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Inhibition of serum angiotensin-converting enzyme in rabbits after intravenous administration of enalaprilat-loaded intact erythrocytes

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Abstract

Encapsulation of drugs in intact erythrocytes, because of the profound characteristics of these natural microspheres, has gained considerable attention in recent years. In this study, the inhibition time courses of serum angiotensin-converting enzyme (ACE) activity after intravenous administration of enalaprilat encapsulated in intact erythrocytes was evaluated and compared with free drug, in a rabbit model. Three groups of animals each received free drug, drug-loaded erythrocytes or sham-encapsulated erythrocytes. Serum ACE activity was determined in each case using the synthetic substrate hippuryl-histidyl-leucine and quantitation of the hippuric acid released by a developed and validated HPLC method. The serum ACE inhibition profiles in the three groups showed that the encapsulated drug inhibited the serum ACE more slowly, more efficiently, over a considerably longer time and in a more reproducible manner, than the free drug or sham-encapsulated erythrocytes. We conclude that the erythrocytes can serve as efficacious slow-release drug carriers for enalaprilat in circulation.

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Introduction

Encapsulation of drugs and other bioactive agents in erythrocytes has been investigated extensively in animal models (Updike et al 1976; Pitt et al 1983b; Alpar & Lewis 1985; Eichler et al 1986; Ropars et al 1987; Field et al 1989; Kravtzoff et al 1990), and the carrier erythrocytes have exhibited a considerable degree of biocompatibility (Summers 1983; Updike & Wakamiya 1983; Lewis & Alpar 1984; Ropars et al 1987; Talwar & Jain 1992) as well as a natural life-span in circulation (Kinosita & Tsong 1978; Jenner et al 1981; Updike & Wakamiya 1983). In addition, the pharmacokinetics of the loaded agents has been evaluated using animal models (Pitt et al 1983b; Lewis 1984; Ropars et al 1987; Field et al 1989).

Enalaprilat is an angiotensin-converting enzyme (ACE) inhibitor, widely used as its esterified oral absorbable prodrug, enalapril, in the management of hypertension and congestive heart failure (Jackson & Garrison 1996; Opie 1992). Considering the widespread clinical use of enalaprilat, and the peptide-like chemical structure of the drug, this study was focused on the efficiency of carrier erythrocytes for intravenous delivery of enalaprilat. For this purpose, the main in-vivo effect of enalaprilat (i.e. ACE inhibition) was selected as an indicator of drug action as well as an indirect measure of drug pharmacokinetics. Given the nature of the UV absorption spectrum of enalaprilat (no discernible peaks and very low E1%), and

the very close similarity between this drug and many endogenous blood constituents, the development of methods for quantitative analysis of this drug in biological fluids is very problematic (Ip & Brenner 1987). As a result, the inhibition of serum ACE activity, having a remarkable correlation with enalaprilat serum concentration (Kubo & Cody 1985; Belz et al 1988), can be evaluated as an indirect measure of drug pharmacokinetics. In this study, the time course of serum ACE inhibition was evaluated after intravenous administration of enalaprilat-loaded erythrocytes to rabbits, and compared with free drug administration.

Materials and Methods

Materials

Enalaprilat (Pharmhispania Co., Spain) was kindly donated by Dr Abidi Pharmaceutical Co. (Tehran, Iran). Hippuryl-histidyl-leucine (HHL; Sigma Co.) and hippuric acid (Sigma Co.) were purchased locally. Other chemicals and solvents were prepared locally and were of chemical laboratory or HPLC grade, when needed.

Animals

Twelve albino rabbits (New Zealand strain), 2.45 ± 0.58 kg, were obtained from the Iranian Pasteur Institute (Tehran, Iran) and were stored in experimental animal cages throughout the study. They were fed with standard food and had free access to water. The animal study protocol was reviewed and accepted by the Review Board of the Postgraduate Studies, Tehran University of Medical Sciences.

Determination of serum ACE activity

To evaluate the ACE activity in serum samples, a method was developed using the synthetic substrate HHL, and the hippuric acid released upon action of serum ACE on this substrate was determined by a simple HPLC method.

Preparation of buffered substrate solution (BSS)

Boric acid (H_3BO_4; 94 mmol) and 150 mmol KCl were dissolved in an approximate volume of 400 mL distilled water and the pH of the resulting solution was adjusted to 8.3 by the addition of 1 M KOH solution. The volume of the solution was adjusted to 500 mL by adding distilled water. Then, 3.6 μmol (1.55 mg) HHL was dissolved in 1 mL of the prepared buffered solution. This solution was kept at 4°C before use.

Enzymatic assay

BSS (100 μL) was added to 20 μL serum and the resulting mixture vortexed for 10 s and incubated in a water bath at 37°C for 30 min. The enzymatic reaction was terminated by the addition of 10 μL perchloric acid solution (60 %, w/v), and the mixture vortexed for 20 s. After centrifuging the suspension at 3000 *g* for 5 min to separate the precipitated serum proteins, 20 μL of the supernatant was injected onto the chromatograph for quantitation of hippuric acid produced upon enzymatic action. To determine the baseline concentrations of hippuric acid in serum samples, when needed, and also for verification of hippuric acid production only by enzyme action, a blank sample was prepared as described for the enzyme assay using drug-free rabbit serum, except that the perchloric acid was added before the incubation period. A unit of ACE activity was defined as 1 μmol hippuric acid produced within 1 min of incubation of serum sample with substrate in the reaction condition.

HPLC method

A reversed-phase HPLC method was developed to determine the hippuric acid concentration produced upon enzymatic reaction. In this method, a mobile phase consisting of the aqueous solution of 10 mM KH_2PO_4 and methanol (80:20, v/v) was adjusted to a final pH of 3 by the addition of H_3PO_4 solution (5 M), and a C_{18} column (μBondapak, 300 × 3.9 mm; Waters, USA) was used for analyte separation. The eluent was delivered using a double-reciprocating pump (model 6000; Waters) with a flow rate of 2 mL min^{-1}, which developed a pressure of approximately 2700 psi. Detection was made using a UV detector (model 486; Waters) at the wavelength of 228 nm, and the resulting chromatograms were recorded and processed using a compatible recorder (model 746; Waters) with a chart speed of 0.25 cm min^{-1}. A Rheodyne injector (Rheodyne, USA) equipped with a 20-μL loop was used for sample injection.

Standard curve

A series of standard solutions were prepared as described for BSS, except that the HHL was replaced by hippuric acid in the following concentrations: 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 and 1 mM. The enzymatic assay procedure was carried out as described, using these solutions. The heights of the resulting peaks of hippuric acid in the

corresponding chromatograms were plotted against the known added concentrations. The standard samples were prepared and analysed in triplicate, and linear regression analysis was carried out for each series of data.

Validation tests

To evaluate the intra- and inter-run variations of the developed method, a total of six concentration series were analysed as described above, within one experimental run, and six different runs, respectively. Then, the coefficients of variations for each concentration were calculated. For absolute recovery (accuracy) testing, three concentration series were analysed within one run, and the percentage ratios of the concentrations, determined using the standard curve of known added concentrations, were calculated. To determine the limits of detection and quantitation, the standard deviation of background peaks determined after injection of approximately 25 blank samples, was multiplied by 3 and 10, respectively. Then, the limit concentrations were determined using the standard curve.

Preparation of rabbit erythrocytes

Blood samples (5 mL) were collected from the ear marginal vein of rabbits, using citrated tubes. After separation of plasma and buffy coat by centrifuging at 600 *g* for 5 min, the erythrocyte fraction was washed three times with phosphate-buffered saline (PBS; NaCl, 150 mmol; KH_2PO_4/K_2HPO_4, 5 mmol L^{-1}).

Enalaprilat-loading of rabbit erythrocytes

The hypotonic pre-swelling method described by Pitt et al (1983b) and extensively evaluated by Tajerzadeh & Hamidi (2000) was used with minor modifications for encapsulation of enalaprilat in rabbit erythrocytes. For this purpose, 8 mL hypotonic K^+-reversed Hank's balanced salt solution (HBSS), with a tonicity 0.67-times that of eutonic solution, was added to 2 mL of washed packed erythrocytes. The K^+-reversed HBSS consisted of (g L^{-1}): KCl, 10.18; KH_2PO_4, 0.1; $NaHCO_3$, 1.273; NaCl, 0.316; Na_2HPO_4, 0.08; and glucose, 2.0. The pH of the solution was adjusted to 7.4 using H_3PO_4 (85%, w/v). The mixture was mixed thoroughly by several gentle inversions and was then centrifuged at 600 *g* for 5 min. The supernatant was discarded and 400 μL of a hemolysate, prepared by diluting a further portion of packed erythrocytes with distilled water (1:1), was poured gently onto the top of the remaining swollen cells. This layer serves both as a reservoir of the intracellular constituents and an osmotic shock barrier for underlying erythrocytes. Then, 500 μL of enalaprilat aqueous solution (8 mg mL^{-1}) was added gently onto the top of the swollen cells and the resulting suspension was inverted several times and centrifuged at 600 *g* for 5 min. The addition of the drug solution and centrifuging steps were repeated once more to bring the cells to the point of lysis, which was detectable by a sudden increase in transparency of suspension and disappearance of the distinct boundary between cells and supernatant on centrifugation. At the point of lysis, the tonicity of the suspension was restored by the rapid addition of 200 μL hypertonic K^+-reversed HBSS, having a tonicity 10-times that of the eutonic solution, followed by gentle mixing of the suspension. The suspension was incubated in a water bath at 37°C for 30 min to reaneal the cells, and the resulting carrier erythrocytes were washed three times with PBS. In some instances, sham-encapsulated erythrocytes were required, and these were prepared as described, except that the enalaprilat aqueous solution was replaced by distilled water.

Loading parameters

Three parameters were determined and used for the evaluation of loading efficiency of enalaprilat into erythrocytes: loaded amount (the amount of enalaprilat encapsulated in 0.1 mL of the final packed erythrocytes); efficiency of entrapment (the percentage ratio of loaded amount of enalaprilat to amount added per 0.1 mL of initial packed cells during the entire loading process); and cell recovery (the percentage ratio of hematocrit value of initial packed cells to that of final loaded cells measured using equal volumes of total suspensions).

Drug administration

A total of 12 rabbits were included in the study in three treatment groups (n = 4). The first group (free drug group) received 1 mL of the enalaprilat solution in K^+-reversed HBSS (2 mg mL^{-1}), injected slowly via the ear marginal vein to each rabbit in the group. For the second group (sham-encapsulation group), 1 mL of the sham-encapsulated erythrocytes from each rabbit in the group was suspended in an equal volume of K^+-reversed HBSS and then re-injected slowly via the ear marginal vein to the same rabbit. For the third group (encapsulated drug group), 1 mL of the drug-loaded erythrocytes from each rabbit in the group was suspended in an equal volume of K^+-reversed HBSS and was re-injected slowly via the ear marginal vein to the same rabbit.

Just before (time 0), and at 1, 12 and 24 h and 2, 4, 8, 15 and 30 days after injection of the drug/erythrocytes to rabbits, 300-μL blood samples were withdrawn from each animal via the ear marginal vein and allowed to stand for 45 min for completion of the coagulation process. The serum fractions were separated by centrifuging at 700 *g* for 15 min. The ACE activity of serum samples was determined and the percentage of serum ACE inhibition was calculated with reference to the activity in the time 0 sample using the formula: ACE inhibition (%) = (ACE activity at the time 0 – ACE activity at the given time/ACE activity at the time 0) × 100

Results and Discussion

Determination of serum ACE activity

Several methods have been reported for the determination of ACE activity (Lieberman 1975; Chiknas 1979; Testoni 1983; Beneteau et al 1986), all of which include the action of ACE on a synthetic substrate, thereby releasing a measurable compound. In this study, a method has been developed using the substrate HHL for determination of ACE activity in rabbit sera. The enzyme action on the substrate resulted in the production of hippuric acid, which was quantitated by a developed HPLC method. The optimum chloride concentration for the activity of ACE in rabbit serum is 300 mM (Ibarra et al 1993), which was considered in the preparation of the reaction mixture in this study. In addition, the pH, temperature and substrate concentration were optimized according to previously reported investigations (Lieberman 1975; Chiknas 1979; Testoni 1983).

A linear relationship was evident between hippuric acid concentrations and the corresponding peak heights over the concentration range from 0.025 to 1 mM (r^2 = 0.999; slope ± s.d. = 79.06 ± 3.09; intercept of regression line = −0.42). The results of validation tests on the method are summarized in Table 1. It can be concluded from the results that the method has an acceptable degree of accuracy, precision and linear response range.

Table 1 Summarized results of validation tests on the method of determination of serum angiotensin-converting enzyme activity.

Parameter	Result
Linear range (mM)	0.025–1.00
Absolute recovery (%)	98.36 (7.14)[a]
Intra-run variations (%)	5.29 (1.34)
Inter-run variations (%)	5.90 (1.05)
Limit of detection (mM)	0.0063
Limit of quantitation (mM)	0.025

[a]Mean (s.d.); n = 6.

Loading parameters

The average loaded amount, entrapment efficiency and cell recovery of the encapsulation procedure was 206.00 ± 11.20 μg, 51.52 ± 2.79% and 68.13 ± 4.30%, respectively. These findings are comparable with results reported for other drugs as well as in other animal species (Pitt et al 1983b; Ropars et al 1987; Talwar & Jain 1992). Furthermore, according to the average loaded amount of drug in 0.1 mL of carrier erythrocytes, 1 mL of the packed carrier cells re-injected into animals contained approximately 2 mg enalaprilat, which, considering the IC50 of the drug, could result in a considerable percentage of ACE inhibition in rabbit serum (Opie 1992).

ACE inhibition profiles

The average baseline serum ACE activity for the rabbits in this study was 8.84 ± 2.63 U L^{-1}. The serum ACE activity exhibited considerable variation among the

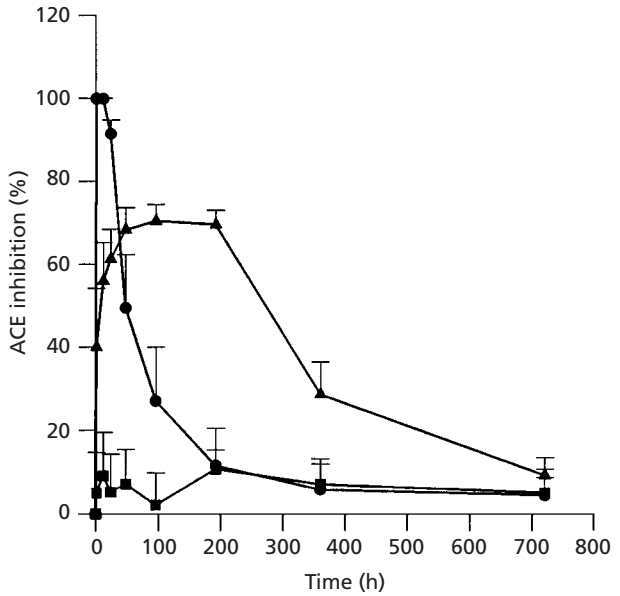

Figure 1 Serum angiotensin-converting enzyme (ACE) inhibition profiles of three groups of rabbits after administration of free enalaprilat, enalaprilat-loaded erythrocytes or sham-encapsulated erythrocytes (n = 4). ●, free enalaprilat; ■, sham-encapsulated erythrocytes; ▲, drug-loaded erythrocytes.

Table 2 Pharmacodynamic parameters of enalaprilat after administration of free and encapsulated enalaprilat (n = 4).

	Free enalaprilat			Encapsulated enalaprilat		
	E_{max} (%)	t_{max} (h)	AUC_{0-720}(% h)	E_{max} (%)	t_{max} (h)	AUC_{0-720} (% h)
Mean	99.96	6.50	10739.74	71.91	144.00	27951.25
s.d.	0.08	6.35	3265.95	3.29	55.43	2160.38
CV%	0.08	97.69	30.41	4.58	38.49	7.73

animals; however, this was not important in this study since the enzyme activity of each rabbit at the different time points was calculated with reference to its own baseline.

The time courses of serum ACE inhibition for the three groups of rabbits, and the pharmacodynamic parameters derived from these profiles, are shown in Figure 1 and Table 2, respectively. From these data, it can be seen that in the rabbits receiving free drug, the percentage of ACE inhibition increased rapidly (t_{max} values in Table 2), followed by a plateau in the curve up to 12 h. Moreover, this curve showed a relatively rapid decreasing trend that approached baseline levels at about Day 8 of the study. This trend was in agreement with the pharmacokinetic profile of enalaprilat and the rate of reconstitution of serum ACE activity (Kubo & Cody 1985; Belz et al 1988). In the case of rabbits receiving encapsulated drug, the enzyme inhibition profile exhibited a slower increase up to 2 days, with a more extended plateau up to Day 8, and a pronounced slower reconstitution rate of ACE activity. In addition, the maximum percentage inhibition was lower in the case of encapsulated drug (E_{max} values in Table 2). The other important parameter was the area under the ACE inhibition–time curve over the entire course of study (AUC_{0-720}), a measure of the total extent of enzyme inhibition throughout the sampling period. As shown in Table 2, the AUC_{0-720} was remarkably higher (approx. 3-times) in the case of encapsulated drug, indicating that the overall efficiency of the drug therapy was greater in this case. Furthermore, the inter-animal variation of this parameter was lower in the case of encapsulated drug, reflecting a more reproducible therapeutic outcome of the drug in this group. As already mentioned, the serum ACE inhibition profile can be regarded as an indirect measure of serum concentration profile of enalaprilat with E_{max}, t_{max} and AUC values being comparable with C_{max}, t_{max} and AUC, respectively.

Since enalaprilat remains within the erythrocytes until the point of cell lysis in the circulation (preliminary in-vitro release studies), the time course shown in Figure 1 is representative of the remarkably long life-span of carrier erythrocytes in the circulation. The inhibition profile depicted for the sham-encapsulated group showed that injection of the processed erythrocytes had no significant effect on the serum ACE activity, and that the inter-day variation of the baseline serum ACE activity of rabbits was not considerable during the course of the study. Considering these findings, it is evident that carrier erythrocytes can serve as reservoirs of enalaprilat in the circulation, as has been reported to be responsible for the modified in-vivo drug action in several studies (Jenner et al 1981; Pitt et al 1983a; Field et al 1989; Garin et al 1996).

References

Alpar, H. O., Lewis, D. A. (1985) Therapeutic efficacy of asparaginase encapsulated in intact erythrocytes. *Biochem. Pharmacol.* **34**: 257–261

Belz, G. G., Kirch, W., Kleinbloesem, C. H. (1988) Angiotensin-converting enzyme inhibitors, relationship between pharmacodynamics and pharmacokinetics. *Clin. Pharmacokinet.* **15**: 295–318

Beneteau, B., Baudin, B., Morgant, G., Giboudean, J., Bauman, F. C. (1986) Automated kinetic assay of angiotensin-converting-enzyme in serum. *Clin. Chem.* **32**: 884–886

Chiknas, S. G. (1979) A liquid chromatography-assisted assay for angiotensin converting enzyme (peptidyl dipeptidase) in serum. *Clin. Chem.* **25**: 1259–1262

Eichler, H. G., Gasic, S., Bauer, K., Korn, A., Bacher, S. (1986) In vivo clearance of antibody-sensitized human drug carrier erythrocytes. *Clin. Pharmacol. Ther.* **40**: 300–303

Field, W. N., Gamble, M. D., Lewis, D. A. (1989) A comparison of treatment of thyroidectomized rats with free thyroxine and thyroxine encapsulated in erythrocytes. *Int. J. Pharm.* **51**: 175–178

Garin, M. I., Lopez, R. M., Sanz, S., Pinilla, M., Luque, J. (1996) Erythrocytes as carriers for recombinant human erythropoietin. *Pharm. Res.* **13**: 869–874

Ibarra, R. M., Gomez, V. L. A., Cruz, C., Pedraza, C. J. (1993) Effect of chloride and diamide on serum angiotensin converting enzyme activity from eight mammalian species. *Comp. Biochem. Physiol. C.* **106**: 495–501

Ip, D. P., Brenner, G. S. (1987) Enalapril maleare. In: Florey, K. (ed.) *Analytical Profiles of Drug Substances*. Vol. 16. Academic Press, London, pp 207–243

Jackson, E. K., Garrison, J. C. (1996) Renin and angiotensin. In: Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W. (eds) *The Pharmacological Basis of Therapeutics*. 9th edn. McGraw-Hill, New York, pp 733–758

Jenner, D. J., Lewis, D. A., Pitt, E., Offord, R. A. (1981) The effect of intravenous administration of corticosteroids encapsulated in intact erythrocytes on adjuvant arthritis in the rat. *Br. J. Pharmacol.* **73**: 212P–213P

Kinosita, K., Tsong, T. Y. (1978) Survival of sucrose-loaded erythrocytes in circulation. *Nature* **272**: 258–260

Kravtzoff, R., Ropars, C., Laguerre, M., Muh, J. P., Chassaigne, M. (1990) Erythrocytes as carriers for L-asparaginase: methodological and mouse in-vivo studies. *J. Pharm. Pharmacol.* **42**: 473–476

Kubo, S. H., Cody, R. J. (1985) Clinical pharmacokinetics of the angiotensin converting enzyme inhibitors. *Clin. Pharmacokinet.* **10**: 377–391

Lewis, D. A. (1984) Red blood cells for drug delivery. *Pharm. J.* **233**: 384–386

Lewis, D. A., Alpar, H. O. (1984) Therapeutic possibilities of drugs encapsulated in erythrocytes. *Int. J. Pharm.* **22**: 137–146

Lieberman, J. (1975) Elevation of serum angiotensin-converting enzyme (ACE) level in sarcoidosis. *Am. J. Med.* **59**: 365–372

Opie, L. H. (1992) *Angiotensin-Converting Enzyme Inhibitors: Scientific Basis for Clinical Use*. John Wiley & Sons, New York, pp 149–193

Pitt, E., Lewis, D. A., Offord, R. (1983a) The use of corticosteroids encapsulated in erythrocytes in the treatment of adjuvant induced arthritis in the rat. *Biochem. Pharmacol.* **132**: 3355–3358

Pitt, E., Johnson, C. M., Lewis, D. A., Jenner, D. A., Offord R. (1983b) Encapsulation of drugs in intact erythrocytes: an intravenous delivery system. *Biochem. Pharmacol.* **132**: 3359–3368

Ropars, C., Chassaigne, M., Nicoulau, C. (1987) Red blood cells as carriers for drugs. In: Price, J., Calborne, J. (eds) *Advances in the Biosciences*. Vol. 67. Pergamon, Oxford

Summers, M. P. (1983) Recent advances in drug delivery. *Pharm. J.* **230**: 643–645

Tajerzadeh, H., Hamidi, M. (2000) Evaluation of hypotonic preswelling method for encapsulation of enalaprilat in human intact erythrocytes. *Drug Dev. Ind. Pharm.* **26**: 1247–1257

Talwar, N., Jain, N. K. (1992) Erythrocytes as carriers of primaquin-preparation: characterization and evaluation. *J. Control. Rel.* **20**: 133–142

Testoni, S. R. (1983) Direct spectrophotometric assay of angiotensin-converting enzyme in serum. *Clin. Chem.* **29**: 1093–1096

Updike, S. J., Wakamiya, R. T. (1983) Infusion of red blood cell-loaded asparaginase in monkey. *J. Lab. Clin. Med.* **101**: 679–691

Updike, S. J., Wakamiya, R. T., Lightfoot, E. N. (1976) Asparaginase entrapped in red blood cells: action and survival. *Science* **193**: 681–683