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Heterogeneity of hypotonically loaded rat erythrocyte populations as detected by counter-current distribution in aqueous polymer two-phase systems

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Abstract

Carrier rat erythrocytes loaded with exogenous substances ([^{125}I]carbonic anhydrase) by hypotonic–isotonic dialysis become heterogeneous cell populations that can be fractionated using the counter-current distribution (CCD) technique. Two well-defined low- and high-partition ratio, *G*, subpopulations are obtained in charge-sensitive dextran–polyethylene glycol two-phase systems. The low-*G* subpopulation, which contains the most fragile and surface-altered cells, as deduced from their osmotic fragility curves and partition behaviour, respectively, presents a high amount of exogenous substance incorporated (134.6 cpm/10^6 cells). The high-*G* subpopulation, that contains cells similar to the control or isotonically dialyzed cells presents a lower amount of exogenous substance incorporated (69.8 cpm/10^6 cells). Cells in this high-*G* subpopulation seem to be fractionated, like the controls, according to ageing as suggested by the decline of the pyruvate kinase specific activity from the left- to the right-hand side of the CCD profile.

Keywords: Carbonic anhydrase; Enzymes

1. Introduction

Erythrocytes have been proposed as carriers for drugs, enzymes and pharmacological agents to enhance treatment efficacy, extend pharmacological life span and reduce toxic side-effects. Hypotonic–isotonic dialysis, which makes it possible to open transitory cell membrane pores to allow the free entry of substances, has been used to turn erythrocytes from different species into carriers [1,2]. However, rat erythrocytes, commonly studied mammalian cells, have not yet been studied widely as carriers. The difficulties associated with the incorporation of different substances into rat erythrocytes seem to be related to hemoglobin gelation during osmotic shock [3,4]. Changing the pH of the dialysis medium from the usual 7.4 to 8.0 improves hemoglobin solubility, so that rat erythrocytes can be loaded with significant proportions of substances and can be used as carrier systems in in vivo studies [5,6].

Human and rat erythrocytes loaded by hypotonic–isotonic dialysis are more heterogeneous than control

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erythrocytes [2], [5–9]. In general, the in vivo survival behaviour of human carriers is characterized by the rapid loss of some hypotonized cells during the first 24 h after reinjection, followed by a normal elimination of the remaining hypotonized cells thereafter [2]. It seems the hypotonic–isotonic dialysis might damage cell membrane structure and impair cell survival capacity [10].

Counter-current distribution (CCD) in aqueous dextran–polyethyleneglycol (D–PEG) two-phase systems fractionates erythrocytes from different species according to subtle changes in their membrane surface properties [11–13]. Specifically, our group has fractionated human and rat erythrocytes, as a function of cell surface changes during cellular ageing [14–17]. This paper reports on the cell heterogeneity in hypotonized (80 mOsm/kg) rat erythrocytes, either unloaded or loaded with [^{125}I]carbonic anhydrase, as detected by the CCD technique. The in vitro CCD separation of the rapidly removed cells would improve the survival of the reinjected carriers in animals. Parallel experiments with rat erythrocytes subjected to isotonic dialysis (300 mOsm/kg) are carried out as controls for the dialysis process.

2. Experimental

Blood from male Wistar rats (150–200 g) was collected into heparinized (10 U/ml) tubes and filtered through α-cellulose-cellulose type 50 (mean size 50 μm) filters (Sigma, St. Louis, MO, USA) to obtain erythrocytes [18]. Two washings with phosphate buffered saline (PBS) (10 m*M* phosphate buffer, 3.5 m*M* KCl, 0.5 m*M* $MgCl_2 \cdot 6H_2O$, 145 m*M* NaCl, pH 8.0) at 400 *g* for 10 min at 4°C and another at 2500 *g* for 5 min at 4°C, were performed to obtain packed erythrocytes.

Dialyzed erythrocytes were prepared under either isotonic (300 mOsm/kg) or hypotonic (80 mOsm/kg) conditions as previously described [6]. Briefly, 0.5 ml of packed erythrocytes (70% hematocrit) were placed in a dialysis bag (Medicell, molecular size cut off, 12–14 kDa) and dialyzed against 40 volumes of isotonic or hypotonic PBS, under rotation at 4°C for 50 min. Dialysis bags were then incubated in isotonic PBS for 10 min at 37°C (annealing). Finally, erythrocytes were resealed by addition of 0.1 vol. of a hypertonic PIGPAC solution (5 m*M* adenine, 100 m*M* sodium pyruvate, 100 m*M* inosine, 100 m*M* NaH_2PO_4, 100 m*M* glucose, 12% (w/v) NaCl) to restore isotonicity and then incubated for 30 min at 37°C. Resealed erythrocytes were washed three times at 160 *g* for 5 min at 4°C, in isotonic Hanks solution (6 m*M* glucose in PBS). The cell recovery after the encapsulation process is around 46%.

Loaded erythrocytes were prepared by adding, together with the cellular suspension, a $34.5 \cdot 10^{-3}$ m*M* final concentration of [^{125}I]carbonic anhydrase to the dialysis bags. Carbonic anhydrase (M_r 29 000, from Sigma) was labeled with ^{125}I by means of chloramine-T immobilized on polystyrene beads (Iodo-Beads, Pierce, Rockford, IL, USA), obtaining a specific activity of $5.8 \pm 0.6 \cdot 10^{11}$ cpm/μmol which was diluted with unlabelled carbonic anhydrase to obtain adequate specific activity for the assay ($18.82 \pm 0.6 \cdot 10^7$ cpm/μmol). The amount of [^{125}I]carbonic anhydrase entrapped within the cells is determined directly by using a gamma counter.

The hematologic parameters (cell number, hemoglobin concentration, hematocrit and cell volume) of the different cell populations studied were systematically analyzed in a Serono-Baker Diagnostic system 9000+ coulter counter.

5.6% (w/w) D–4.6% (w/w) PEG, 0.03 *M* sodium chloride and 0.09 *M* sodium phosphate buffer charge-sensitive two-phase systems were prepared by weight from the following stock solutions: 20% (w/w) dextran T-500 (D) (Pharmacia, Uppsala, Sweden) standardized by polarimetry, 40% (w/w) polyethyleneglycol 6000 (Serva, Heidelberg, Germany), 0.2 *M* sodium phosphate buffer (pH 6.8) and 1 *M* sodium chloride [11–13].

For partition experiments 5 μl of packed erythrocytes were added to a two-phase system formed by 2 g of bottom phase and 2 g of top phase from the above equilibrated phases. The system was mixed by 60 inversions and allowed to settle for 20 min at 4°C [19]. Duplicate aliquots were removed from the top phase to measure hemoglobin concentration and cell number in a Serono-Baker Diagnostics System 9000+ coulter counter. The partition ratio, *P*, defined as the quantity of cells in the top phase, at the time

of sampling, as a percentage of the total cells added, was calculated.

A thin-layer counter current distribution apparatus CCD (Bioshef TLCCD, MK 3) with two thin layer units (60 concentric cavities) was used for cell fractionation [16,17]. The volume capacity of the bottom units was 0.85 ml (unit 1) and 0.81 ml (unit 2). Three adjacent cavities (0–2 and 30–32) each received a mixture of 0.46 ml (unit 1) or 0.42 ml (unit 2) of D-rich bottom phase plus 0.1 ml of the erythrocyte suspension, whereas the remaining cavities each received 0.56 ml (unit 1) or 0.52 ml (unit 2) of the D-rich bottom phase. Thirty partition steps were performed at 4°C. For more detailed CCD experimental conditions see [17]. The cell distribution profile is given by the hemoglobin absorbance values at 540 nm vs. the cavity number. Cells with an affinity for the top phase (high-partition ratio, $G=r_{max}/(n-r_{max})$ where r_{max} is the cavity number of the peak of a CCD curve and n is the number of transfers) were distributed as fast-moving cells in the cavities with the highest number. Cells with an affinity for the interface (lower-G values) tended to remain in the fractions with the lowest number as slow-moving cells [11–13]. CCD experiments were repeated at least four times with different erythrocytes.

Pyruvate kinase (EC 2.7.1.40; PK) activity was assayed in hemolysates prepared by hypo-osmotic shock with two volumes of stabilizing solution (2.7 m*M* EDTA–Na_2 and 0.7 m*M* mercaptoethanol, pH 7), followed by freezing and thawing, as previously described [20]. The specific enzyme activity was expressed as U/g Hb or U 10^{-10}/cell. Reagents, substrates, cofactors and auxiliary enzymes were from Sigma and Boehringer. PK activity was measured in duplicate for each CCD experiment. Results are expressed as mean ± S.E.M.

3. Results and discussion

Partitioning in D–PEG two-phase systems allows the differentiation of charge and non-charge (hydrophobicity) cell surface properties by the appropriate selection of polymer and salt concentrations while maintaining physiologic isotonicity and pH [11–13], [19]. Phosphate ion has an affinity for the D-rich bottom phase resulting in a Donnan potential between the two-phases (PEG-rich top phase is positive), that produces charge-sensitive two-phase systems. Since NaCl has equal affinity for the two phases the addition of this salt produces non-charge sensitive systems [11–13].

The partitioning behaviour in a 5.6% D–4.6% PEG charge-sensitive two-phase system of control, isotonically (D-300) and hypotonically (D-80) dialyzed rat erythrocytes (Fig. 1), is characterized by the relatively high affinity of the cells (70%, 70% and 40%, respectively) for the top phase. However, these cells did not show any affinity for the top phase in an equivalent non-charge sensitive system ($P <$ 10%, results not shown), i.e., when phosphate is substituted by NaCl. Thus, the increase in the amount of cells in the top phase going from non-charge- to charge-sensitive systems can only be due to cell surface charge properties [11–13,19]. On the other hand, since CCD subfractionation is best when about 50% of the cells are in the top phase [12,13], getting 40–70% of the erythrocytes in the top phase of a charge sensitive system would give good cell resolution by CCD. Therefore, the 5.6% D–4.6% PEG charge-sensitive two-phase system was chosen to study the CCD fractionation of the different erythrocyte populations as a function of cell surface charge properties.

The CCD fractionation profile of control rat erythrocytes, after 30 partitioning steps, shows a single homogeneous peak located between cavities 5 and 25, with the highest cellularity around cavity 17

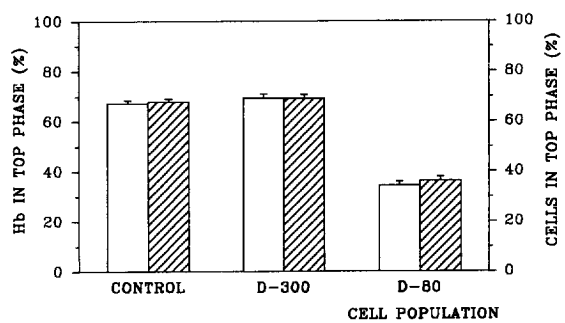

Fig. 1. Partition ratio, P, in a 5.6% D–4.6% PEG charge-sensitive aqueous two-phase system for control, isotonically (D-300) and hypotonically (D-80) dialyzed rat erythrocytes, expressed as percentage of hemoglobin (white bars) or cells (hatched bars) in top phase. Vertical bars represent the mean ± S.E.M. (n=5).

(Fig. 2, top). The cell fractionation obtained was characterized by the age-dependent PK specific activity [21] measured in five pooled CCD fractions (shadowed zones in Fig. 2, top). The highest activity, which is related to younger erythrocytes [16,21], is observed in cells located towards the right-hand side of the CCD profile whereas the lowest PK activity, which is related to older erythrocytes [16,21], is observed in cells progressively located towards the left-hand side of the CCD profile (Fig. 2, bottom). These results suggest an age-related CCD fractionation of control rat erythrocytes in agreement with previous studies carried out by our group [15–17]. Although the decrease of PK specific activity from the right- to the left-hand side of the CCD profile is more significant after 60 partitioning steps [17], which lead to a more efficient cell fractionation, only 30 partition steps were done in this study in order to reduce the CCD fractionation time and improve cell viability given the higher fragility of hypotonized cells.

As a control of the dialysis process, rat erythrocytes subjected to isotonic dialysis (D-300) were studied first. The resulting cells presented normal hematological parameters and slightly increased osmotic fragility (Fig. 3, top), as previously observed by our group [6]. The CCD fractionation of these cells produces homogeneous profiles located between cavities 6 and 27, with a PK specific activity that decreases from the right- to the left-hand side of the CCD profile in a similar way to that observed for control erythrocytes (Fig. 2), suggesting that the dialysis process under isotonic conditions does not damage the erythrocyte surface charge properties. The osmotic fragility of this D-300 cell population after CCD fractionation was also similar to that of the control cells, however, both populations showed

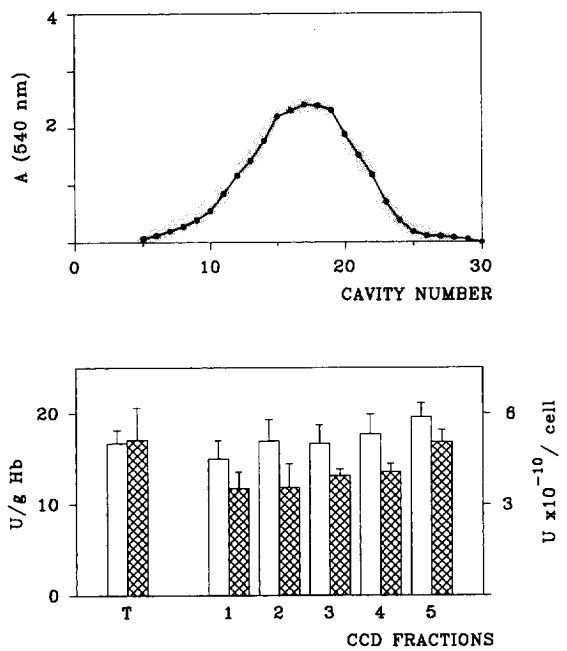

Fig. 2. Representative CCD fractionation profile (hemoglobin absorbance at 540 nm against cavity number), in a 5.6% D–4.6% PEG charge-sensitive two-phase system, of a control rat erythrocyte population (top). PK specific activity (U/g Hb, white bars, and U 10^{-10}/cell, hatched bars) in the unfractionated control erythrocyte population (T) and in the five pooled CCD fractions (bottom). Vertical bars represent the mean ± S.E.M. (n=5).

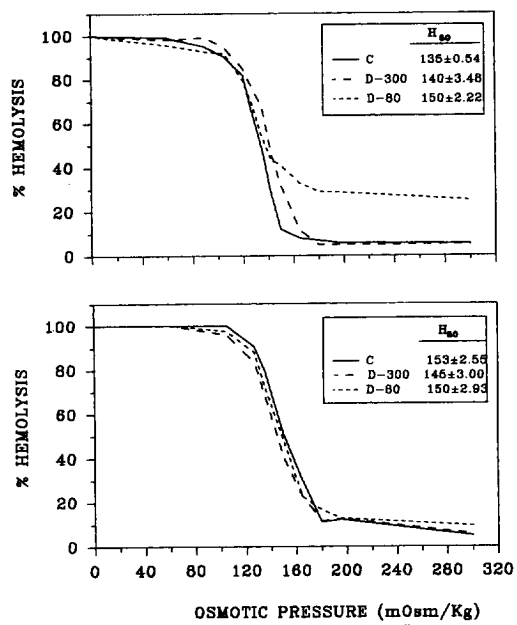

Fig. 3. Osmotic fragility curves of control (C), isotonically (D-300) and hypotonically (D-80) dialyzed rat erythrocytes unfractionated (top) and fractionated (bottom) by CCD. In the bottom graphs, D-80 curve only corresponds to the high-G subpopulation obtained by CCD. Curves shown are the mean of at least three different experiments. H_{50} values correspond to the osmotic pressure producing 50% hemolysis.

an osmotic fragility slightly higher after (Fig. 3 bottom) than before (Fig. 3 top) the CCD procedure.

Rat erythrocytes subjected to hypotonic dialysis (D-80) have abnormal hematological parameters (increased cell volume) [6], possibly due to their high osmotic fragility compared with the control and isotonically dialyzed erythrocytes (Fig. 3, top), indicating some influence of the hypotonic dialysis process on cell structure. When these erythrocytes are fractionated by CCD, some cell heterogeneity is observed (Fig. 4, top): two well-defined subpopulations of low (cavities 1 to 10) and high (cavities 10 to 27) partition ratio, G, with the highest cellularity being obtained around cavities 3 and 20, respectively. The high-G cell subpopulation is found in cavities equivalent to the control or isotonically dialyzed cells (Fig. 2, top) and has a similar osmotic resistance to these two erythrocyte populations (Fig. 3, bottom), suggesting that this high-G subpopulation contains the cells least altered by the hypotonic dialysis process. In contrast, the low-G subpopulation, located towards the first cavities, contains cells with a high degree of hemolysis and cell debris (as observed by optical microscopy in fresh preparations), suggesting that the most altered cells are found in this low-G subpopulation. Moreover, because of its low partitioning (cells located in the first cavities of the CCD rotor), these cells have a decreased surface charge.

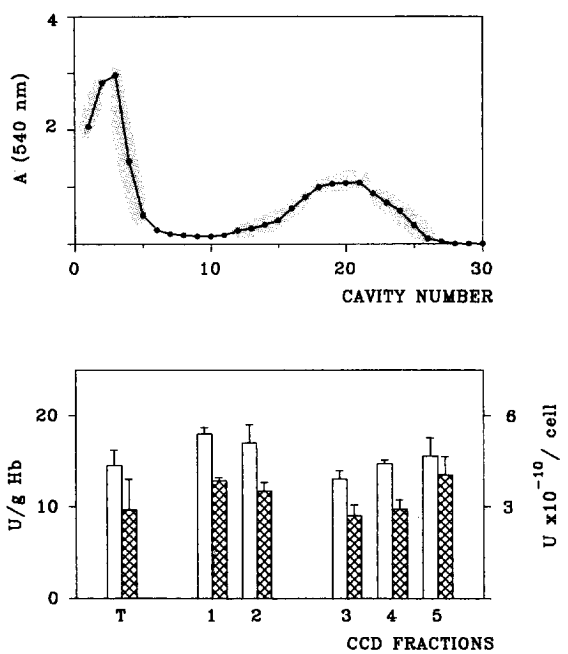

Fig. 4. Representative CCD fractionation profile (hemoglobin absorbance at 540 nm against cavity number), in a 5.6% D–4.6% PEG charge-sensitive two-phase system, of rat erythrocytes subject to hypotonic dialysis (D-80) (top). PK specific activity (U/g Hb, white bars, and U 10^{-10}/cell, hatched bars) in the unfractionated control erythrocyte population (T) and in the five pooled CCD fractions (bottom). Vertical bars represent the mean ± S.E.M. (n=5).

To check cell surface alterations, fractionation as a function of cell ageing was studied in the two subpopulations obtained by CCD. The PK specific activity in two pooled CCD fractions from the low-G subpopulation and in three pooled CCD fractions from the high-G subpopulation (shadowed zones in Fig. 4, top) was analyzed. Pools 1 and 2, with some cell surface alterations, have considerable PK activity, which suggests that in spite of the surface, morphologic and osmotic alterations observed, these cells have apparently maintained their metabolic capacity. In the high G-subpopulation, PK activity decreases from pool 5 to pool 3 suggesting age-related cell fractionation which also shows the similarity between these cells and the control cells.

Once demonstrated that the whole hypotonically dialyzed rat erythrocyte population contains a mixture of normal (high-G subpopulation) and surface-altered (low-G subpopulation) cells that can be easily and completely separated by CCD, the potential capacity as carriers for active substances of these two different cell subpopulations was studied. [^{125}I]carbonic anhydrase-loaded erythrocytes made by hypotonic-isotonic dialysis were used. [^{125}I]carbonic anhydrase was chosen as an encapsulation marker because its molecular mass and solubility properties allow it to be acceptably incorporated (30%) into rat erythrocytes during hypotonic dialysis. As expected, CCD fractionation of the loaded erythrocyte population (containing approx. $1 \cdot 10^9$ cells and $1.5 \cdot 10^5$ cpm), is similar to that of the erythrocytes subjected to hypotonic dialysis in the absence of the marker (Fig. 4, top). Thus, two well-defined low- and high-G subpopulations are obtained, which means that substance incorporation per se does not modify the charge-related cell surface properties. The low-G subpopulation contains a higher percentage of [^{125}I]carbonic anhydrase (134.6

Table 1
Cellularity and [^{125}I]carbonic anhydrase (cpm) content in the low- and high-*G* erythrocyte subpopulations obtained after CCD fractionation of rat erythrocytes loaded by hypotonic dialysis.

	Cells		cpm		cpm/10^6 cells
	$\times 10^9$	%	$\times 10^4$	%	
Low-*G* subpopulation	0.50±0.18	63.5±5.9	7.03±2.47	82.2±4.8	134.6±18.0
High-*G* subpopulation	0.19±0.05	36.5±7.9	1.18±0.18	17.8±4.8	69.8±6.0

Results represent the mean ± S.E.M. (n=3).

cpm/10^6 cell, i.e., 82% of the total recovered radioactivity after CCD, Table 1), than the high-*G* subpopulation (69.8 cpm/10^6 cell, i.e., 17% of the total recovered radioactivity after CCD, Table 1). This suggests that membrane alterations detected in the low-*G* subpopulation allow a higher protein incorporation into the cells. These results allow us to suggest that the most altered cells (the ones in the low-*G* subpopulation), that incorporate the most substance, could be the first to be removed by the mononuclear phagocytic system after reinjection in animals, whereas the cells most like the controls (the ones in the high-*G* subpopulation), that incorporate less substance, might remain in the circulating blood in an indistinguishable manner from control erythrocytes. Both cell subpopulations would then have potential pharmacological applications and could perform selective therapeutic actions; the low-*G* subpopulation could direct carrier erythrocytes towards different organs in the mononuclear phagocytic system, and the high-*G* subpopulation could maintain carriers in circulating blood.

In summary, CCD fractionation in aqueous two-phase systems makes it possible to easily reduce cell heterogeneity of rat erythrocyte carrier populations prepared by hypotonic dialysis and produce two separate and well-defined cell subpopulations which differ in their cell charge surface properties, osmotic resistance and quantity of incorporated substance, and which can be considered as separate carrier systems for in vivo studies.

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References

[1] J.R. DeLoach and J.L. Way (Editors), Carrier and Bioreactor Red Blood Cells for Drug Delivery and Targeting, Pergamon, Oxford, 1994.
[2] L. Chiarantini and J.R. DeLoach, Adv. Exper. Med. Biol., 326 (1992) 55.
[3] J.R. DeLoach, Med. Res. Rev., 6 (1986) 487.
[4] C. Bailleul, M.Ch. Borrelly-Villereal, M. Chassaigne and C. Ropars, Biotechnol. Appl. Biochem., 11 (1989) 31.
[5] J. Luque, M.I. Garín, S. Sanz, P. Ropero and M. Pinilla, in M. Magnani and J.R. DeLoach (Editors), The Use of Resealed Erythrocytes as Carriers and Bioreactors, Plenum Press, New York, 1992, p. 81.
[6] M.C. Tejedor, C.E. Alvarez, F.J. Alvarez, A. Herraez and J. Luque, in J.R. Deloach and J.L. Way (Editors), Carrier and Bioreactor Red Blood Cells for Drug Delivery and Targeting, Pergamon, Oxford, 1994, pp. 73–83.
[7] U. Sprandel and N. Zöllner, Res. Exp. Med., 48 (1985) 785.
[8] D. Scott, F.A. Kuypers, P. Butikofer, R.M. Bookchin, O.E. Ortiz and B.H. Lubin, J. Lab Clin. Med., 115 (1990) 470.
[9] M. Garín, R. Kravtzoff, N. Chestier, S. Sanz, M. Pinilla, J. Luque and C. Ropars, Biochem. Mol. Biol. Int., 33 (1994) 806.
[10] M.A. Castellana, M.R. DeRenzis, G. Piccinini, G. Minetti, C. Seppi, C. Balduini and A. Broveli, Adv. Exp. Med. Biol., 326 (1992) 91.
[11] P.A. Albertsson, Partition of Cell Particles and Macromolecules, Wiley, New York, 1986.
[12] H. Walter, in H. Walter, D.E. Brooks and D. Fisher (Editors), Partitioning in Aqueous Two-phase Systems: Theory, Methods, Uses and Applications to Biotechnology, Academic Press, New York, 1985, p. 327.
[13] H. Walter and G. Johansson (Editors), Methods in Enzymology, Vol. 228, Academic Press, New York, 1994.

[14] J. Mendieta, A. Herraez, P. Sancho and J. Luque, Biosci. Rep., 9 (1989) 541.
[15] M. Martin, C. Tejero, M. Gálvez, M. Pinilla and J. Luque, Acta Biol. Med. Germ., 40 (1981) 979.
[16] P. Jimeno, A.I. García-Pérez, J. Luque and M. Pinilla, Biochem. J., 279 (1991) 237.
[17] L. Lucas, A.I. García-Pérez, P. Jimeno, M.T. Pérez, M. Pinilla, P. Sancho and J. Luque., J. Chromatogr. B., 664 (1995) 137.
[18] E. Beutler, C. West and K.G. Blume, J. Lab. Clin. Med., 88 (1976) 328.
[19] A.I. García-Pérez, P. Sancho and J. Luque, J. Chromatogr., 504 (1990) 79.
[20] W.N. Valentine and K.R. Tanaka, Methods Enzymol., 9 (1966) 468.
[21] G. Bartosz, Gerontology, 37 (1991) 33.