International Journal of Pharmaceutics, 43 (1988) 161–166
Elsevier

IJP 01460

Characterisation of freeze-dried albumin microspheres containing the anti-cancer drug adriamycin

N. Willmott and P.J. Harrison

Department of Pharmacy, Pharmaceutics Division, University of Strathclyde, Glasgow, Scotland (U.K.)

(Received 5 October 1987)
(Accepted 30 October 1987)

Key words: Microsphere; Storage; Embolisation; Adriamycin; Release-rate

Summary

Information regarding in vitro characteristics and in vivo behaviour of microspherical drug delivery systems in animal models has accumulated and many have been developed to the stage of clinical assessment. A vital requirement for clinical trial is a stable product with a long shelf-life and reproducible properties. Thus, we have investigated whether adriamycin-loaded albumin microspheres can be freeze-dried without deterioration of important characteristics. The following properties of this delivery system were compared in the freeze-dried and freshly prepared state: particle size and state of aggregation (by Coulter Counter); drug content (by HPLC) and drug release rate (in a flow-through system). It was observed that freeze-drying and subsequent storage of adriamycin-loaded albumin microspheres resulted in no substantial difference in these properties compared to freshly prepared product. Thus, the freeze-dried product, with its considerable advantages as regards prolonged storage, appears suitable for clinical use.

Introduction

The need to divert cytotoxic anti-cancer agents away from organs and tissues in which toxicity arises and towards the desired site of action has long been recognised. Albumin microspheres were first suggested as a drug-delivery system for cytotoxics by Kramer in 1974. The original concept was that micron-sized particles could be "targetted" to solid tumours in a manner analogous to that suggested for liposomes. The intervening years have seen this concept founder on the prodigious capacity of the reticuloendothelial system to sequester particles of this size, essentially restricting their deposition to phagocytic cells of organs such as liver and spleen (Bradfield, 1984).

More recently, colloidal systems with particle sizes $> 7\ \mu m$ have been used as carriers for cytotoxic drugs. Organ selectivity by this technique, termed chemoembolisation, is achieved by passive filtration by capillaries of intra-arterially injected particles. Deposition of particles in tumour deposits harboured in the organ can be achieved with vasoactive agents (Willmott, 1987).

Various combinations of carrier and drug have been suggested for chemoembolisation. For example, ethylcellulose/mitomycin C (Kato et al., 1981); polylactide/cis-platinum (Spenlehauer et al., 1986) and 5-fluorouracil/carnauba wax (Benita et al., 1986). Our group has concentrated on

Correspondence: N. Willmott, Department of Pharmacy, Pharmaceutics Division, University of Strathclyde, Glasgow, Scotland, U.K.

adriamycin-loaded albumin microspheres that are currently undergoing evaluation in patients with liver metastases from colorectal cancer. An important prerequisite for clinical use is a stable product with reproducible characteristics. At present, microspheres are manufactured in suspension form for use in patients within a few hours. It will be important to increase the shelf-life of the product so that large-scale clinical studies can be conducted. Freeze-drying is a technique that could achieve this aim; however, it is desirable that freeze-dried microspheres exhibit release characteristics comparable to those prepared in suspension and used freshly made, on which our previous pre-clinical studies are based. In this report we compare the properties of freeze-dried adriamycin-loaded albumin microspheres with freshly prepared material.

Materials and Methods

Microsphere preparation

Albumin microspheres were prepared by the stabilisation, through cross-linking of protein by glutaraldehyde, of water in oil emulsion droplets containing protein and drug. The technique was first described by Lee et al. (1981), and has been modified by us to produce aggregate-free suspensions of adriamycin-loaded albumin microspheres in aqueous buffers (Willmott et al., 1985a) for intravascular administration. Previous in vivo and in vitro studies from our laboratory have always used microspheres freshly prepared on the same day that they were used in an experiment in order to avoid potential sources of variability due to storage of reactive drug in contact with protein.

To examine whether freeze-drying of drug-loaded microspheres affected their characteristics, the following protocol was adopted: 600 mg human serum albumin (Immuno, Sevenoaks, Kent), in a vol. of 3 ml, was lyophilised and dissolved in 1.2 ml 1 mM phosphate buffer containing 0.1% SDS and a solution of adriamycin (30 mg) (Farmatalia Carlo Erba, Italy) in 1.6 ml water added. After heating to effect complete dissolution, this served as the discontinuous phase of the water in oil emulsion. Oil phase consisted of 60 ml petroleum ether (120–160 °C), 40 ml cottonseed oil plus 0.5 ml Span 80. After stirring at 1300 rpm for 30 s, stabilisation of microspheres was achieved by addition of 200 μl of 25% glutaraldehyde. Stirring was continued for a further 60 min.

Drug-loaded particles were washed $\times 3$ in petroleum ether, $\times 2$ in isopropanol and $\times 3$ in phosphate-buffered saline +0.5% Tween 80. At this point microspheres were suspended in phosphate-buffered saline containing 6% mannitol. The weight of product (filtration through 0.22 μm filters), particle size (Coulter Counter), drug content and drug release rate of a 5 ml sample of the freshly prepared product were ascertained.

The remainder of the product was filled into 10 ml freeze-drying vials as 5 ml aliquots. These aliquots were swirled and flash-frozen, to ensure adequate dispersion of particles, in a mixture of acetone/solid CO_2. The frozen vials were freeze-dried in an Edwards Minifast (Model DO.1) over 2 days at −25 °C and then secondary dried for one day at +10 °C. A placebo mixture of mannitol/saline freeze-dried under identical conditions gave a moisture content of 0.7% w/w after primary drying and 0.2% after secondary drying as determined using a Karl Fischer Apparatus (Model 652KF). Mannitol was included at 6% w/v to prevent aggregation of microspheres during freeze-drying, to aid redispersion on reconstitution and ensure that an acceptable "cake" was produced. The freeze-dried product was stored at 4 °C for 11 days until reconstituted in 5 ml water for the comparative studies.

Assessment of drug content

Following trypsin digestion of a known weight of albumin microspheres, drug was extracted with chloroform/isopropanol (2 : 1), evaporated to dryness and adriamycin measured by HPLC with fluorescence detection. The techniques are described in detail elsewhere (Willmott et al., 1985a).

Drug release rate

The continuous-flow system used to assess rate of adriamycin release from microspheres is described at length elsewhere (Willmott et al., 1985b). Briefly, either freshly prepared or freeze-dried microspheres were immobilised on a glass column

containing glass-wool as support material and maintained at 37°C with a water jacket. The column was subjected to a constant flow of 60 mM phosphate buffer with 0.1% benzalkonium chloride as preservative. Using a fraction collector, samples were collected every hour for 27 h by which time adriamycin-content of fractions was near the limit of detection. Measurement of drug in fractions was by HPLC with fluorescence detection. To promote reproducibility, column packing and sample application were ostensibly identical for each run and flow rate was always 5.5 ml/h.

Data analysis

Release profiles were plotted in the form μg incorporated adriamycin remaining in microspheres (A) versus time (t) and it was found that curves were multiphasic. To determine the number of phases, a Fortran program was used that involved fitting either 2 or 3 linear regression lines of the form $A = K_n t + C_n$ (K_n is the zero-order rate constant and C is the zero time intercept for that phase). K and C for the first two phases were obtained by the Method of Residuals (Gupta et al., 1986)). The judgement as to whether curves were biphasic or triphasic was made on the basis of which had the least total sum of squares computed from individual sums of squares of each regression lines. The duration of each phase was decided on the same basis.

Results

The characteristics of adriamycin-loaded microspheres that were examined in the freshly prepared and freeze-dried state were: particle size, drug content and drug release rate. It was found that freeze-drying of drug-loaded microspheres had no substantial effect. Thus, as regards size, freshly prepared microspheres were 33.1 μm in diameter whereas after freeze-drying they were 33.7 μm (50% weight average). Moreover, the size distributions (whether on the basis of number or weight of particles) were virtually identical (Fig. 1). On the addition of water the freeze-dried product could be easily dispersed to give a suspension of aggregate-free microspheres.

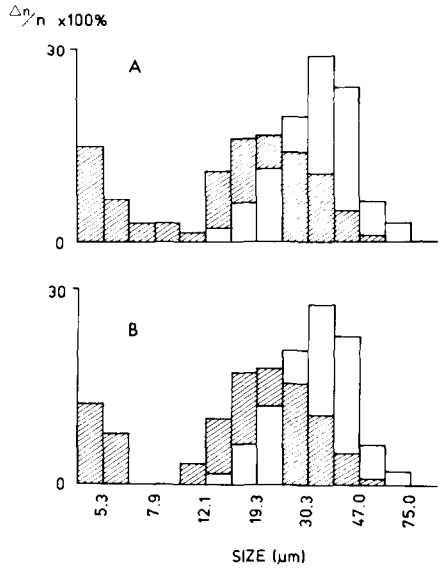

Fig. 1. Size distribution of freshly prepared and freeze-dried adriamycin-loaded albumin microspheres. Microspheres were prepared as described in text and size distribution obtained using a Coulter Counter. A: freeze-dried product; B: freshly prepared product. Hatched areas represent particle size distribution on a number basis, open areas represent particle size distribution on a weight basis. Δn = number or weight of particles in a size increment; n = total number or weight of particles.

Content of pure adriamycin following freeze-drying (7.3 μg/mg) was similar to freshly-prepared microspheres (7.6 μg/mg). Drug release rate of the systems was assessed using a flow-through apparatus. Fig. 2 shows results for freshly prepared adriamycin-loaded microspheres. The release profile for the freeze-dried product was virtually superimposable and is not shown here although zero-order rate constants for freshly prepared and freeze-dried microspheres are shown in Table 1. For comparison the rate of elution from the column of adriamycin in solution is also shown in Fig. 2. The weight of microspheres in the freshly prepared suspension and in the reconstituted freeze-dried form were virtually identical (13.5 and 13.7 mg/ml, respectively). Thus, the weight of each product used in the release assay was strictly comparable since 5 ml was used in each case.

The data from 1 h onwards were fitted to a model in which release of drug is considered to

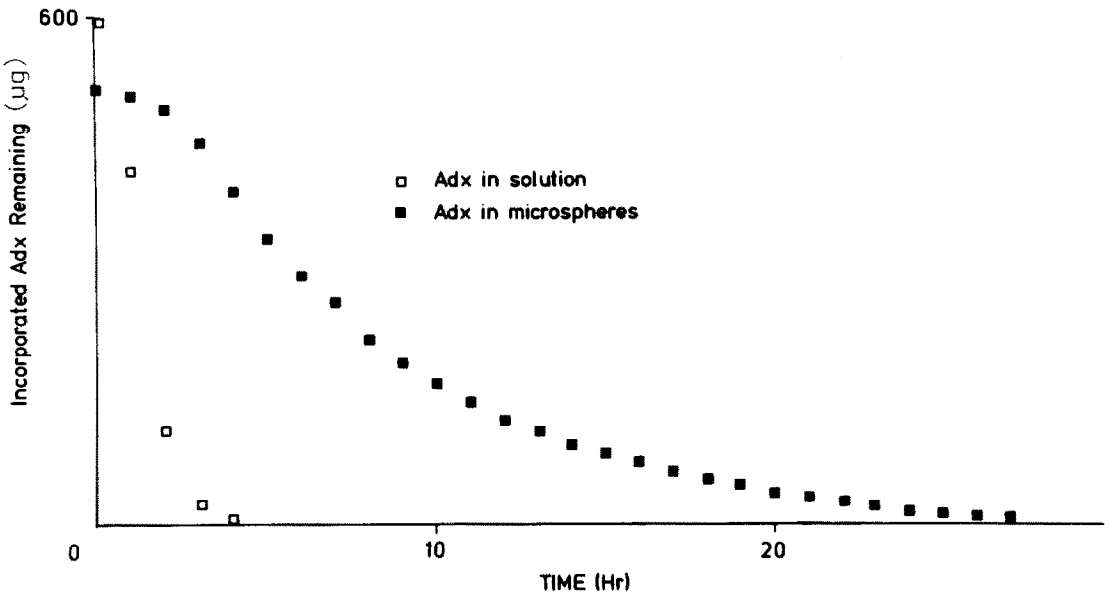

Fig. 2. Release rate of adriamycin from freshly prepared microspheres. Drug-loaded microspheres were prepared as described in the text and adriamycin release-rate examined using a flow-through system. Fractions were collected at hourly intervals and adriamycin content measured by HPLC. Elution from the column of drug in solution is shown for comparison. The release profile of adriamycin from microspheres was described by 3 consecutive linear regression lines (phases). The number and duration of phases was assigned by computer-aided curve-fitting (see Table 1).

TABLE 1

Characteristics of freshly prepared and freeze-dried microspheres

Microspheres	Size (µm) [a]	Drug content (µg/mg) [b]	Release rate constants (µg/h) [c]
Freshly prepared	33.1	7.6	$K_1 = -24.4$ $K_2 = -13.7$ $K_3 = -6.8$
Freeze-dried	33.7	7.3	$K_1 = -21.9$ $K_2 = -13.8$ $K_3 = -7.1$

[a] 50% weight average when assessed by Coulter Counter.

[b] Following digestion of microspheres in 0.4% trypsin, samples were extracted with chloroform/isopropanol (2/1), the extract evaporated to dryness and reconstituted in methanol for analysis of adriamycin by HPLC.

[c] K_1, K_2, K_3 are slopes of regression lines representing initial, intermediate and final phases of release profile (Fig. 2). The point of demarcation between initial and intermediate phases was 8 h (for both freshly prepared and freeze-dried microspheres). Between intermediate and final phases it was 14 h (freshly prepared) and 15 h (freeze-dried).

take place in 3 linear phases. It can be seen from Table 2 that the 3 zero-order rate constants for freshly prepared microspheres were similar to those for the freeze-dried product. The data from 1 h onwards for elution of adriamycin in solution was best described as first-order with a rate constant of -0.6^{-1} h, which is similar to that described in previous work (Willmott et al., 1987).

Discussion

Protein microspheres containing cytotoxic agents have many potential advantages for cancer chemotherapy. For example, they can be targetted to selected organs and even diseased areas of organs (Willmott, 1987); they can release drug at predictable rates (Gupta et al., 1986; Chen et al., 1987b; Willmott et al., 1987); they can be prepared from a variety of proteins such as albumin and transferrin (Chen et al., 1987a) to take ad-

vantage of receptors on endothelial cells (Ghitescu et al., 1986) and tumour cells (Yeh et al., 1984), respectively; the protein carriers themselves are innocuous, being both biocompatible and biodegradable; their manufacture is relatively simple and lends itself to scale-up and performance under sterile conditions.

We have recently succeeded in preparing adriamycin-loaded albumin microspheres on a scale that enables administration to humans and in a pharmaceutically acceptable condition as regards sterility. The last remaining obstacle to phase I clinical trials was the production of a product that could be stored (and thereby be readily accessible) without loss of desirable characteristics. Here we report the effects of freeze-drying on particle size, native drug content and drug release rate.

Our results clearly show that the freeze-drying process had little effect on the particle size (> 99.9% of the weight of the microspheres being of a size necessary for embolisation). Likewise, the content of pure adriamycin was not compromised by the freeze-drying process. We have recently detected an adriamycin complex within microspheres that is chromatographically separable, in trypsin digests of drug-loaded microspheres, from pure drug. The formation of the complex was attributed to a covalent bond, via a glutaraldehyde bridge, between adriamycin and protein (manuscript submitted). We have not directly examined the effect of freeze-drying on drug in this compartment but in view of the fact that native drug is unaffected it appears likely that a similar situation will prevail for complexed adriamycin.

Release of pure drug from freeze-dried microspheres was virtually identical to that from freshly prepared product. For analysis of adriamycin release data we have followed the example of Gupta et al. (1986). These authors found that none of the simple mathematical models (i.e. zero-order, first-order, Higuchi) adequately described release of adriamycin from heat-stabilised albumin microspheres over the entire period of observation. This was also our experience with adriamycin-loaded albumin microspheres prepared by glutaraldehyde stabilisation (Willmott et al., 1985b). On dividing the release rate curve into phases, analogous to the procedure adopted when modelling plasma pharmacokinetics, the data was adequately represented by a series of either zero-order, first-order to Higuchi equations; however, on theoretical grounds the zero-order model is preferred (Gupta et al. 1986). On applying this analysis to release rate profiles generated from both freshly prepared and freeze-dried adriamycin-loaded albumin microspheres 3 zero-order rate constants were obtained using the Method of Residuals. It was observed that corresponding rate constants for the two systems were similar.

In summary, characteristics of adriamycin-loaded albumin microspheres relevant for targetting and drug efficacy have been studied in the freshly prepared and freeze-dried state. No substantial differences were noted. This study validates the use of freeze-drying in the preparation of this material for clinical use.

Acknowledgements

Both authors are grateful to Agnes Hughes for skilled technical assistance to Mrs. Pat Morrison for typing the manuscript. The computing assistance of Mr. Don Evans of the Computing Centre, University of Strathclyde and Dr. Naiming Chu of the Department of Ship and Marine Technology, University of Strathclyde, is also gratefully acknowledged. The work was supported by Medical Research Council of Great Britain.

References

Benita, S., Zouai, O. and Benoit, J.P., 5-fluorouracil: carnauba wax microspheres for chemoembolisation: an in vitro evaluation. *J. Pharm. Sci.*, 75 (1986) 847–851.

Bradfield, J.W.B., The reticulo-endothelial system and blood clearance. In Davis, S.S., Illum, L., McVie, J.G. and Tomlinson, E. (Eds.), *Microspheres and Drug Therapy, Pharmaceutical, Immunological and Medical Aspects*, Elsevier, Amsterdam, 1984, pp. 25–37.

Chen, Y., Willmott, N., Anderson, J. and Florence, A.T., Haemoglobin, transferrin and albumin/polyaspartic acid microspheres as carrier for the cytotoxic drug adriamycin. I. Ultrastructural appearance and drug content. *J. Controlled Release*, (1987a) in press.

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

Ghitescu, L., Fixman, A., Simionescu, M. and Simionescu, N., Specific binding sites for albumin restricted to plasmalemmal vesicles of continuous capillary endothelium: receptor mediated transcytosis. *J. Cell Biol.*, 102 (1986) 1304–1311.

Gupta, P.K., Hung, C.T. and Perrier, D.G. Albumin microspheres. I. Release characteristics of adriamycin. *Int. J. Pharm.*, 33 (1986) 137–146.

Kato, T., Nemoto, R., Mori, H., Takahashi, M. and Harada, M., Arterial chemoembolisation with mitomycin C microcapsules in the treatment of primary and secondary carcinoma of the kidney, liver, bone and intrapelvic organs. *Cancer*, 48 (1981) 674–680.

Kramer, P.A., Albumin microspheres as vehicles for achieving specificity in drug delivery. *J. Pharm. Sci.*, 63 (1974) 1646–1647.

Lee, T.K., Sokoloski, T.D. and Royer, G.P., Serum albumin beads: an injectable biodegradable system for the sustained release of drugs. *Science*, 213 (1981) 223–235.

Spenlehauer, G., Veillard, M. and Benoit, J.P., Formation and characterisation of cis platin loaded poly (d,l-lactide) microspheres for chemoembolisation. *J. Pharm. Sci.*, 75 (1986) 750–755.

Willmott, N., Chemoembolisation in cancer chemotherapy – a rationale. *Cancer Treat. Rev.*, in press.

Willmott, N., Cummings, J., Stuart, J.F.B. and Florence, A.T., Adriamycin-loaded albumin microspheres: preparation, in vivo distribution and release in the rat. *Biopharm. Drug Disp.*, 6 (1985a) 91–104.

Willmott, N., Cummings, J. and Florence, A.T., In vitro release of adriamycin from drug-loaded albumin and haemoglobin microspheres. *J. Microencapsulation*, 2 (1985b) 293–304.

Willmott, N., Chen, Y. and Florence, A.T., Haemoglobin, transferrin and albumin/polyaspartic acid microspheres as carriers for the cytotoxic drug adriamycin. II. In vitro drug release rate. *J. Controlled Release*, (1987) in Press.

Yeh, C.G. and Page Faulk, W., Killing of human tumor cells in culture with adriamycin conjugates of human transferrin. *Clin. Immunol. Immunopathol.*, 32 (1984) 1–11.