.

Fluorescein-labelled BSA (1–2 μm) and CAS/MS (1–5 μm) were dispersed in distilled water by mild sonication. A 1 mL aliquot of the 10 mg mL^{-1} microsphere suspension and 4 mL culture medium was added to each flask, followed by incubation for 5, 10, 15, 30 or 60 min. After incubation, the supernatant was again removed from each flask, and the adherent cells were washed twice with 2·4 mL Hanks' buffered salt solution to remove any free spheres. After the addition of 5 mL culture medium, the cells were scraped from the bottom of the flask, and a small sample was removed for viability assessment using Trypan blue as the vital stain. The remaining cells were fixed in 10% neutral buffered formalin and examined with epi-fluorescent microscopy for the presence of fluorescein-labelled spheres within the cells. The percentage of cells containing one or more spheres was divided by the viability of the flask to give the percent of viable cells containing microspheres. Two flasks were treated and analysed per incubation time for both CAS and BSA/MS.

In-vivo evaluation of mitoxantrone-loaded casein spheres

Male $B6D_2F_1$ mice were implanted subcutaneously with 1 mm^3 fragments of fresh Lewis lung carcinoma tissue in the right axilla. Tumours were sized with Vernier calipers 14 days after implantation and the volumes calculated as for an oblate spheroid, i.e. volume $= \pi/6 \cdot (\text{width})^2(\text{length})$. Each treatment group consisted of seven animals, and injections were performed with a 23 gauge needle. On day 0, each animal received three 0·15 mL intratumour injections perfusing the centre and periphery of the tumour. On day 1, each animal received an additional four 0·15 mL injections. A total of 3 mg mitoxantrone was administered to each animal either as free drug or incorporated in microspheres. Drug-loaded microspheres were completely dispersed in phos-

phate-buffered saline by sonication before injection. Tumour volumes and animal weights were monitored for 14 days.

Results and Discussion

Mitoxantrone is a new anticancer agent with a wide spectrum antitumour activity comparable with that of doxorubicin, cyclophosphamide, methotrexate and cytosine arabinoside against P388 and L1210 leukaemias, B16 melanoma and colon tumour 26 in mice. It is reported to be well tolerated in man, with a low incidence of severe nausea, vomiting, phlebitis, alopecia and cardiotoxicity. The pharmacokinetics of mitoxantrone in man and laboratory animals has been reviewed by Batra et al (1986).

Analysis of the drug content in 1–2 μm casein spheres showed that when the drug was incorporated by in-situ synthesis, i.e. mixed with the protein solution and cross-linked, approximately 8% drug loading was achieved. However, approximately 25% mitoxantrone was incorporated by post-synthesis loading of CAS/MS containing PGA. Using a similar post-loading procedure, 18% doxorubicin was also readily incorporated into the CAS/MS. PGA has previously been shown to form a stable drug-complex with basic antitumour agents such as adriamycin (Goldberg et al 1981), facilitating high drug payloads in microspheres. Additional later reports by Goldberg et al (1984) and Willmott et al (1988) confirm enhanced basic drug loading by microspheres containing anionic polypeptides. However, it should be noted that the syntheses by Goldberg et al (1981, 1984) and in this study involved not only in-situ incorporation of basic drugs (during microspheres synthesis and cross-linking) as reported by others, but also post-loading from aqueous drug solutions (after preparing the protein microspheres). Using our post-loading procedure, high drug payloads can be achieved, drug is not exposed directly to the glutaraldehyde cross-linker, and any opportunity for chemical modification during microsphere synthesis is eliminated. Although insoluble and stable in water, the drug-polypeptide complexes have been shown to dissociate readily in physiological saline to release the free drug. Since mitoxantrone is a basic drug, this ionic complex formation is presumably responsible for the higher payload observed in the case of CAS-PGA/MS.

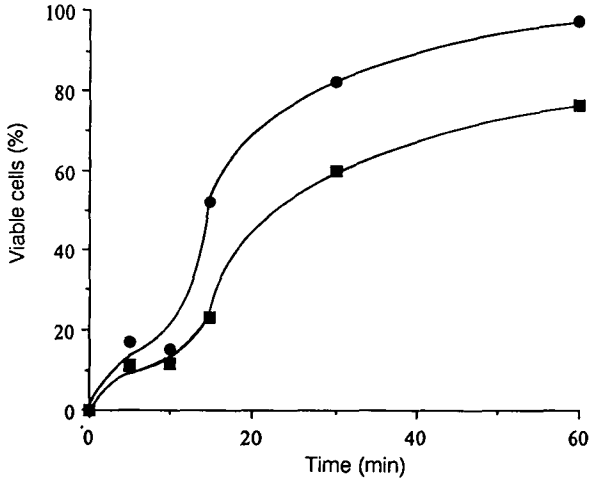

FIG. 3. Phagocytic uptake of microspheres. ■ 1–5 μm casein microspheres, ● 1–2 μm albumin microspheres.

The in-vitro release profiles of mitoxantrone incorporated during microsphere synthesis from CAS/MS and CAS-PGA/MS are shown in Fig. 2. For comparison, drug release from similarly prepared BSA/MS containing mitoxantrone is also shown in the same figure. The release profiles were comparable for both casein and BSA systems (no PGA). Most of the mitoxantrone released over a period of 24 h

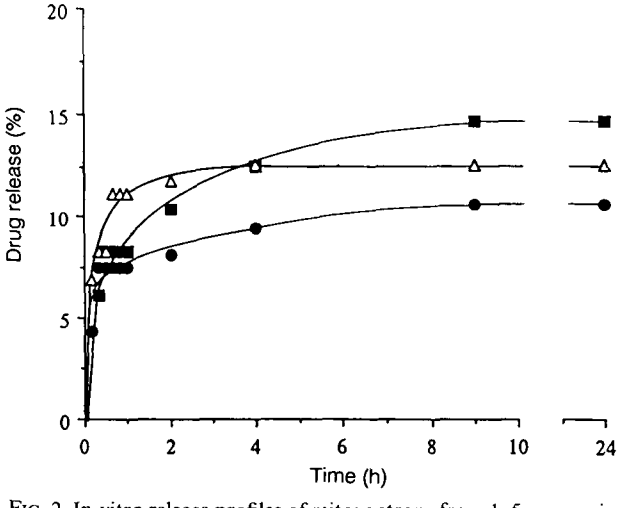

FIG. 2. In-vitro release profiles of mitoxantrone from 1–5 μm casein and 1–2 μm albumin microspheres. △ Albumin microspheres, ■ casein microspheres, ● post-synthesis loaded casein/20% poly-L-glutamic acid microspheres.

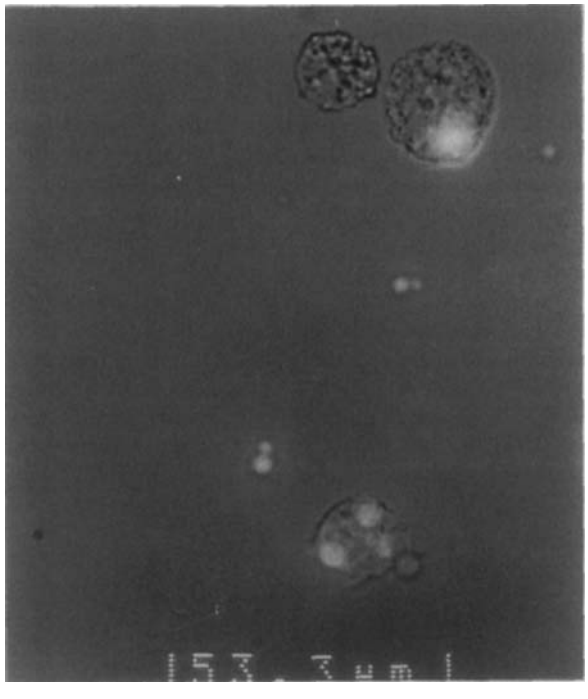

FIG. 4. Optical micrograph of macrophage-like cells containing 1–5 μm casein microspheres.

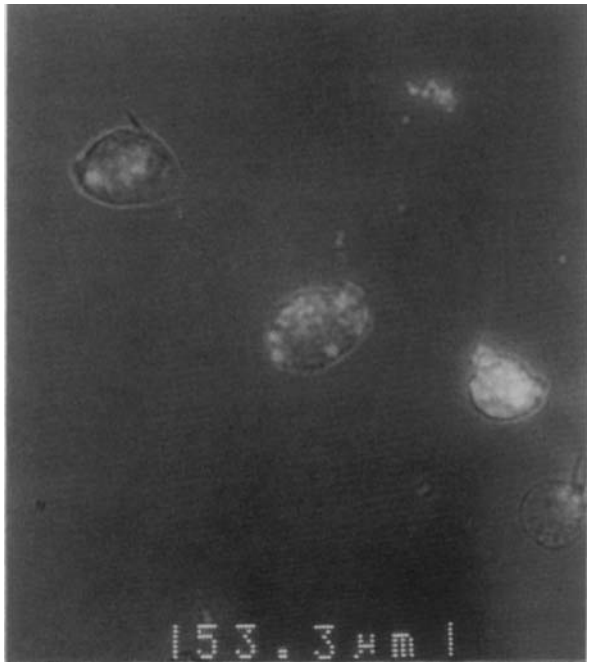

FIG. 5. Optical micrograph of macrophage-like cells containing 1-2 albumin microspheres.

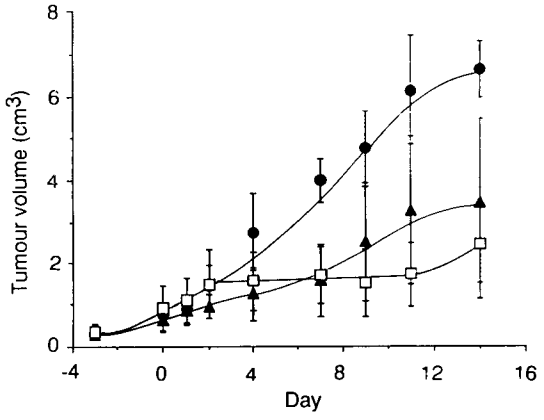

FIG. 6. Intra-tumour chemotherapy of subcutaneous mouse Lewis lung carcinoma with 1-5 µm microspheres. ● Untreated control group, ▲ 3 mg/mouse (free drug), □ 3 mg mitoxantrone/mouse in casein/20% poly-L-glutamic acid microspheres. Day 0 was 24 days after tumour implantation when average tumour size was ca. 0·8 cc; n = 7 mitoxantrone per group.

appeared to be drug which was physically associated with the microsphere. The major portion of mitoxantrone was more strongly bound to the matrix, perhaps covalently via glutaraldehyde, and could be released only upon enzymatic degradation of the matrix. In this regard, facile in-vitro biodegradation of albumin and casein microspheres has been shown by us following macrophage uptake (Hoffman et al 1985) and in-vivo by Willmott et al (1989) for microspheres embolized in various organs. The in-vitro mitoxantrone release from post-loaded CAS-PGA/MS was similar to in-situ loaded microspheres in spite of the significantly higher drug loading for post-loaded microspheres. Here too, only a fraction of the drug was released in 24 h, suggesting that either the ionic drug complex is fairly stable or that some drug was bound to the protein matrix via available reactive residual aldehyde groups (Longo et al 1982).

It is known that particles less than 5–10 µm in diameter are rapidly cleared from the circulation by the fixed and floating macrophages of the reticuloendothelial system, but the rate of removal is also dependent on the surface characteristics of the particle (Davis & Illum 1983; Illum et al 1986). We have also shown that hydrophilic albumin microspheres are avidly taken up by bovine macrophages in-vitro (Hoffman et al 1985). It was thus of interest to compare the phagocytic uptake of the hydrophilic CAS/MS with BSA/MS. Fluorescein labelled microspheres less than 5 µm in diameter were used in all experiments with a mouse myelomonocyte leukaemia cell line. The results of the study are shown in Fig. 3. There were only small differences in the uptake of the casein and BSA microspheres at 5 and 10 min. However, for incubation times of 15 min or longer, approximately 20% more cells contained BSA/MS compared with the CAS/MS. This is a small difference and may not be significant since both microsphere compositions were avidly phagocytized. Figs 4 and 5 are typical optical photomicrographs of macrophages containing the BSA and casein microspheres. Since both casein and albumin microspheres are so avidly phagocytized by macrophages, the use of such microspheres for microphage-targeted therapies and for treatment of intracellular parasitic, bacterial and viral diseases appears promising.

The problems and opportunities for intratumoural therapy have been reviewed by McLaughlin & Goldberg (1983). This therapeutic modality remains somewhat controversial for general clinical oncological use, except for epithelial tumours. Our preliminary results for intratumoural chemotherapy with mitoxantrone administered as free drug and in post-synthesis loaded CAS-PGA/MS are shown in Fig. 6. Intratumour injections of free drug inhibited tumour growth but the drug-loaded microspheres were even more effective, showing no change in tumour volume from day 2 to day 11 for this group. Drug toxicity was assessed by monitoring animal deaths and weight. No drug toxicity was apparent for either mitoxantrone or mitoxantrone-loaded microsphere groups given 3 mg/mouse intratumourally; this dose is an order of magnitude greater than the LD50 for systemically administered mitoxantrone.

References

Batra, V. K., Morrison, J. A., Woodward, D. L., Siverd, N. S., Yacobi, A. (1986) Pharmacokinetics of mitoxantrone in man and laboratory animals. Drug Metab. Rev. 17: 311–329

Chen, Y., Willmott, N., Anderson, J., Florence, A. T. (1987) Comparison of albumin and casein microspheres as a carrier for doxorubicin. J. Pharm. Pharmacol. 39: 978–985

Davis, S. S., Illum, L. (1983) The targeting of drugs using polymeric microspheres. Br. Polymer J. 15: 160–164

Goldberg, E. P., Terry, R. N., Levy, M. (1981) Polymeric drugs with tissue binding properties for localized chemotherapy. Proc. Am. Chem. Soc. Div. Org. Coatings Plastics Chem. 44: 132–136

Goldberg, E. P., Iwata, H., Longo, W. (1984) Hydrophilic albumin

and dextran ion-exchange microspheres for localized chemotherapy. In: Davis, S. S. (ed.) Microspheres and Drug Therapy. Elsevier Science Publishers, B.V., pp 309–325

Gupta, P.K., Hung, C.-T. (1990) Targeted delivery of low dose doxorubicin hydrochloride administered via magnetic albumin microspheres in rats. J. Microencap. 7: 85–94

Hoffman, E. M., Longo, W. E., Goldberg, E. P. (1985) Macrophage uptake and degradation of streptomycin albumin microspheres. Proc. Int. Symp. Control. Rel. Bioact. Mater. 12: 335

Illum, L., Hunneyball, I. M., Davis, S. S. (1986) The effect of hydrophilic coatings on the uptake of colloidal particles by the liver and by peritoneal macrophages. Int. J. Pharm. 29: 53–65

Kim, C.-K., Oh, Y.-K. (1988) Development of hydrophilic human serum albumin microspheres using a drug-albumin conjugate. Int. J. Pharm. 47: 163–169

Longo, W. E., Iwata, H., Lindheimer, T. A., Goldberg, E. P. (1982) Preparation of hydrophilic albumin microspheres using polymeric dispersing agents. J. Pharm. Sci. 71: 1323–1328

McLaughlin, C. A., Goldberg, E. P. (1983) Local chemo- and immunotherapy by intratumor drug injection: opportunities for polymer-drug compositions. In: Goldberg, E. P. (ed.) Targeted Drugs. John Wiley & Sons, New York, pp 231–268

Morimoto, Y., Fujimoto, S. (1985) Albumin microspheres as drug carriers. In CRC Critical Reviews in Therapeutic Drug Carrier Systems 1: 19–63

Willmott, N., Chen, Y., Florence, A. T. (1988) Haemoglobin, transferrin and albumin. II. In vitro release rate. J. Contr. Rel. 8: 103–109

Willmott, N., Chen, Y., Goldberg, J., McArdle, C., Florence, A. T. (1989) Biodegradation rate of embolized protein microspheres in lung, liver and kidney of rats. J. Pharm. Pharmacol. 41: 433–438