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Note

Adriamycin-loaded albumin microspheres: qualitative assessment of drug incorporation and in vitro release by high-performance liquid chromatography and high-speed multi-diode array spectrophotometric detection

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Microspherical particles are receiving increasing attention as drug carriers [1]. Their capacity to deliberately alter the site of drug disposition and the rate of drug release could theoretically confer a degree of target specificity on otherwise non-specific cytotoxic anti-cancer drugs. The first particulate system used was liposomes and although many clinically active anti-cancer agents can be incorporated the reaction of sub-micrometre particles with the reticuloendothelial system is still a problem [2]. This has lead to the use of drug-loaded protein microspheres that either by capillary blockade [3] or by extra-corporal guidance [4] can circumvent the reticuloendothelial system. Such microspheres are made under conditions that could impair the incorporated drug (either glutaraldehyde cross-linking or temperature in excess of 100°C), so it is perhaps suprising that few attempts have been made to examine the chemical purity of incorporated drug using high-performance chromatographic (HPLC) techniques.

In this report we have combined the resolving power of an HPLC assay [5] with high-speed and accurate scanning spectrophotometric detection in order to determine the purity of the anthracycline anti-cancer drug adriamycin

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(ADR) incorporated into and released from albumin microspheres prepared by glutaraldehyde cross-linking.

MATERIALS AND METHODS

Experimental design

Drug-loaded microspheres (0.5—1 mg in a final volume of 2.3 ml phosphate buffer 0.9% sodium chloride, pH 7.2) were digested with the proteolytic enzyme trypsin for 15 h (0.4%, w/v, Difco Labs., Surrey, U.K., 1:250). Antibiotics, penicillin and streptomycin were included in incubation media to prevent possible drug degradation by bacterial growth. Immediately following digestion drug was extracted with a five-fold excess of the solvent mixture chloroform—propan-2-ol (2:1) according to the method described by Cummings et al. [5], after which samples were ready for chromatography. The above treatment was applied to the microspheres in order to artificially release incorporated drug in a form amenable to analysis. ADR actually released from carrier particles was obtained by immobilising 30 mg of drug-loaded microspheres on a glass wool column (20 cm × 3 cm I.D.) and eluting the free drug off the column in 60 m*M* phosphate buffer, pH 6.0 (flow-rate 5 ml/h, containing 0.1% of the preservative benzalkonium chloride, B.P.). Fractions were collected hourly and the quantity of drug in each was usually sufficient for direct injection into a liquid chromatograph. When fractions were extracted the technique was as described above.

Preparation of adriamycin-loaded microspheres

A 10-mg amount of adriamycin · HCl (as supplied for administration to patients, Farmitalia, Milan, Italy) was encapsulated with 200 mg of bovine serum albumin (prepared by method IV of Cohn, supplied by Sigma, Poole, U.K.) using 1% (v/v) glutaraldehyde (Grade 1, Sigma) by the method of Willmott et al. [3].

Drug analysis techniques

HPLC equipment and the separation of ADR and its metabolites by HPLC have already been described in detail [5]. The method distinguishes between at least five ADR metabolites, including a number of aglycones which may well represent in vitro chemical degradation products [6]. The multi-diode array detector was an Hewlett-Packard Model 1040A and was controlled by a Hewlett-Packard Model 85B computer with peripheral twin disc drive unit (Model 9121) and high-resolution data plotter (Model 7470A, all from Hewlett-Packard, Manchester, U.K.). The multi-diode array detector was connected to the output line of a convential isocratic liquid chromatograph directly without the need for specialised equipment. A fluorescence detector was connected in series with the multi-diode array detector for increased sensitivity of fluorescent ADR-related species.

RESULTS AND DISCUSSION

Chromatographic peaks were identified by simultaneous multi-wavelength

detection at 210, 233, 254, 320 and 480 nm and fluorescence detection at an excitation wavelength of 480 nm and emission wavelength of 560 nm. Four replicate trypsin digests of ADR-loaded microspheres, four replicate trypsin incubations of ADR without microspheres and fifteen consecutive column fractions were analysed. In all cases only one chromatographic peak was identified which could be related to ADR and it eluted with a retention time almost identical to native ADR standard. Spectrophotometric purity of this peak and its UV—visible spectrum were obtained by the multi-diode array detection. The multi-diode array detector, because it measures absorbances simultaneously from 190 to 600 nm every 10 msec, can effectively scan a compound eluting from a high-performance liquid chromatograph at several points of its peak. It was pre-programmed, in this study, to memorise a spectrum at the beginning of a peak, at its apex and near the end of a peak, and to superimpose the three spectra (supplying suitable attenuation). If the peak was pure, spectra would superimpose perfectly, if not, spectral shifts were identified and could even be attributed to particular parts of the peak. The first case is illustrated in Fig. 1 with 10 μg/ml ADR standard. Exact times when spectra were memorised are indicated on the time axis of its chromatogram (Fig. 1a) detected at 233 nm. Clearly, the three different spectra superimposed well confirming peak purity (Fig. 2a). Absorption maxima occurred at 233, 253, 290, 478, 492 and 530 nm, and these values corresponded virtually identically to the absorption maxima of ADR obtained by convential scanning spectrophotometry under stringent analytical conditions [7]. A representative chromatogram (233 nm) of a tryptic digest of ADR-loaded microspheres is shown in Fig. 1b included with the times when spectra were recorded on the time axis. Several peaks were always resolved and these were identified as

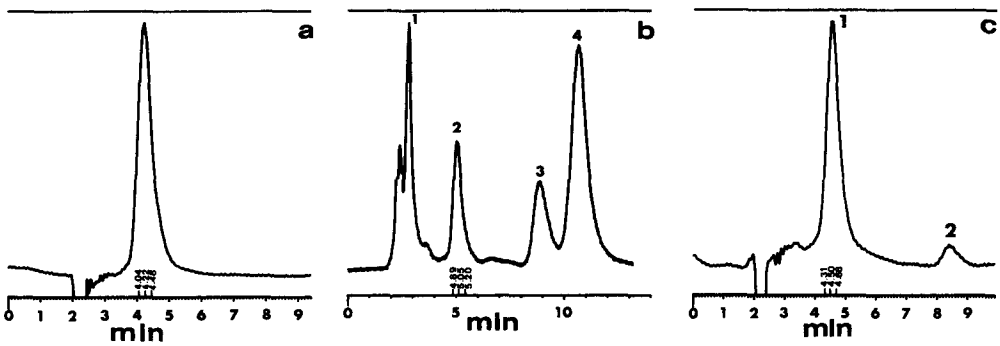

Fig. 1. Chromatograms of a pure adriamycin (ADR) standard, ADR after incorporation into, and release from albumin microspheres. For chromatographic conditions see Materials and methods; detection was by UV absorbance at 233 nm. (a) 200 ng (injected on column) of a methanolic solution of pure ADR standard; the times when spectra were recorded by the multi-diode array detector (see Fig. 2) are indicated on the time axis. (b) Approximately 200 ng of ADR extracted from drug-loaded albumin microspheres after particles were digested with the proteolytic enzyme trypsin (incorporated drug). Peaks: 1 = digested microspheres and penicillin; 2 = ADR (times when spectra were recorded are indicated on the time axis); 3 = daunorubicin (internal standard); 4 = streptomycin. (c) Approximately 200 ng of ADR released from albumin microspheres obtained by immobilising drug-loaded particles on a column and eluting free drug off the column. Peaks: 1 = ADR; 2 = ADR aglycone.

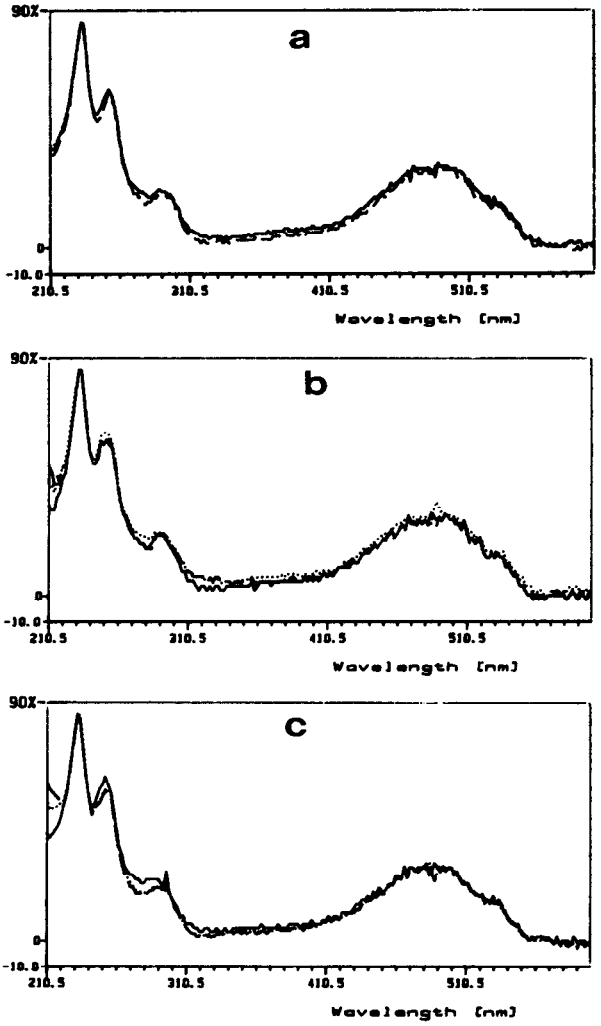

Fig. 2. Spectral analysis of chromatographic peaks of pure ADR standard, ADR after incorporation into and release from albumin microspheres. Each of the figures is composed from three individual UV—visible spectra which were taken at different points of the ADR chromatographic peak (for times when spectra were recorded, see Fig. 1). The continuous line represents the spectrum taken at the beginning of the peak, the dotted line the spectrum at the apex of the peak and the broken line the spectrum near the end of the peak. (a) Pure ADR standard; (b) ADR extracted from microspheres (incorporated drug); (c) ADR released from microspheres.

follows: (1) digested microspheres and penicillin; (2) ADR; (3) daunorubicin (internal standard) and (4) streptomycin. Spectral analysis of the peak that co-eluted with ADR yielded a UV-visible spectrum identical to the native compound with all the characteristic absorption maxima intact (Fig. 2b). All three spectra superimposed also implying purity. A representative chromatogram (233 nm) of a column fraction is shown in Fig. 1c and spectral analysis again indicated pure, native ADR (Fig. 2c). A trace amount of ADR aglycone was also present (peak 2).

Tokes et al. [8] have shown that ADR can react with aldehyde groups on polyglutaraldehyde microspheres probably by a Schiff base condensation with the amino group of the sugar moiety of ADR to form a stable imino complex. Removal of the C_7 linked sugar from ADR would produce a series of aglycones which are considerably less polar than the parent drug and therefore easily resolved by HPLC [5]. Such species, which would be identified during drug analyses, were not detected either inside or being released from the albumin microspheres in substantial concentrations.

Another quite different situation is possible. Alterations in the structure of the benzanthraquinone nucleus of ADR can give rise to forms with UV—visible spectra quite distinct from native ADR but which, nevertheless, co-chromatograph with the parent drug [9]. Also, ADR can react with several small biomolecular species and even itself to form drug complexes where the visible end of the ADR spectrum has invariably shifted. Chromatographically, these complexes may be indistinguishable from non-complexed drug. In this situation even HPLC is inadequate and another analytical technique is required. However, the use of high-speed scanning spectrophotometric detection has enabled us to establish that ADR incorporated into and released from albumin microspheres is spectrophotometrically pure.

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REFERENCES

1 E. Tomlinson, Int. J. Pharm. Tech. Prod. Manuf., 4 (1983) 49.
2 J.N. Weinstein and L.D. Leserman, Pharmacol. Ther., 24 (1984) 207.
3 N. Willmott, J. Cummings, J.F.B. Stuart and A.T. Florence, Biopharm. Drug Dispos., 6 (1985) 91.
4 K.J. Widder, H.E. Senyei and B. Sears, J. Pharm. Sci., 71 (1982) 379.
5 J. Cummings, J.F.B. Stuart and K.C. Calman, J. Chromatogr., 311 (1984) 125.
6 M. Israel, W.J. Pegg, P. Wilkinson and M.B. Garnick, J. Liq. Chromatogr., 1 (1978) 795.
7 F. Arcamone, G. Cassinelli, G. Franceschi, S. Penco, C. Pol, S. Redaelli and A. Selva, in S.K. Carter, A. Di Marco, M. Ghione, I.H. Krakoff and G. Mathe (Editors), Proceedings of the International Symposium on Adriamycin, Milan, September 9—10, 1971, Springer, New York, 1972, pp. 9—22.
8 Z.A. Tokes, K.E. Rogers and A. Rembaum, Proc. Nat. Acad. Sci. U.S., 79 (1982) 2026.
9 J. Cummings, J. Chromatogr., 341 (1985) 401.