Thermochimica Acta, 193 (1991) 287–297
Elsevier Science Publishers B.V., Amsterdam

Flow microcalorimetry for human erythrocyte hemolysis induced by ionic drug binding

Magobei Yamamoto and Hatsumi Aki

Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-Ku, Fukuoka 814-01 (Japan)

(Received 7 December 1990)

Abstract

The interactions of the ionic drugs chlorpromazine (CPZ), promethazine (PMZ), flufenamic acid (FA) and mefenamic acid (MA) with human erythrocyte membranes were compared by flow microcalorimetry, with respect to hemolytic activity and membrane uptake. Cationic drugs such as CPZ and PMZ bound to and/or penetrated the intramembranes, with small negative enthalpy and large positive entropy changes arising from hydrophobic interactions, and then induced hemolysis immediately as a result of drug binding, forming mixed micelles with the membrane lipids and disrupting the membrane structure. Anionic drugs such as FA and MA had two kinds of binding sites on the membrane surface; one strong binding site with large negative enthalpy and positive entropy changes, and some weak sites with a higher capacity and small negative entropy change, reflecting not only hydrophobic interaction but also ionic and van der Waals interactions. The first class of binding sites was already saturated when these drugs caused hemolysis.

INTRODUCTION

Ionic drugs frequently stabilize erythrocytes against hypotonic hemolysis at lower concentrations, cause hemolysis at higher concentrations [1–5], and change the shape of the cells. Phenothiazines (as cationic drugs) give rise to the stomatocytic (cupped) erythrocytes whereas non-steroidal anti-inflammatory drugs (as anionic drugs) generally produce echinocytic (crenated) cells [6–10], at which erythrocyte hemolysis is caused by the drug interaction [7,8,11–16]. For a better understanding of the hemolysis, more information on the interaction between drugs and erythrocyte cells over a large range of drug concentrations is required.

We have recently studied drug interactions with blood components by using differential flow calorimetry [17–20], a useful technique to determine the stoichiometry and nature of the reactions. As optically clear solutions are not required, a large concentration range of materials or suspensions can be measured without any further destruction and separation of bound and free fractions. In the present study, we investigated the thermodynamic

characteristics of drug-induced hemolysis, which we expected to be different for the anionics flufenamic acid and mefenamic acid, and the cationics chlorpromazine and promethazine. Furthermore, comparable studies on the drug binding action, cell uptake, and the effect of these drugs on the membrane structure were performed.

EXPERIMENTAL

Materials

Chlorpromazine hydrochloride (CPZ), promethazine hydrochloride (PMZ), flufenamic acid sodium salt (FA) and mefenamic acid sodium salt (MA) were purchased from Sigma (USA). [^{14}C]CPZ (50 mCi mmol^{-1}) and [^{14}C]FA (55 mCi mmol^{-1}) were obtained from Amersham International (UK) and Commissariat á L'Energie Atomique (France), respectively. Human erythrocytes, donated by Fukuoka Red Cross Blood Center (Japan), were washed with isotonic sodium phosphate buffer solution (PBS) (NaCl 90.0 g l^{-1}, $NaH_2PO_4 \cdot 2H_2O$ 3.43 g l^{-1} and $Na_2PO_4 \cdot 12H_2O$ 34.425 g l^{-1}; pH 7.4), and resuspended in the same buffer as a stock solution. Before each use, erythrocytes were washed a few times with PBS until the supernatant was clear and colorless, to obtain packed cells with a 100% erythrocyte concentration. Hemoglobin-free erythrocyte ghosts were prepared according to the procedure of Dodge et al. [21]. Protein concentration in the ghost solution was determined by the method Lowry et al. [22] with human serum albumin (Sigma, HSA Fraction V) as a standard. The number of erythrocytes or ghosts in each experimental suspension was measured by a Model TA-2 Coulter Counter (USA).

Microcalorimetry

Calorimetric measurements were carried out at 37 °C for a differential flow microcalorimeter. The instrument was calibrated both electrically and chemically. The procedures used in the flow experiments (Fig. 1) have been described in detail elsewhere [23]. The binding heat of drug to intact erythrocytes was measured using mode (1) because of the high viscosity and the large dilution heat of the erythrocyte suspension (E). The heat effect during hemolysis was continuously measured using mode (2). A base-line was established with erythrocyte suspension and PBS solution, and then drug solution was added to the suspension by a microsyringe. The signal during erythrocyte hemolysis corresponded to the heat of dilution of hemoglobin released from the erythrocyte cells. These differential measurements could eliminate the heat of dilution and the thermal effect of friction and turbulence of the flowing solutions.

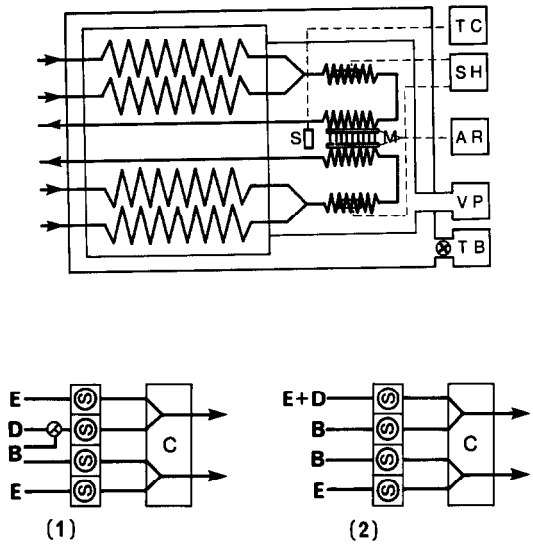

Fig. 1. Schematic design of flow calorimeter and experimental modes of calorimetric measurements. Top: flow calorimeter: TC, temperature controller; SH, standard heat generator; AR, amplifier recorder; VP, vacuum pump; TB, bath thermostat; S, thermometer; M, thermopile. Bottom: experimental modes: C, calorimeter; ⓢ, peristaltic pump; B, buffer; D, drug; E, erythrocyte.

Processing the binding data

The heat of drug binding to the erythrocyte membrane was measured as a function of drug concentration (D_t) when the concentration of the ghost suspension was constant at 40% (v/v). The reaction heat was proportional to the recorded steady-state value, which was equal to the sum of the heat of dilution of the components and the heat of reaction. The heat of dilution of the ghost solution can be instrumentally eliminated. After subtraction of the heat of dilution of the drug measured separately, the experimental heat of reaction rate ϕ is proportional to the quantity of drug–protein complex formed with the protein concentration fixed at P_t

$$\phi = \Delta H D_b F \tag{1}$$

where ΔH is the binding enthalpy per mole of a drug and D_b is the bound concentration of the drug at a constant flow rate F. The equilibrium aspect of such interactions was correlated through the mass law, yielding the familiar equation

$$D_b = P_t\left(\sum_{i=1}^{m} n_i K_i D_f\right)/(1 + K_i D_f) \tag{2}$$

where m is the number of classes of independent binding sites such that

each class (i) has n_i sites with binding affinity K_i, and D_f is the free drug concentration, given by the equation

$$D_t = D_b + D_f \tag{3}$$

In the simplest case of drug binding where only one class of binding sites is involved ($m = 1$), eqn. (1) can be expressed as a function of D_t by use of eqns. (2) and (3), as follows:

$$\phi = \Delta HF\left[A - \sqrt{\left(A^2 - 4n_1P_tD_t\right)}\right]/2 \tag{4}$$

where

$$A = (1/K_1) + n_1P_t + D_t \tag{5}$$

For a two-class binding model ($m = 2$), eqn. (2) can be expressed in the form

$$(D_t - D_f)/P_t = \left[n_1K_1/(1 + K_1D_f)\right] + \left[n_2K_2/(1 + K_2D_f)\right] \tag{6}$$

This expression yields

$$D_f^3 + AD_f^2 + BD_f + C = 0 \tag{7}$$

where

$$A = P_t(n_1 + n_2) + (1/K_1) + (1/K_2) - D_t$$

$$B = P_t\left[(n_1/K_2) + (n_2/K_1)\right] - D_t\left[(1/K_1) + (1/K_2)\right] + (1/K_1)(1/K_2)$$

$$C = -D_t(1/K_1)(1/K_2) \tag{8}$$

The value of D_f can be found by solving the cubic eqn. (7). The binding and thermodynamic parameters K_i, n_i and ΔH were computed from actual measurements with a computer program for the non-linear least-squares regression method. An initial value of ΔH was estimated from the slope of the initial linear plots of the titration curve. The program was run on a FACOM M-380R digital computer [19].

Drug-induced hemolysis

An aliquot of 0.2 ml of erythrocyte suspension was added to 9.8 ml of drug solution at various concentrations and mixed immediately. The mixture was incubated for 90 min at 37 °C, and after centrifugation at 1000g for 10 min, the absorbance $E^{1\,\mathrm{cm}}_{543\,\mathrm{nm}}$ of hemoglobin in the supernatant was determined. The per cent hemolysis is expressed by comparison with complete hemolysis of erythrocytes in water.

Drug uptake by erythrocyte membranes

Two milliliters of 20% ghost suspension was incubated with varying concentrations of FA and CPZ for 30 min at 37 °C (containing 0.2 μCi ml^{-1}

each of labeled compound). At the end of the incubation, ghost cells were washed with isotonic saline four times by repeated centrifugation (1000*g* for 30 min) at room temperature. At the end of the last washing, the ghost cells were hemolyzed in 5 volumes of distilled water. One milliliter of hemolysate was digested in 1.5 ml of Soluene-350–iso-propanol (1 : 1, v/v), and the mixture was allowed to stand at room temperature for 10 min, then warmed to 40 °C for 20 min. After addition of 15 ml of Insta-Gel–0.5 M HCl (9 : 1, v/v) mixture and vigorous shaking, the sample was ready for counting, after temperature and light equilibration, using the liquid scintillation system. The radioactivities were measured by a Packard Tri-Carb 2660 liquid scintillation counter (USA). The amount of drug uptake by ghost membranes was estimated from the radioactivities in the membranes.

RESULTS AND DISCUSSION

Heat effect of drug-induced hemolysis

Hemolytic profiles of the heat effect (upper) and per cent hemolysis in the calorimetric solution (lower) during incubation of the 4% (v/v) erythrocyte suspension with 2.0 mM FA and 1.0 mM CPZ, as a typical example, are shown in Fig. 2. Hemolysis induced by CPZ was rapid and complete within several minutes, and the heat effect increased endothermically with the

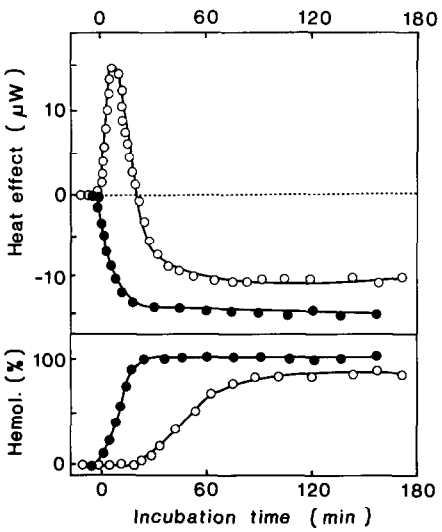

Fig. 2. Time courses of heat effect (upper) and per cent hemolysis of the calorimetric solution (lower) during human erythrocyte hemolysis induced by drugs at 37 °C. Human erythrocytes in 4% (v/v) suspensions were incubated with 1.0 mM CPZ (●) and 2.0 mM FA (○). The percentage of hemolysis was expressed as the ratio of the absorbance at 543 nm in the final calorimetric solution to the absorbance after complete hemolysis in water.

degree of hemolysis. However, a different pattern was observable in FA-induced hemolysis. There was a lag phase of about 30 min and then hemolysis gradually resulted. It took more than 60 min to cause complete hemolysis. The calorimetric profile observed during hemolysis showed two stages. The first stage was characterized as an extremely exothermic process and after 30 min the second one proceeded to an endothermic process. The endothermic heat effect of hemolysis was proportional to the quantity of free hemoglobin released from the erythrocyte cells.

Heat effect of drug binding to erythrocyte membranes

To investigate the interaction between drugs and erythrocytes, the heat of reaction between drugs and erythrocyte ghost membranes was measured as a function of drug concentration. The results are shown in Fig. 3. The calorimetric titration curves of ghosts with CPZ and PMZ were divided clearly into three stages. The first stage of titration (up to about 0.2 mM of CPZ and 0.1 mM of PMZ) was exothermic and the heat of binding increased with increasing drug concentration. In the second stage (titration in the range 0.2–0.7 mM of CPZ and 0.1–0.4 mM of PMZ), the heat effect reached a plateau and then reversed to an endothermic process in the third stage (over 0.7 mM of CPZ and 0.4 mM of PMZ). This suggests that CPZ and PMZ induce not only the ghost binding reaction but also other reactions such as conformation changes and/or destruction of membranes, whereas

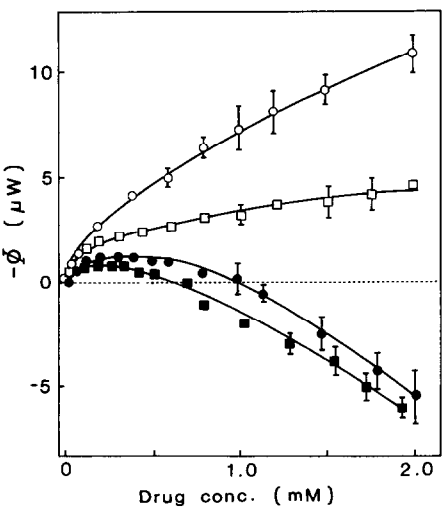

Fig. 3. Calorimetric titration curves of drug binding to erythrocyte ghosts at pH 7.4 and 37°C. The initial concentrations of ghosts were prepared in 40% (v/v) suspension and the protein concentrations in the final calorimetric solutions were in the range $(2.029–2.111)\times10^{-5}$ M. Each point shows the mean value of three measurements. ●, CPZ; ■, PMZ; ○, FA; □, MA.

TABLE 1

Binding and thermodynamic parameters for drug binding to human erythrocyte ghosts at 37 °C

Drug	Class	K (10^4 M^{-1})	n	N [a]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
CPZ	1	5.959	2.5	0.381	−5.774	−28.35	72.8
PMZ	1	1.180	2.2	0.349	−4.602	−24.17	63.2
FA	1	11.07	0.9	0.165	−20.85	−29.94	28.9
	2	0.1014	6.4	1.158		−17.84	−9.6
MA	1	2.991	1.2	0.296	−18.28	−26.56	26.8
	2	0.1826	10.0	1.719		−19.36	−3.3

[a] N, Number of drug molecules bound to all binding sites of a ghost cell (10^{-16} mol per cell).

the calorimetric titration curves of FA and MA were monophasic. The heat of binding increased exothermically with the increasing concentration of drugs and did not reach a plateau within the range of concentrations used.

Calorimetric data for CPZ and PMZ clearly fit the one-class binding model, and FA and MA fit the two-class binding model. The most probable values of the binding and thermodynamic parameters are listed in Table 1. Free energy changes (ΔG) were calculated from the K values in the table, according to the equation $\Delta G = -RT \ln K$, where R is the gas constant and T is the temperature in Kelvin. The entropy changes (ΔS) were then calculated using the relationship $\Delta S = (\Delta H - \Delta G)/T$. CPZ and PMZ bound to only one class of binding sites ($n = 2$–3) with a binding affinity of the order of 10^4 M^{-1}. The binding was characterized by small negative ΔH and large positive ΔS, reflecting hydrophobic interactions between drug and lipid bilayers and/or hydrophobic membrane proteins. On the other hand, FA and MA were bound to two classes of membrane binding sites. The first class of binding sites, with a high affinity ($K_1 = 10^4$–10^5 M^{-1}) and a low capacity ($n_1 = 1$), was characterized by large negative ΔH and positive ΔS values. The second class of sites had a lower affinity, K_2, of the order of 10^3 M^{-1} and a higher capacity ($n_2 = 6$–10) with negative ΔS, indicating that not only hydrophobic interaction but also other forces such as ionic interaction and van der Waals interaction contribute to the binding process of anionic drugs.

Relationship between hemolytic activity and binding activity of drugs to ghost membranes

Figure 4 shows the hemolytic activities of drugs on human erythrocytes in isotonic solution. The initial concentration at which hemolysis was initiated (C_1), determined from the intercept obtained by extrapolating the plot to 0% hemolysis, and the concentration inducing 50% hemolysis (C_{50}) are listed in Table 2.

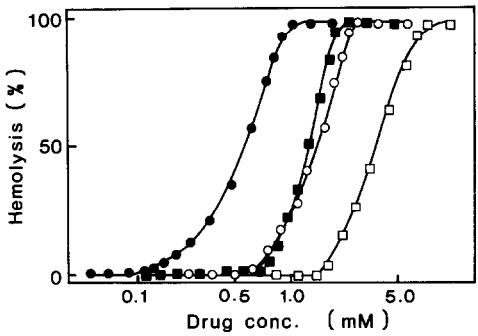

Fig. 4. Hemolytic effect of drugs on human erythrocytes. The initial concentration of erythrocyte suspensions was 2% (v/v) in PBS solution at pH 7.4 and 37 °C. (For key to drugs see Fig. 3 legend.)

As the erythrocytes have an age distribution, the amount of drug bound to the membrane was compared with hemolytic concentrations at C_1 and C_{50}. The amount of bound drug per ghost cell (r_1 at C_1 and r_{50} at C_{50}) was calculated from calorimetric data by using eqn. (1). The ratios of r_1 and r_{50} to the total amount of drug bound to all binding sites of a cell (N) (i.e. r_1/N and r_{50}/N), indicating the degree of saturation of binding sites, were obtained and are shown in Table 2. CPZ and PMZ show the same degree of saturation at the binding sites as at the concentration that induced hemolysis. However, with FA and MA, the first class of binding sites was already saturated when hemolysis occurred but the degree of saturation at the second class increased with increasing hemolysis, indicating that the second site plays an important role in hemolysis.

Comparison of uptaken drugs taken up by erythrocyte membranes

To determine whether drug uptake by erythrocytes was due to a penetration of the drug into the membrane or to bound accumulation of drug

TABLE 2

Hemolytic concentrations and amount of bound drug per ghost cell at 37 °C [a]

Drug	class	C_1 (mM)	r_1	r_1/N (%)	C_{50} (mM)	r_{50}	r_{50}/N (%)
CPZ	1	0.25	0.280	73.5	0.52	0.293	76.9
PMZ	1	0.84	0.250	71.6	1.27	0.252	72.2
FA	1	0.70	0.163	98.7	1.45	0.164	99.4
	2		0.459	39.6		0.674	58.2
MA	1	1.70	0.290	98.1	1.70	0.293	99.0
	2		1.264	73.6		1.469	85.4

[a] C_1 and C_{50}, concentrations inducing hemolysis initially and 50% hemolysis, respectively; r_1 and r_{50}, amounts of bound drug per ghost cell at C_1 and C_{50}, respectively (10^{-16} mol per cell).

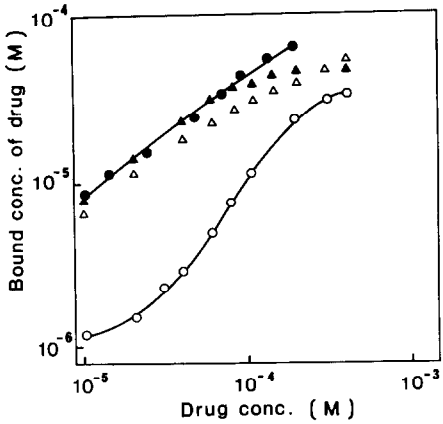

Fig. 5. Uptake of drugs by erythrocyte ghost cells at pH 7.4 and 37 °C. Circles and triangles show the amounts obtained from the ghost cell uptake experiment using labeled drug as a tracer, and the membrane-bound drug concentration calculated from calorimetric data using the parameters given in Table 1, respectively. ● and ▲, CPZ; ○ and △, FA.

molecules on the membrane, the concentrations of the drug taken up and the bound drug concentrations were compared for CPZ and FA. Results are shown, as a function of initial drug concentration, in Fig. 5. Up to a concentration of 0.08 mM, the membrane uptake of CPZ was almost equal to the amount of bound CPZ calculated from the calorimetric data by using eqn. (2), indicating that CPZ was bound to and/or penetrated the membrane [24], whereas it decreased at higher concentration, possibly as a result of formation of mixed micelles with membrane lipids [25], resulting in the hemolytic or disruptive effect.

FA was less penetrative than CPZ at the same concentration, and the membrane uptake of FA was only 10–30% of the amount bound to the ghosts. It was evident that FA was bound to and accumulated on the surface of the membrane rather than in a pocket. The binding sites of FA may exhibit a positively charged group(s) close to the hydrophobic surface with electron-donor groups on the erythrocyte membrane such as band 3 protein [26]. Thus, upon binding, the negative charge of the carboxylate anion of the drug is neutralized by a cationic locus on the protein; the complexed anion behaves then as a neutral molecule with respect to hydrophobic interactions.

REFERENCES

1 P. Seeman and J. Weinstein, I. Erythrocyte membrane stabilization by tranquilizers and antihistamines. Biochem. Pharmacol., 15 (1966) 1737–1752.
2 J. Dausset and L. Contu, Drug-induced hemolysis. Annu. Rev. Med., 18 (1967) 55–70.
3 A.D. Inglot and E. Wolna, Reactions of non-steroidal anti-inflammatory drugs with the erythrocyte membrane. Biochem. Pharmacol., 17 (1968) 269–279.

4 J. Santos-Martinez, T.A. Aviles and J.A. Laboy-Torres, The hemolytic effect of some phenothiazine derivatives in vitro and in vivo. Arch. Int. Pharmacodyn. Ther., 196 (1972) 83–92.
5 Y. Mizushima, S. Sasaki and M. Yamamura, Mode of stabilizing action of non-steroid anti-inflammatory drugs on erythrocyte membrane. Biochem. Pharmacol., 19 (1970) 227–234.
6 B. Deuticke, Transformation and restoration of biconcave shape of human erythrocytes induced by amphiphilic agents and changes of ionic environment. Biochim. Biophys. Acta, 163 (1968) 494–500.
7 M.P. Sheetz and S.J. Singer, Biological membranes as bilayer couples. A molecular mechanism of drug–erythrocyte interactions. Proc. Natl. Acad. Sci. U.S.A., 71 (1974) 4457–5561.
8 N. Mohandas and C. Feo, A quantitative study of the red cell shape changes produced by anionic and cationic derivatives of phenothiazine. Blood cells, 1 (1975) 375–384.
9 T. Fujii, T. Sato, A. Tamura, M. Wakatsuki and Y. Kanaho, Shape changes of human erythrocytes induced by various amphipathic drugs acting on the membrane of the intact cells. Biochem. Pharmacol., 28 (1979) 613–620.
10 A. Nwafor and W.T. Coakley, Charge-independent effects of drugs on erythrocyte morphology. Biochem. Pharmacol., 34 (1985) 3329–3336.
11 I.P. Lee, A possible mechanism of ellipticine-induced hemolysis. J. Pharmacol. Exp. Ther., 196 (1976) 535–535.
12 G. Zorgafi and M.V. Munshi, Effect of chemical modification on the surface activity of some phenothiazine derivatives. J. Pharm. Sci., 59 (1970) 819–822.
13 D. Papahadjopoulos, Studies on the mechanism of action of local anesthetics with phospholipid model membranes. Biochim. Biophys. Acta, 265 (1972) 169–189.
14 T. Ogiso, M. Iwaki and K. Mori, Fluidity of human erythrocyte membrane and effect of chlorpromazine on fluidity and phase separation of membrane. Biochim. Biophys. Acta, 649 (1981) 325–335.
15 G.V.R. Born and G.M. Housley, Effects of modification of the membranes of intact erythrocytes on the anti-haemolytic action of chlorpromazine. Br. J. Pharmac., 79 (1983) 481–487.
16 M.R. Lieber, Y. Lange, R.S. Weinstein and T.L. Steck, Interaction of chlorpromazine with the human erythrocyte membrane. J. Biol. Chem., 259 (1984) 9225–9234.
17 H. Aki and M. Yamamoto, Biothermodynamic characterization of erythrocyte hemolysis induced by phenothiazine derivatives and anti-inflammatory drugs. Biochem. Pharmacol., 39 (1989) 396–398.
18 H. Aki and M. Yamamoto, Drug binding to human erythrocytes in the process of ionic drug-induced hemolysis. Flow microcalorimetric approaches. Biochem. Pharmacol., 41 (1990) 133–138.
19 H. Aki and M. Yamamoto, Thermodynamics of the binding of phenothiazines to human plasma, human serum albumin and α_1-acid glycoprotein: a calorimetric study. J. Pharm. Pharmacol., 41 (1989) 674–679.
20 H. Aki and M. Yamamoto, Microcalorimetric study for the binding of ionic drugs to human erythrocytes and the ghost membranes. J. Pharm. Pharmacol., 42 (1990) 637–641.
21 J. T. Dodge, C. Mitchell and D.J. Hanahan, The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch. Biochem. Biophys., 100 (1963) 119–130.
22 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, Protein measurement with folin phenol reagent. J. Biol. Chem., 193 (1951) 265–275.
23 M. Yamamoto and H. Aki, Application of differential flow microcalorimetry for study of drug interactions in the blood system. J. Biochem. Biophys. Methods, 16 (1988) 271–282.
24 M.J. Conrad and S.J. Singer, The stability of amphipathic molecules in biological

membranes and lipid bilayers and its implications for membrane structure. Biochemistry, 20 (1981) 808–818.

25 M. Luxnat and H.J. Gralla, Partition of chlorpromazine into lipid bilayer membranes: the effect of membrane structure and composition. Biochim. Biophys. Acta, 856 (1986) 274–282.

26 J.L. Cousin and R. Motais, Inhibition of anion transport in the red blood cell by anionic amphiphilic compounds. II. Chemical properties of the flufenamate-binding site on the band 3 protein. Biochim. Biophys. Acta, 687 (1982) 147–155, 156–164.