ELSEVIER

International Journal of Pharmaceutics 115 (1995) 259–263

international journal of pharmaceutics

A comparison of alternative methods for the determination of the levels of proteins entrapped in poly(lactide-co-glycolide) microparticles

Sameena Sharif *, Derek T. O'Hagan

Department of Pharmaceutical Sciences, University of Nottingham, University Park, Nottingham NG7 2GD, UK

Received 13 May 1994; accepted 31 August 1994

Abstract

Two alternative techniques, one involving extraction into dichloromethane (DCM) and the other polymer hydrolysis by sodium hydroxide (NaOH), and extraction by sodium dodecyl sulphate (SDS), were assessed for their ability to determine the levels of three model proteins, bovine serum albumin (BSA), ovalbumin and lysozyme, entrapped in poly(lactide-co-glycolide) microparticles. In addition, the two techniques were compared with two more widely used techniques for the quantitation of proteins and peptides, polyacrylamide gel electrophoresis (SDS-PAGE) followed by scanning densitometry, and amino acid analysis (AAA). As a control, BSA was radiolabelled to allow an accurate determination of the total level of protein entrapped in microparticles. The results showed that NaOH/SDS extraction resulted in accurate determinations of the levels of proteins entrapped in microparticles, but that extraction into DCM resulted in underestimations of the total amount of protein present. AAA also resulted in accurate determinations of the levels of entrapped proteins, but SDS-PAGE resulted in underestimations.

Keywords: Protein; Poly(lactide-co-glycolide) microparticle; Quantitative determination

In recent years, a large number of recombinant proteins and synthetic peptides have been developed as potential therapeutic agents (Holden, 1990). Since in general, these materials are poorly absorbed following oral delivery and are not well absorbed from alternative routes, the delivery of proteins and peptides using controlled release delivery systems has become an important area of investigation. Controlled release from parenterally administered devices offers potential for reducing the total number, or frequency, of injections required for effective therapy. The systems most commonly employed for the controlled delivery of proteins and peptides comprise microparticles prepared from the poly(lactide-co-glycolide) (PLG) polymers. Microparticles have also been shown to be effective for the delivery of antigens by the oral (Eldridge et al., 1990; O'Hagan et al., 1993a) and parenteral routes (Eldridge et al., 1991; O'Hagan et al., 1993b). Hence, the entrapment of antigens into PLG microparticles is an area of considerable interest,

* Corresponding author.

SSDI 0378-5173(94)00318-1

which offers potential for the development of single dose vaccines (Eldridge et al., 1991; O'Hagan et al., 1993b). Nevertheless, a crucial issue in the development of such formulations is the accurate determination of the levels of proteins and peptides entrapped in microparticles. Although accurate determinations may be performed during in vitro release studies, since the rate of release of peptides and proteins from microparticles is mainly dependent on polymer degradation, such studies are slow to provide data. Therefore, two alternative techniques have been developed to more rapidly remove entrapped proteins from PLG microparticles. One such technique involves the dissolution of the microparticles in dichloromethane (DCM), followed by extraction of the entrapped proteins into water (Ogawa et al., 1988; Cohen et al., 1991; Eldridge et al., 1990, 1991). A second technique involves alkaline hydrolysis of the microparticles and extraction of the entrapped protein into SDS (Hora et al., 1990; Jeffery et al., 1993).

In early studies with a model protein, ovalbumin (OVA), it became clear that the DCM extraction method resulted in an underestimation of the total level of protein entrapped in microparticles (Jeffery, 1992). Therefore, the current studies were undertaken to compare the two methods of protein extraction from PLG microparticles, using three different model proteins, OVA, BSA and lysozyme (LYS). In addition, two alternative techniques, SDS-PAGE, followed by analysis of the stained gel using scanning densitometry, and amino acid analysis (AAA) were also assessed for their ability to determine the levels of proteins entrapped in microparticles. As a control, the amount of BSA entrapped in PLG microparticles was accurately determined following radiolabeling (^{125}I-bovine serum albumin).

Microparticles (mean size of each batch < 5 μm) were prepared with entrapped OVA, BSA and LYS (Sigma Chemical Co., Dorset), using the method described by Jeffery et al. (1993). For each of the model proteins, at least three individual batches of microparticles were prepared as follows; 10 ml of a 6% w/v solution of PLG (co-polymer composition 50:50, Resomer RG 505, Boehringer Ingelheim, Germany) in DCM (May and Baker, Essex) was emulsified together with 1 ml of a 6% w/v solution of protein using a Silverson homogeniser (Silverson Instruments, Chesham, Bucks) at 12400 rpm. The resulting water in oil (w/o) emulsion was then emulsified with 20 ml of a 10% w/v solution of polyvinyl alcohol (PVA) (Aldrich Chemical Co., Dorset) to produce a water in oil in water (w/o/w) emulsion. This emulsion was stirred overnight using a magnetic stirrer, under ambient temperature and pressure, to allow solvent evaporation and microparticle formation. The microparticles were collected by centrifugation, washed three times in double-distilled water and freeze dried.

The microparticles were sized by laser diffractometry using a Malvern Mastersizer S3.00 (Malvern Instruments, Malvern, UK) prior to the assessment of their protein content. The protein content of the microparticles was determined following extraction of the entrapped proteins using two different methods. The first method involved a DCM extraction process and the second, an alkaline hydrolysis extraction method.

The DCM extraction method was performed as previously described (Ogawa et al., 1988; Cohen et al., 1991; Eldridge et al., 1990, 1991). Briefly, 20–25 mg of microparticles, accurately weighed, were dissolved in 5.0 ml of DCM with the aid of overnight shaking. Three 3-ml volumes of distilled water were each shaken with the samples for 20 min; following centrifugation, they were then collected and adjusted to a final volume of 10 ml. The protein content of the combined water fractions was then determined using a Bicinchoninic protein assay (BCA) (Sigma).

The alkaline hydrolysis method was performed as previously described (Hora et al., 1990; Jeffery et al., 1993). Briefly, 10–15 mg of microparticles, accurately weighed, were shaken overnight with 3 ml of 5% w/v SDS (Sigma) in 0.1 M sodium hydroxide solution (NaOH/SDS). Following centrifugation, the protein content of the supernatant was determined using a BCA assay.

For each batch of microparticles containing each protein, the protein extraction techniques were performed in triplicate. Blank microparticles without entrapped protein were also extracted using each technique, to provide back-

ground readings for the BCA assays. The BCA assay (Smith et al., 1985) was performed as described in the manufacturer's information. Briefly, standard curves were constructed using defined concentrations (50–1000 μg/ml) of the relevant protein, OVA, BSA and LYS. The 100 μl of the extracted protein samples and standards were placed in borosilicate tubes and 2 ml of the BCA protein determining reagent was added. The tubes were then incubated in a water bath at 60°C for 10–20 min. After cooling, the absorbances of the samples were read at 562 nm using a UV spectrophotometer (LKB biochrom).

Two additional techniques for the quantitation of proteins were also used to determine the levels of proteins entrapped in PLG microparticles. One of these techniques involved SDS-PAGE followed by analysis of the stained gel (Coomassie blue) by scanning densitometry (SD). The other technique involved AAA, which was performed after complete hydrolysis and derivatisation of the protein entrapped in the microparticles. SDS-PAGE was performed following extraction of the protein from the microparticles by shaking the microparticles overnight in reducing sample buffer, which contained SDS (2% w/v) and mercaptoethanol (5% v/v), and boiling for 3 min. The samples were loaded into a 12% gel alongside protein standards (2–12 μg), which were used to construct a calibration curve and electrophoresed (Bio-Rad mini protean dual slab cell, Bio-Rad, California) for 45 min (Laemmli, 1970). The proteins were stained with Coomassie blue and destained overnight, prior to determining the sample concentrations by SD (Shimadzu dual-wavelength fly spot densitometer, Shimadzu, Japan). AAA was performed in three steps as follows; the protein in the microparticles was hydrolysed to yield free amino acids using 6 N hydrochloric acid in the vapour phase. The samples were then derivatised with phenylthiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acids, and the PTC amino acids were quantified by HPLC.

As a control, to accurately determine the total level of protein entrapped in microparticles, BSA

Table 1
Percent protein entrapment (w/w) obtained using DCM water and NaOH/SDS extraction methods compared to AAA and SDS/PAGE followed by scanning densitometry

Batch	Extraction methods		Amino acid analysis	SDS/PAGE gel densitometry
	NaOH/SDS	DCM/H_2O		
Bovine serum albumin				
A	5.0 (0.12)	4.5 (0.26)		
B	4.6 (0.05)	3.6 (0.03)		
C	4.4 (0.95)	2.6 (0.30)		
D	5.9 (0.29)	2.2 (1.38)	6.6	3.4 (0.50)
E	5.9 (0.40)	1.6 (0.44)	6.7	6.8 (2.30)
F	5.6 (0.35)	1.7 (0.38)	6.9	4.5 (1.00)
Ovalbumin				
G	7.0 (1.80)	0.7 (0.53)		
H	5.5 (0.14)	0.4 (0.05)		
I	5.4 (0.02)	0.5 (0.10)		
J	6.5 (0.99)	0.4 (0.13)	6.5	4.1 (0.98)
K	6.4 (0.94)	0.3 (0.06)	8.8	4.4 (1.12)
L	5.4 (0.98)	0.7 (0.25)	5.6	3.0 (1.60)
Lysozyme				
M	7.9 (0.40)	5.9 (0.22)		
N	6.0 (0.10)	1.0 (0.04)		
O	3.1 (0.10)	1.3 (0.29)		
P	6.7 (0.26)	1.0 (0.59)	5.1	2.8 (0.70)
Q	5.7 (0.68)	0.7 (0.49)	5.3	2.1 (0.70)
R	5.7 (2.10)	0.8 (0.54)	3.4	4.3 (1.02)

was radiolabelled with I^{125} (Amersham International, Amersham, UK) as described by Hudson and Hay (1989). The radiolabelled BSA (1 MBq initial activity per batch) was then mixed with unlabelled BSA, and entrapped in PLG microparticles as described above. After preparation, the protein content of the microparticles was determined by counting the radioactivity associated with 10–15 mg, accurately weighed, samples of the microparticles using a gamma counter (LKB 182 Compugamma LS, LKB Wallac, Finland). To confirm the efficiency of the NaOH/SDS protein extraction method, this extraction procedure was performed on the microparticles with entrapped radiolabelled BSA and the radioactivity in the supernatant was counted as described above.

The levels of the different proteins (% w/w loading) entrapped in the microparticles, which were determined using the different extraction techniques and the alternative quantitative assay methods are shown in Table 1. Three batches of microparticles were prepared using radiolabelled BSA, these batches contained 6.7, 6.2 and 7.8% w/w BSA, as determined by direct counting of the microparticles in the gamma counter. The NaOH/SDS protein extraction technique was applied to these microparticles, and gamma counting of the supernatants showed that this method extracted, respectively, 91, 93 and 95% of the entrapped BSA.

The current studies served to confirm and expand our previous observation, which indicated that DCM extraction of proteins from PLG microparticles results in an underestimation of the total level of entrapped protein (Jeffery, 1992). The apparent underestimation is probably due to the inability of some proteins to completely partition into the aqueous phase from the DCM, following extraction. This is perhaps not surprising, since it has been shown on a number of occasions that due to their amphiphilic nature, proteins tend to associate with interfaces (Norde and Lykelma, 1991). The current studies indicated that the extent of underestimation of the protein content of PLG microparticles following DCM extraction, appears to be protein dependent. In general, the extent of underestimation would be expected to be greater for more hydrophobic proteins, which would remain in the DCM, and would not partition into the aqueous phase during extraction. In the current studies, DCM extraction only appeared to determine about 10% of the total amount of OVA entrapped in PLG microparticles. However, in our previous studies, using the same technique, the extent of underestimation of the levels of OVA entrapped in microparticles was considerably less (Jeffery, 1992). Hence, this finding illustrates an additional problem with DCM extraction, which became apparent during the current studies. DCM extraction of proteins from microparticles showed a lack of reproducibility when the same technique was performed on different days (data not shown). In contrast to DCM extraction, NaOH/SDS extraction showed itself to be an efficient and reproducible technique for the extraction of proteins from PLG microparticles. Moreover, using radiolabelled BSA, it was shown that NaOH/SDS extraction resulted in the recovery of $93 \pm 2\%$ of BSA entrapped in PLG microparticles.

As expected, AAA, which involved the complete hydrolysis of proteins and the determination of derivatised amino acids by HPLC, appeared to result in the complete recovery of proteins from PLG microparticles. However, this technique is relatively difficult and expensive to perform, and consequently, is only available in a relatively small number of specialized laboratories. Therefore, this technique would be difficult to adopt and use as a routine assay method for determination of the levels of proteins entrapped in PLG microparticles.

SDS-PAGE followed by SD is a process which is relatively difficult and time consuming to perform. Moreover, it is also dependent on the availability of a highly specialized piece of equipment, a scanning densitometer, to allow quantitative determinations to be performed. Furthermore, in the current studies, SDS-PAGE followed by SD tended to underestimate the total amount of protein present in PLG microparticles.

In conclusion, NaOH/SDS extraction, followed by BCA assay, is an accurate, simple and rapid technique for the determination of the lev-

els of proteins entrapped in PLG microparticles. In contrast, DCM extraction does not appear to recover all of the proteins entrapped in microparticles. Nevertheless, the DCM extraction technique was performed directly as described in the literature (Ogawa et al., 1988; Cohen et al., 1991; Eldridge et al., 1990, 1991), the addition of SDS to the aqueous phase would result in a more complete recovery of the protein using this method. AAA also allows an accurate determination of the levels of proteins entrapped in PLG microparticles, but it is an expensive technique, which is not readily available in most laboratories. In the current studies, SDS-PAGE followed by SD appeared to result in an underestimation of the total levels of protein entrapped in PLG microparticles. Therefore, it is our conclusion that NaOH/SDS extraction, followed by an assay for total protein (e.g., BCA assay) should be adopted as the standard technique for the determination of the levels of proteins entrapped in PLG microparticles.

Acknowledgements

The amino acid analysis was carried out at the Department of Biochemistry, Queens Medical Centre, Nottingham, by K. Bailey. We would also like to thank N. Kanji, L.Y. Ng and J. Allsopp for their technical assistance. S.S. is funded through a research agreement with SmithKline Beecham, Epsom.

References

Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H. and Langer, R., Controlled delivery systems for proteins based on poly(lactic/glycolic) microspheres. *Pharm. Res.*, 8 (1991) 713–720.

Eldridge, J.H., Hammond, C.J., Meulbroek, J.A., Staas, J.K., Gilley, R.M. and Tice, T.R., Controlled vaccine release in the gut-associated lymphoid tissues: I. Orally administered biodegradable microspheres target the Peyer's patches. *J. Controlled Release*, 11 (1990) 205–214.

Eldridge, J.H., Staas, J.K., Meulbroek, J.A., Tice, T.R. and Gilley, R.M., Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralising antibodies. *Infect. Immun.*, 59 (1991) 2978–2986.

Holden, C., Drugs and biotechnology. *Science*, 248 (1990) 964.

Hora, M.S., Rana, R.K., Nunberg, J.H., Tice, T.R., Gilley, R.M. and Hudson, M.E., Release of human serum albumin from poly(lactide-co-glycolide) microspheres. *Pharm. Res.*, 7 (1990) 1190–1194.

Hudson, L. and Hay, F.C., *Practical Immunology*, 3rd Edn, Blackwell, Oxford, 1989, pp. 52–53.

Jeffery, H., The preparation and characterisation of biodegradable microparticles for use in antigen delivery. Ph.D. Thesis, Nottingham (1992).

Jeffery, H., Davis, S.S. and O'Hagan, D.T., Preparation and characterisation of poly(lactide-co-glycolide) microparticles: II. Water-in-oil-in-water emulsion solvent evaporation. *Pharm. Res.*, 10 (1993) 362–368.

Laemmli, U.K., Cleavage of structual proteins during the assembly of the head of bacteriophage T4. *Nature*, 227 (1970) 680–685.

Norde, W. and Lykelma, J., Why proteins prefer interphases. *J. Biomater. Sci. Polym.*, 2 (1991) 183–202.

Ogawa, Y., Yamamoto, M., Okada, H., Yashiki, T. and Shimamoto, T., A new technique to efficiently entrap leuprolide acetate into microcapsules of polylactic acid or copoly(lactic/glycolic) acid. *Chem. Pharm. Bull.*, 36 (1988) 1095–1103.

O'Hagan, D.T., Jeffery, H. and Davis, S.S., Long term antibody responses in mice following subcutaneous immunisation with ovalbumin entrapped in biodegradable microparticles. *Vaccine*, 9 (1993b) 965–969.

O'Hagan, D.T., McGee, J.P., Holmgren, J.P., Mowat, A. McI., Donachie, A.M., Mills, K.G.H., Gaisford, W., Rahman, D. and Challacombe, S.J., Biodegradable microparticles as oral vaccines. *Vaccine*, 11 (1993a) 149–154.

Smith, P.K., Krohn, R.I., Hermanson, A.K., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Geoke, N.M., Olsen, B.J. and Klenk, D.C., Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150 (1985) 76–85.