International Journal of Pharmaceutics 401 (2010) 41–46

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Study of the factors influencing the encapsulation of zidovudine in rat erythrocytes

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ARTICLE INFO

Article history:
Received 1 October 2009
Received in revised form 6 September 2010
Accepted 14 September 2010
Available online 18 September 2010

Keywords:
Zidovudine
Carrier erythrocytes
Hypotonic dialysis

ABSTRACT

Antiretroviral-loaded erythrocytes offer a promising therapy against HIV owing to their potential to deliver this kind of drugs to macrophages and reticulo-endothelial (RES) tissues. The aim of the present work was to develop and optimize a hypotonic dialysis method for the encapsulation of the antiretroviral Zidovudine (AZT) in rat erythrocytes. The influence of several factors in the encapsulation was also evaluated. Variables such as the initial AZT concentration, the dialysis time, and the dialysis bag/buffer volume ratio exhibited statistically significant differences in the encapsulation of the drug in erythrocytes. The amount of drug encapsulated was related to the different values of the variables by multiple linear regression. Osmotic fragility and haematological parameters were estimated as indicators of erythrocyte viability. No statistically significant differences in the osmotic fragility profiles of the control and carrier erythrocytes were observed, and this parameter was also independent of the dialysis concentration of AZT, the hypo-osmotic dialysis time, and the dialysis bag/buffer volume ratio. The *in vitro* release of AZT from carrier erythrocytes pointed to a fast leakage of the drug; however, around 30% of the drug remained encapsulated for a prolonged period of time. Pre-dialysis diamide treatment did not have a significant effect on the encapsulation and release of AZT in erythrocytes.

1. Introduction

Owing to the broad range of drugs available to treat HIV infection, the HIV-associated disease-acquired immunodeficiency syndrome (AIDS) has been reduced to a chronic infection, at least in most developed countries. Nevertheless, highly active antiretroviral therapy (HAART) still has important limitations, such as the high cost of the treatments, the adverse effects of certain antiretroviral drugs, drug resistance, drug interactions, non-compliance problems, etc. . . (Kalkut, 2005; Lanao et al., 2007). In recent years, the struggle against HIV/AIDS has boosted the development of different therapeutic strategies, including the use of drug delivery systems to overcome these drawbacks.

Erythrocytes are potential biocompatible vectors for different bioactive substances, such as drugs, enzymes and other macromolecules. They have properties which make them suitable as drug carriers, such as their ability to provide a controlled-release effect or to target drugs to the reticulo-endothelial system, avoiding adverse effects in other organs, due to their rapid clearance from the blood by the monocyte–macrophage system. In comparison with other carrier systems, they have the advantage of higher biocompatibility, especially when autologous erythrocytes are used (Hamidi and Tajerzadeh, 2003; Gutiérrez et al., 2004b; Hamidi et al., 2007a,b,c; Briones et al., 2008; Patel et al., 2008).

Antiretroviral drugs show insufficient macrophage penetration capacities and low bioavailability, giving rise to the appearance of resistance and toxic effects due to their accumulation in other organs. The monocyte–macrophage system is the first to be infected by HIV. This supports the intracellular replication of the virus and acts as a reservoir, which favours the dissemination of the infection and protects the virus against antiretroviral treatment. The specific delivery of these agents into macrophages by means of drug delivery systems is therefore of huge therapeutic interest (Hu et al., 2000; Fraternale et al., 2002; Lanao et al., 2007; Briones et al., 2008).

The hypo-osmotic dialysis procedure is the method most frequently used for erythrocyte drug encapsulation because of its simplicity and relatively high yield of encapsulation (DeLoach and Ihler, 1977; Dale, 1987; DeLoach, 1987; Álvarez et al., 1998; Bax et al., 1999; Bax et al., 2000a,b; Gutiérrez et al., 2004a,b). The aim of this work was to study and optimize the factors that influence the encapsulation of zidovudine in rat erythrocytes, using a hypo-osmotic dialysis method.

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doi:10.1016/j.ijpharm.2010.09.006

2. Materials and methods

2.1. Materials

Zidovudine was kindly supplied by GlaxoSmithKline. All other chemicals and solvents were of analytical grade.

2.2. Blood collection and erythrocyte preparation

The housing and experimental treatment of the animals was in accordance with current Spanish and European Union legislation and complied with the "Principles of Laboratory Animal Care". Fresh blood was obtained from male Wistar rats by retro-orbital puncture, using EDTA (1.5 mg/ml) as anticoagulant. Plasma was removed after centrifugation of the blood (600 × *g*, 5 min, 4 °C). Packed erythrocytes were washed twice with isotonic Hanks-PBS buffer (pH 8).

2.3. Encapsulation of zidovudine in carrier erythrocytes

The encapsulation of AZT in rat erythrocytes was accomplished using a hypotonic dialysis method (Eichler et al., 1986; Sanz et al., 1999; Gutiérrez et al., 2005). Packed erythrocytes were resuspended in an AZT solution in Hanks-PBS buffer to obtain a cell suspension of 70% hematocrit. The cell suspension was placed in the dialysis bag (Medicell, molecular size cut-off, 12–14 KDa.) and incubated against 50 ml of a hypotonic buffer (15 mM $NaH_2PO_4{\cdot}2H_2O$, 15 mM $NaHCO_3$, 20 mM glucose, 2 mM ATP, 3 mM reduced glutathione, 5 mM NaCl, 90 mOsm/Kg, pH 8) at 4 °C. Then, the dialysis bag was transferred to 20 ml of the resealing buffer (250 mM NaCl, 12.5 mM glucose, 12.5 mM sodium pyruvate, 12.5 mM inosine, 12.5 mM $NaH_2PO_4{\cdot}2H_2O$, 0.63 mM adenine, 500 mOsm/Kg, pH 8) for 15 min at 37 °C. Finally, drug-loaded erythrocytes were washed twice in Hanks-PBS buffer to remove unencapsulated drug. Different conditions were tested in order to optimize the encapsulation method. The conditions assayed were: (1) initial AZT concentrations in the cell suspension of 1, 2, 3, 6 and 10 mg/ml for a dialysis time of 45 min and a bag/buffer ratio of 1:50 ml, (2) dialysis times of 45, 60 and 90 min for an initial AZT concentration of 10 mg/ml and a bag/buffer ratio of 1:50 ml, (3) dialysis bag/hypotonic buffer ratios, using dialysis bag volumes of 1, 1.5 and 2 ml, against a fixed hypotonic buffer volume of 50 ml, for an initial AZT concentration of 10 mg/ml and a dialysis time of 45 min. Eight replicates of each experiment were carried out. In addition, the influence of diamide treatment on AZT encapsulation in erythrocytes was studied. To this end, washed erythrocytes were resuspended in 2 mM diamide in Tris/ClH buffer (40 mM Tris/ClH, 5 mM KCl, 116 mM NaCl, 0.2 mM $MgCl_2$, 5 mM glucose, pH 7.4) (Jordán et al., 2001; Lotero et al., 2001), and incubated at 37 °C for 1 h. Then, cells were washed twice with the same buffer and hypotonic dialysis was carried out as described above.

2.4. Scanning electron microscopy (SEM)

In order to evaluate the existence of morphological changes in erythrocytes after the loading process, control and zidovudine-loaded ghost erythrocytes were prepared for scanning electron microscopy (SEM) as follows. Briefly, samples were incubated with poly-L-lysine for 1 h and then fixed with 25% glutaraldehyde steam overnight. Samples were washed with phosphate buffer and dehydrated using a concentration gradient of acetone from 30 to 100%, and finally dry acetone. Then, critical point drying in liquid carbon dioxide and metallization with gold particles was carried out. The samples thus prepared were analyzed using a Zeiss DSM 940 electron microscope.

2.5. Measurement of haematological parameters

Haematological parameters were determined in an ADVIA TM 120 Hematology System analyzer. The parameters evaluated were haematocrit (HCT), released haemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC).

2.6. Osmotic fragility

Osmotic fragility was determined with Dacieĭs method (Dacie and Vaughan, 1938). Twenty-microlitre aliquots of carrier erythrocytes were incubated in 0.5 ml of a NaCl solution with a concentration ranging from 0 to 0.9% (w/v) for 30 min at room temperature, and then centrifuged. Haemoglobin release into supernatant was determined by spectrophotometry (λ = 418 nm).

The amount of haemoglobin released was expressed as a percentage of the maximum haemolysis, which was determined as the amount of haemoglobin released into distilled water.

The osmotic fragility index was calculated for control and carrier erythrocytes as the NaCl concentration (% w/v) required to obtain 50% of haemolysis. Osmotic fragility was determined in triplicate for the different experimental conditions of this study.

2.7. In vitro release

The *in vitro* release of AZT from carrier erythrocytes was studied in erythrocytes prepared with 10 mg/ml as the initial AZT concentration, a dialysis time of 45 min, and a dialysis bag volume of 2 ml, with and without diamide treatment.

For these studies, carrier erythrocytes were resuspended in autologous plasma at a final haematocrit of 30%, separated into ten aliquots, and incubated at 37 °C with gentle stirring. At different times, aliquots were removed and centrifuged at 10 900 rpm for 10 min. Sampling times were 5, 10, 15, and 30 min, and 1, 2, 4, 8, 12, 24 and 48 h. The amount of AZT released into the supernatant was determined by an HPLC technique, as described below. Five replicates of this assay were carried out.

2.8. Quantification of encapsulated zidovudine

AZT concentrations in loaded erythrocytes were determined by a validated reverse-phase HPLC technique with UV detection (λ = 265 nm). Chromatographic analysis was performed with a Shimadzu LC 10 AD chromatograph. The analytical column used was a RP-18 LichroCart 5 cm × 4 mm I.D., 3 μm particle size. The mobile phase was 25 mM KH_2PO_4 pH 7/acetonitrile (91:9%) at a flow rate of 1 ml/min. The retention time of AZT was 3.6 min. Sample pretreatment was performed by protein precipitation with perchloric acid. A 25 μl aliquot of erythrocytes was diluted with 75 μl of saline, and 5 μl of 60% perchloric acid was added. The mixture was vortexed and centrifuged at 10 900 rpm for 5 min. Then, 20 μl of the supernatant was injected into the chromatograph. The technique was validated according to FDA specifications (Guidance for Industry: Bioanalytical Method Validation, 2001). The method showed linearity in the concentration range used. The CV estimated for precision and accuracy were less than 6%.

2.9. Data analysis

Statistical analysis of data corresponding to AZT encapsulation, osmotic fragility and the haematological parameters was performed using SPSS 15.0 statistical software (Weitzman, 1999).

Analysis of variance (ANOVA) was performed, with dialysis time, the initial AZT concentration and dialysis bag volume as the three independent variables. In addition multilinear regression analysis

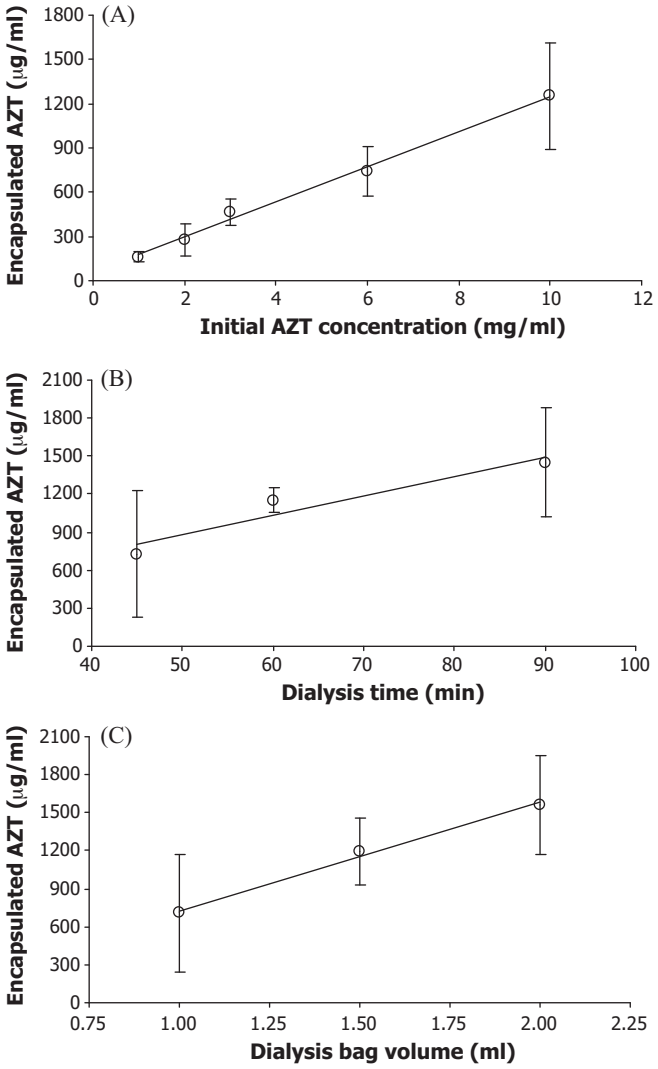

Fig. 1. Linear regression between the amount of encapsulated AZT and the different factors assayed: (A) initial AZT concentration; (B) dialysis time; (C) dialysis bag volume.

was carried out. To assess the reliability of the parameters estimated, the estimated standard error, the 95% confidence limits, and the two-tail p value for a t test were evaluated.

Statistical comparison of the haematological parameters and osmotic fragility among the different conditions assayed were performed using non-parametric analysis. In addition, multivariate hierarchical cluster analysis with haematological parameters was carried out.

The haematological parameters evaluated were hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

3. Results

The results obtained demonstrate that higher initial concentrations of AZT, prolonged dialysis times, and a higher dialysis bag volume improve the encapsulation of AZT in erythrocytes. Analysis of variance (ANOVA) revealed statistically significant differences in the amount of AZT encapsulated for the different values of the factors tested ($p < 0001$).

Table 1 shows the results of the multilinear regression analysis. The amount of AZT encapsulated in erythrocytes was considered as the dependent variable and the initial AZT concentration, dialysis time and dialysis bag volume were considered as the independent variables. The p values obtained indicated that all the independent variables considered were statistically significant ($p < 0.001$).

Fig. 1 (A–C) shows the linear relationships obtained between the amount of AZT encapsulated in erythrocytes and the initial AZT concentration, dialysis time and the dialysis bag volume respectively, generated using the multilinear regression equation included in Table 1.

Fig. 2 shows the results of scanning electron microscopy (SEM), where no significant changes in the shape or size of the erythrocytes can be observed.

Table 2 shows the haematological parameters of loaded erythrocytes in comparison with native erythrocytes as a function of the initial AZT concentrations, dialysis times and dialysis bag volumes.

Fig. 3 shows the results of the cluster analysis of the haematological parameters using Euclidean distance and square root transformation. Euclidian distance was used as it is the more common measure in cluster analysis when unstandardised variables (raw data) are computed. The Euclidian distance of 2 was the best distance to determine the existence of homogeneous groups in our set of haematologic data in the process of data reduction.

Fig. 4 shows the osmotic fragility curves and the osmotic fragility index of loaded erythrocytes in comparison with native erythrocytes as a function of the initial AZT concentrations (A), dialysis times (B) and dialysis bag volumes (C). The comparison of osmotic fragility curves made by an ANOVA test showed non-statistically significant differences ($p > 0.05$).

Fig. 5 shows the *in vitro* release curve of AZT from drug-loaded erythrocytes *versus* time. A rapid leakage of AZT from erythrocytes was observed. However, around 30% of the encapsulated drug remained inside the cell for prolonged periods of time. Previous

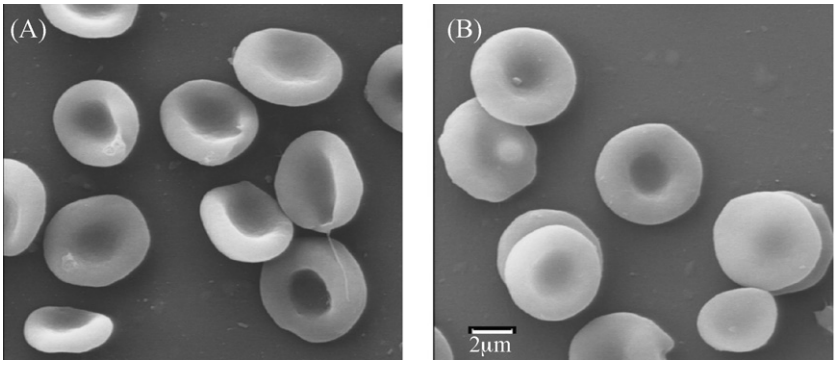

Fig. 2. SEM micrographs of native (A) and AZT-loaded erythrocytes (B) (500 MAG) (bar 2 μm).

Table 1
Results of the multiple linear regression between the amount of AZT encapsulated in erythrocytes and the initial AZT concentration, the dialysis time, and the dialysis bag volume.

Parameter	Value	95% Confidence limits		CV (%)	*p*	*r*
Constant	−909.510	−1241.060	−577.960	18.291	<0.001	
Initial AZT conc. (mg/ml)	80.882	62.411	99.353	14.459	<0.001	0.855
Dialysis time (min)	10.983	6.439	15.528	20.759	<0.001	0.454
Dialysis bag volume (ml)	569.578	371.289	767.868	17.468	<0.001	0.546

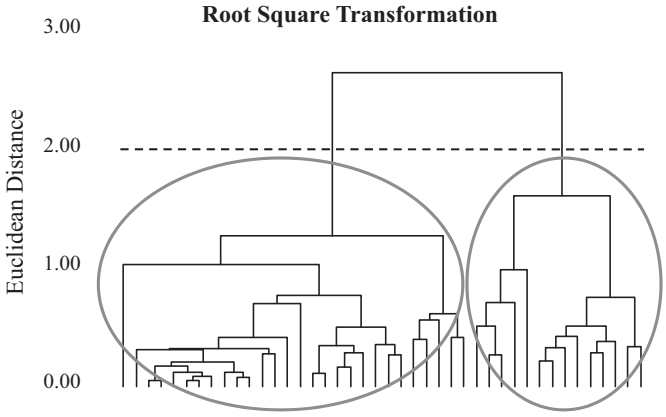

Fig. 3. Cluster analysis of haematological parameters.

diamide treatment of loaded erythrocytes did not lead to significant differences in the amount of drug released ($p > 0.05$).

4. Discussion

The hypotonic dialysis method is the technique that best preserves the initial biochemical and physiological characteristics of the erythrocytes. For this reason it is widely used for the encapsulation of drugs and therapeutic substances in this cells (Gutiérrez et al., 2004b). Despite its frequent use, however, the conditions used by different researchers to implement the method differ widely (Sanz et al., 1999; Gutiérrez et al., 2004a,b, 2008; Murray et al., 2006).

In this study, an optimized method of zidovudine erythrocyte encapsulation aimed at achieving the highest encapsulated concentration without compromising the viability of the erythrocytes was developed. Considering the lipophilic character of zidovudine (oil/water partition coefficient = 1.1) (Enting et al., 1998), the uptake of the drug by carrier erythrocytes can be considered a passive process. According to the results shown in Table 1, a simultaneous dependence on the initial concentration of drug, dialysis time and dialysis bag volume in the yield of the encapsulation can be observed. An increase in the concentration of the drug in the dialysis bag will facilitate encapsulation into cells by means of a passive process (Lotero et al., 2003; Murray et al., 2006). Another essential feature in the process of drug encapsulation is the duration of the dialysis; this varies with the different substances to be encapsulated and ranges between 20 and 180 min, although dialysis times between 45 and 75 min are the most frequent (Gutiérrez et al., 2004a,b). In this study, a progressive increase in dialysis time increased the amount of AZT encapsulated.

The influence of these factors—a high initial drug concentration and prolonged hypo-osmotic dialysis time on encapsulation efficiency has been described previously for amikacin (Gutiérrez et al., 2008). However, the influence of the dialysis bag volume in the encapsulation of drugs has not yet been reported. As shown in Table 1 and Fig. 1, a progressive increase in dialysis bag volume from 1 to 2 ml incubated against the same volume of buffer improved the amount of drug encapsulated. This phenomenon may be related to an alteration of the drug gradient in the dialysis bag. Also, the superficial area increases when the dialysis bag volume is higher. This should facilitate the passive incorporation of zidovudine into the erythrocytes. Further studies with higher dialysis bag/hypotonic buffer volume ratios would be of interest to improve the yield of zidovudine encapsulation.

Although the analysis by SEM showed no significant changes in the shape or size of the erythrocytes, the analysis of haematological parameters showed statistically significant differences ($p < 0.05$) with respect to the native erythrocyte controls for some of the conditions assayed (Table 2), especially when high dialysis times ($\geq$60 min) were tested. The incubation time affected the viability of the erythrocytes, because statistically significant differences were observed in haematological parameters such as HCT, MCV, MCH and MCHC ($p < 0.05$) for an incubation time of 60 and 90 min with respect to the native erythrocytes.

Statistically significant differences in some haematological parameters were found for the dialysis bag volumes used in this assay with respect to the control group. With multivariate hierarchical cluster analysis, and using a Euclidean distance of 2, the results revealed that the 5 variables led to the formation of two

Table 2
Mean hematological parameters of control and loaded erythrocytes and statistical significance using different conditions of encapsulation (*$p < 0.05$).

Parameter	HGB (g/dl)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)
Initial AZT concentration (dialysis time 45 min, dialysis volume 1 ml)					
Control	13.00 ± 0.92	37.50 ± 2.08	54.10 ± 4.42	18.73 ± 1.44	34.73 ± 2.80
1 mg/ml	13.70 ± 0.89	26.28 ± 6.77	70.18 ± 8.20	39.15 ± 13.18*	54.78 ± 14.14*
2 mg/ml	12.47 ± 1.33	24.77 ± 7.54	75.37 ± 0.42	39.50 ± 8.23*	52.40 ± 10.69
3 mg/ml	13.15 ± 0.66	30.85 ± 6.42	66.68 ± 9.85*	29.75 ± 9.47*	43.80 ± 7.70*
6 mg/ml	13.18 ± 0.46	29.45 ± 8.17	67.98 ± 6.45*	32.58 ± 0.75*	47.23 ± 12.11
10 mg/ml	13.68 ± 0.67	35.63 ± 2.49	62.03 ± 4.34	23.88 ± 2.35*	38.48 ± 2.08*
Dialysis time (initial AZT concentration 10 mg/ml, dialysis volume 1 ml)					
45 min.	13.68 ± 0.67	35.63 ± 2.49	62.03 ± 4.34	23.88 ± 2.35*	38.48 ± 2.08*
60 min.	12.90 ± 1.57	27.43 ± 8.24*	70.60 ± 6.40*	35.88 ± 12.48*	50.00 ± 13.85*
90 min.	14.10 ± 2.62	25.90 ± 6.54 *	76.2 ± 0.91*	42.78 ± 11.11*	56.23 ± 14.91*
Dialysis bag volume (initial AZT concentration 10 mg/ml, dialysis time 45 min)					
1 ml	13.68 ± 0.67	35.63 ± 2.49	62.03 ± 4.34*	23.88 ± 2.35*	38.48 ± 2.08*
1.5 ml	13.75 ± 0.41	35.27 ± 1.59	66.37 ± 8.06*	25.92 ± 3.18*	39.03 ± 1.23*
2 ml	13.78 ± 0.82	36.90 ± 0.66	61.50 ± 4.4*	23.05 ± 2.96*	37.35 ± 2.48

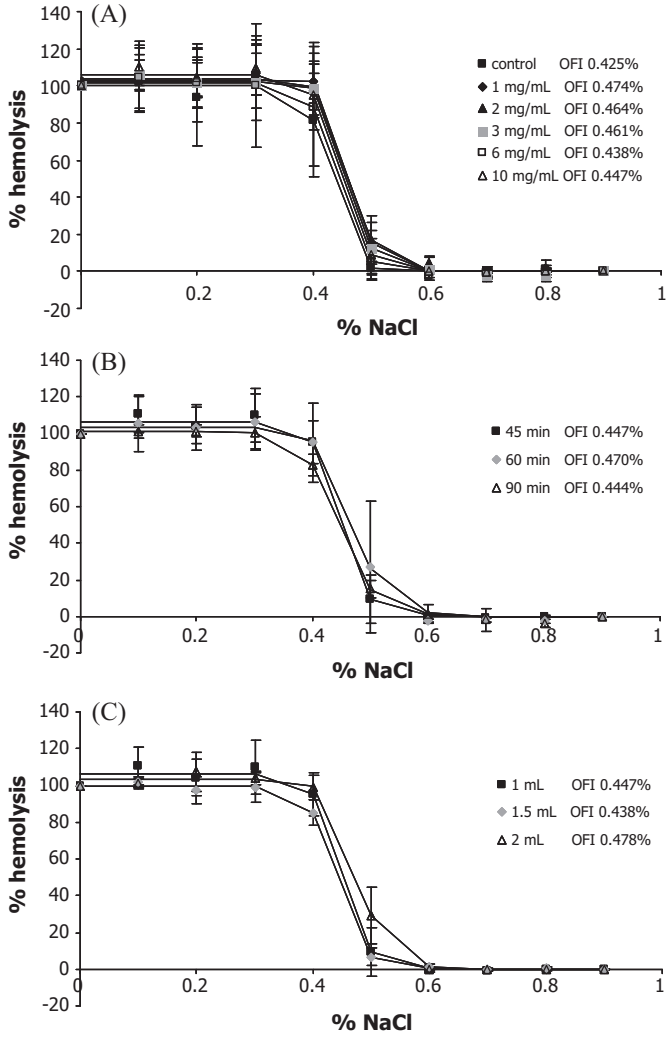

Fig. 4. Osmotic fragility curves and osmotic fragility index (OFI) of control and carrier erythrocytes prepared using different initial AZT concentrations (A), dialysis times (B) and dialysis bag volumes (C).

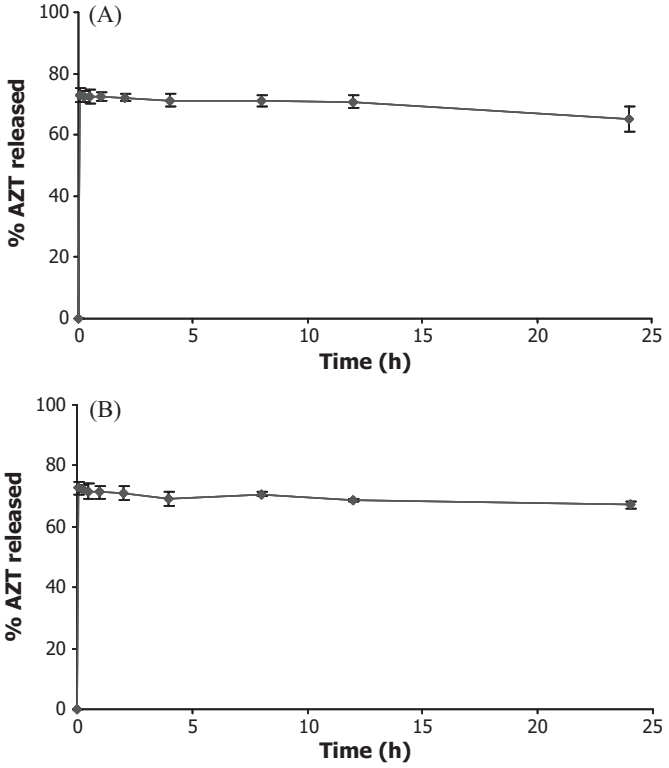

Fig. 5. Release of zidovudine from carrier erythrocytes as a function of time: (A) AZT-erythrocytes, (B) AZT-erythrocytes with pre-dialysis diamide treatment.

discrete clusters, as shown in Fig. 3, confirming that extreme dialysis conditions, such as a dialysis time ≥60 min, elicit different haematologic behaviour.

As can be observed in Fig. 4 for the different NaCl concentrations tested, no statistical differences in the release of haemoglobin with the initial concentration of the drug ($p > 0.05$), the hypo-osmotic dialysis time ($p > 0.05$) and the dialysis volume ($p > 0.05$) were observed. The osmotic fragility index is slightly lower for native erythrocytes than the index estimated for AZT-loaded erythrocytes. The results for the haematological parameters and osmotic fragility of carrier erythrocytes in the different conditions tested suggest that for drug encapsulation in extreme conditions with very long dialysis times (e.g.: 60 min or more) a shortened life span of loaded erythrocytes is expected.

The results about the release kinetics of zidovudine from carrier erythrocytes shown a fast leakage of AZT from carrier erythrocytes considering the lipophylic nature of the drug. However a 20–30% of the encapsulated amount remains inside the cell system for prolonged periods of time suggesting a very slow zero-order release *in vitro* as was previously stated by other authors (Lizano et al., 1998). This prolonged stay of a fraction of the encapsulated drug inside the erythrocyte may be related with covalently linking to red cell surface proteins. This type of behaviour allows the system to work as a drug reservoir, providing sustained release into the body and selectively directing the drug to the RES tissues (monocyte–macrophage system), such as the liver, spleen and bone marrow, which constitute the usual sites for the destruction of erythrocytes and are a reservoir for HIV viruses (Briones et al., 2008).

Diamide is an oxidizing agent that induces changes in the erythrocyte membrane. Such modifications can modulate recognition by macrophages, their survival in the circulation, and their removal by organs such as the liver and spleen (Lotero et al., 2001). Here, its influence in the encapsulating process and release of zidovudine was evaluated. Pre-dialysis diamide treatment failed to modify either the amount of drug encapsulated or the release kinetics of the drug.

According to the results obtained concerning encapsulation yield, haematologic parameters and osmotic fragility, the conditions finally selected for encapsulation were 10 mg/ml as the initial zidovudine concentration, an incubation time of 45 min and a dialysis bag/buffer volume ratio of 2:50 ml. The concentration of AZT encapsulated was 1.56 ± 0.39 mg/ml, and the encapsulation yield was 15.6%.

5. Conclusion

Evaluation of the influence of some *in vitro* conditions in the encapsulation of zidovudine in rat erythrocytes using a hipo-osmotic dialysis method was made. Changes in the initial drug concentration, dialysis times and dialysis bag/hypotonic buffer ratio led to statistically significant differences in the drug encapsulated. Also the viability of erythrocytes is affected by the values of this variables specially the dialysis times. A multilinear regression equation between the encapsulated zidovudine amount and the different conditions tested was made. The *in vitro* release of AZT from carrier erythrocytes pointed to a fast leakage of the drug; however, around 30% of the drug remained encapsulated for a prolonged period of time.

Acknowledgement

This work was supported by the Projects I + D + i PI060152 and SA049A07.

References

Álvarez, F.J., Jordán, J.A., Herráez, A., Díez, J.C., Tejedor, M.C., 1998. Hypotonically loaded rat erythrocytes deliver encapsulated substances into peritoneal macrophages. J. Biochem. 123, 233–239.

Bax, B.E., Bain, M.D., Fairbanks, L.D., Simmonds, A.H., Webster, A.D., Chalmers, R.A., 2000a. Carrier erythrocyte entrapped adenosine deaminase therapy in adenosine deaminase deficiency. Adv. Exp. Med. Biol. 486, 47–50.

Bax, B.E., Bain, M.D., Fairbanks, L.D., Webster, A.D., Chalmers, A.D., 2000b. In vitro and in vivo studies of human carrier erythrocytes loaded with polyethylene glycol-conjugated and native adenosine deaminase. Br. J. Haematol. 109, 549–554.

Bax, B.E., Bain, M.D., Talbot, P.J., Parker-Williams, E.J., Chalmers, R.A., 1999. Survival of carrier erythrocytes in vivo. Clin. Sci. 96, 171–178.

Briones, E., Colino, C.I., Lanao, J.M., 2008. Delivery systems to increase the selectivity of antibiotics in phagocytic cells. J. Control. Release 125, 210–227.

Dacie, J.V., Vaughan, J.M., 1938. The fragility of the red blood cells: its measurement and significance. J. Pathol. Bacteriol. 46, 341–356.

Dale, G., 1987. High-efficiency entrapment of enzymes in resealed red cell ghosts by dialysis. In: Green, R., Widder, K.J. (Eds.), Methods in Enzymology, 149. Academic Press, San Diego, pp. 229–234.

DeLoach, J.R., 1987. Dialysis method for entrapment of proteins into resealed red blood cells. In: Green, R., Widder, K.J. (Eds.), Methods in Enzymology, 149. Academic Press, San Diego, pp. 235–242.

DeLoach, J.R., Ihler, G., 1977. A dialysis procedure for loading erythrocytes with enzymes and lipids. Biochim. Biophys. Acta 496, 136–145.

Eichler, H.G., Rameis, H., Bauer, K., Korn, A., Bacher, S., Gasic, S., 1986. Survival of Gentamicin-loaded carrier erythrocytes in healthy human volunteers. Eur. J. Clin. Invest. 16, 39–42.

Enting, R.H., Hoetelmans, R.M.W., Lange, J.M.A., Burger, D.M., Beijnen, J.H., Portegies, P., 1998. Antiretroviral drugs and the central nervous system. AIDS 12, 1941–1955.

FDA, 2001. Guidance for Industry. Bioanalytical Method Validation, http://www.fda.gov/cder/guidance/index.htm.

Fraternale, A., Casabianca, A., Orlandi, C., Cerasi, A., Chiarantini, L., Brandi, G., Magnani, M., 2002. Macrophage protection by addition of glutathione (GSH)-loaded erythrocytes to AZT and DDI in a murine AIDS model. Antiviral Res. 56, 263–272.

Gutiérrez, C., Arévalo, M., Zarzuelo, A., González, F., Sayalero, M.L., Lanao, J.M., 2005. Encapsulation and in vitro evaluation of amikacin-loaded erythrocytes. Drug Deliv. 12, 409–416.

Gutiérrez, C., Bax, B.E., Zarzuelo, A., Sayalero, M.L., Lanao, J.M., 2008. In vitro studies of amikacin-loaded human carrier erythrocytes. Transl. Res. 152, 59–66.

Gutiérrez, C., Sayalero, M.L., Zarzuelo, A., Lanao, J.M., 2004a. Drug, enzyme and peptide delivery using erythrocytes as carriers. J. Control. Release 95, 27–49.

Gutiérrez, C., Zarzuelo, A., Sayalero, M.L., Lanao, J.M., 2004b. Factors associated with the performance of carrier erythrocytes obtained by hypotonic dialysis. Blood Cells Mol. Dis. 33, 132–140.

Hamidi, M., Tajerzadeh, H., 2003. Carrier erythrocytes: an overview. Drug Deliv. 10, 9–20.

Hamidi, M., Zarei, N., Zarrin, A.H., Mohammadi-Samani, S., 2007a. Preparation and in vitro characterization of carrier erythrocytes for vaccine delivery. Int. J. Pharm. 338, 70–78.

Hamidi, M., Zarrin, A., Foroozesh, M., Mohammadi-Samani, S., 2007b. Applications of carrier erythrocytes in delivery of biopharmaceuticals. J. Control. Release 118, 145–160.

Hamidi, M., Zarrin, A.H., Foroozesh, M., Zarei, N., Mohammadi-Samani, S., 2007c. Preparation and in vitro evaluation of carrier erythrocytes for RES-targeted delivery of interferon-alpha 2b. Int. J. Pharm. 341, 125–133.

Hu, J., Liu, H., Wang, L., 2000. Enhanced delivery of AZT to macrophages via acetylated LDL. J. Control. Release 69, 327–335.

Jordán, J.A., Álvarez, F.J., Lotero, L.A., Herráez, A., Díez, J.C., Tejedor, M.C., 2001. In vitro phagocytosis of carrier mouse red blood cells is increased by Band 3 cross-linking or diamide treatment. Biotechnol. Appl. Biochem. 34, 143–149.

Kalkut, G., 2005. Antiretroviral therapy: an update for the non-AIDS specialist. Curr. Opin. Oncol. 17, 479–484.

Lanao, J.M., Briones, E., Colino, C.I., 2007. Recent advances in delivery systems for anti-HIV1 therapy. J. Drug Target 15, 21–36.

Lizano, C., Sanz, S., Luque, J., Pinilla, M., 1998. In vitro study of alcohol dehydrogenase and acetaldehyde dehydrogenase encapsulated into human erythrocytes by an electroporation procedure. Biochim. Biophys. Acta 1425, 328–336.

Lotero, L.A., Jordán, J.A., Olmos, G., Álvarez, F.J., Tejedor, M.C., Diez, J.C., 2001. Differential in vitro and in vivo behavior of mouse ascorbate/Fe^{3+} and diamide oxidized erythrocytes. Biosci. Rep. 21, 857–871.

Lotero, L.A., Olmos, G., Diez, J.C., 2003. Delivery to macrophages and toxic action of etoposide carried in mouse red blood cells. Biochim. Biophys. Acta 1620, 160–166.

Murray, A.M., Pearson, I.F., Fairbanks, L.D., Chalmers, R.A., Bain, M.D., Bax, B.E., 2006. The mouse immune response to carrier erythrocyte entrapped antigens. Vaccine 24, 6129–6139.

Patel, P.D., Dand, N., Hirlekar, R.S., Kadam, V.J., 2008. Drug loaded erythrocytes: as novel drug delivery system. Curr. Pharm. Des. 14, 63–70.

Sanz, S., Lizano, C., Luque, J., Pinilla, M., 1999. In vitro and in vivo study of glutamase dehydrogenase encapsulated into mouse erythrocytes by a hypotonic dialysis procedure. Life Sci. 65, 2781–2789.

Weitzman, E.A., 1999. Analyzing qualitative data with computer software. Health Serv. Res. 34, 1241–1263.