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A critical analysis of microcalorimetric methodology for drug-induced haemolysis

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Abstract

The present work reports a study on the interaction of three drugs, trifluoperazine (TFP), dibucaine (DBC) and praziquantel (PZQ), with erythrocyte membranes. Drug–erythrocyte interaction was studied by measuring the drug partition coefficient between membrane and water, and also by monitoring their haemolytic activity. Microcalorimetric techniques were employed to study drug-induced haemolysis. TFP presented the highest membrane partition and best haemolytic efficacy, followed by DBC and PZQ. The heat effects of drug-induced haemolysis obtained by using a thermal activity monitor (TAM) were −18.2 and −3.6 pJ/cell for TFP and DBC, respectively. We were unable to obtain measurable heat effect for PZQ-induced haemolysis. The discrepancy in heat effect values of drug-induced lysis indicates that different pathways are involved in haemolysis establishment. Misunderstandings concerning the effect of drug-induced haemolysis, found in recent literature, lead us to present a critical analysis concerning microcalorimetric methodology and a comparison between the heat effect values obtained in this work with the values available in literature.

Keywords: Trifluoperazine; Dibucaine; Praziquantel; Erythrocytes; Haemolysis; Partition coefficient; Microcalorimetry

1. Introduction

The erythrocyte (red blood cell) membrane is the most studied biological membrane since it can be copiously obtained by venous puncturing and also easily isolated by low speed centrifugation. Consequently, the erythrocyte membrane has become the most used model for drug–membrane interaction and has provided a large quantity of information of clinical interest.

Haemolysis is the disruption of the red cell which can be caused by the interaction of chemical compounds with the membrane. Many substances have been described as haemolytic agents, including important groups such as anaesthetics, anti-inflammatory and neuroleptic drugs [1,2].

The effect of a drug on the erythrocyte membrane can be attributed to two main steps: (i) the entrance into the bilayer and (ii) the intensity of the membrane-perturbing action of the molecule once incorporated

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[3]. Some factors are considered determinant in haemolysis establishment: the compound's hydrophobicity, the presence of an ionizable group on the drug and the size of the compound.

Although drug-induced haemolysis has been studied since 1937 [4], the nature of drug–membrane interaction is not completely elucidated. We have been studying haemolysis caused by drugs in order to discriminate the different possible pathways that culminate in cell lysis [5–7]. The comparative analysis of drug partition coefficients between different membrane systems and water can discriminate possible drug interaction with specific components of the membrane (lipid or protein).

In the present report, we have used a pure lipid phase (liposomes), a membrane with a high content of protein (70%) (microsomes) and the erythrocyte membrane to compare the drug partition coefficients between them and water. We also have investigated the different drug membrane interaction by monitoring their haemolytic activity. The drugs studied are trifluoperazine (TFP), a potent antipsychotic agent, dibucaine (DBC), a local anaesthetic, and praziquantel (PZQ), an antihelmintic compound.

Microcalorimetry is a useful tool that has been increasingly employed in medical and biological areas [8]. In this work we observed significant differences between heat effect values of drug-induced lysis. These differences indicate that different pathways are involved in haemolysis establishment, which are consistent with other haemolytic parameters (kinetic, lytic efficacy and partition coefficient).

However, besides erythrocyte metabolism (basal erythrocyte heat production), which has been calorimetrically studied by certain research groups [9–14], there are very few reports in the literature describing thermodynamic parameters in drug–erythrocyte interactions. These few published results present some conceptual contradictions: Aki and Yamamoto [15] calorimetrically measured the drug–erythrocyte interaction denoting the endothermic heat effect obtained as enthalpy of haemoglobin released from one cell (ΔH hemol), while in their next article [16], they call the same process as heat effect of haemoglobin release. Although in module, the heat effect values presented in this report are equal to Aki and Yamamoto [16], the signal is the opposite (exothermic). Since our results are not in agreement with some recent literature data cited above [15,16], we also present some methodological considerations based on our experimental work that can be useful for subsequent work.

2. Materials and methods

TFP hydrochloride, DBC dihydrochloride and egg phosphatidylcholine (EPC) were obtained from Sigma, St. Louis, MO. PZQ was donated by Merck SA Indústrias Químicas, RJ, Brazil.

2.1. Membrane preparation

Mouse liver microsomes were prepared as described previously [5].

EPC multilamellar vesicles were prepared by evaporating stock chloroform EPC solutions under a stream of wet nitrogen. The samples were left under vacuum for at least 2 h. Vesicles were obtained by the addition of phosphate buffered saline, PBS (0.15 M NaCl, 5 mM phosphate, pH 7.4), and vortexing for 5 min.

Erythrocytes. Freshly obtained mouse blood was collected into Alsever's solution (27 mM sodium citrate/72 mM NaCl/114 mM glucose/2.6 mM citric acid) and washed three times in PBS.

Erythrocyte ghost membranes were prepared as described in [17].

Protein determination. Membrane protein concentration was measured using bovine serum albumin as a standard [18].

Phospholipid determination. Membrane phospholipid concentration was determined according to Ref. [19].

2.2. Partition coefficient (P) determination by phase separation

Membranes were prepared as described above and kept in PBS. A known amount of drug was incubated with the membranes for 10 min at room temperature. The drug concentration remaining in the supernatant after centrifugation at $105\,000 \times g$ for 1 h was optically detected against the respective control (membrane in PBS). The drug absorbance was measured at 256 nm (ϵ_M=23 500) for TFP [19], at 324 nm (ϵ_M=3770) for

DBC (experimental data) and 260 nm (ϵ_M=320) for PZQ (experimental data). For erythrocytes, ghosts were used rather than whole cells because haemoglobin released during the experiment overlapped with the optical spectra of the drugs, preventing their precise determination in the supernatant. The amount of drug bound to the apolar phase was obtained by subtracting the supernatant concentration from the total drug concentration measured before phase mixing [20]. The partition coefficient, *P*, was determined according to Eq. (1)[21]:

$$P = \frac{n_m(s)/V_m}{n_w(s)/V_w}, \quad (1)$$

where s denotes the solute (TFP, DBC or PZQ), *n* is the number of moles of solute, *V* is the volume and the subscripts m and w refer to the membrane and aqueous phase, respectively. For erythrocytes, ghosts and microsomes, the apolar phase volume (V_m) was calculated assuming a lipid density of 1 g/ml [22–24]. For EPC multilamellar vesicles, *P* was determined after four freeze-thawing cycles, increasing the incubation time up to 30 min.

2.3. Haemolytic assay

TFP (1–400 μM) and PZQ (0.01–20 mM) previously dissolved in dimethylsulphoxide, DMSO, with a maximal DMSO concentration of 5%, were prepared in PBS, pH 7.4. DBC (0.1–10 mM) was dissolved in PBS, pH 6.8, in order to increase its solubility. Erythrocytes (1% haematocrit) were added and the samples were kept at room temperature (22–25°C) until total lysis occurred. The incubation time was 10 min for TFP, 30 min for DBC and 90 min for PZQ. The samples were then centrifuged at 260×*g* for 3 min and the haemoglobin released into the supernatant was detected at 540 nm.

The haemolytic effect, measured as percent relative haemolysis (RH), was determined on the basis of released haemoglobin, according to the following formula:

$$RH = \frac{A_s - A_{c1}}{A_{c2} - A_{c1}}, \quad (2)$$

where *A* means absorbance, s denotes the sample, c1, the mechanical haemolysis control (erythrocytes in PBS), and c2, the 100% haemolysis (erythrocytes in water).

2.4. Microcalorimetric assay

Heat of interaction between drugs and erythrocyte membranes were carried out in a model 2277 thermal activity monitor (TAM) system (Thermometric AB, Sweden), which consists of four independent isothermal heat-conduction microcalorimeters [25]. The heat flow signal (dq/dt) in μW was monitored as a function of time. The TAM microcalorimeter was previously calibrated using the protonation reaction of tris-(hydroxymethyl)aminomethane (Tris) solution with HCl solution as recommended in the literature [26]. The heat effect of drug dilution (Q_{dil}) and of drug–erythrocyte membrane reaction (Q_{react}) were obtained by integrating the heat flow curve over the reaction time. All experiments were performed at 25°C using a stirring speed of 120 rpm. Two ml of erythrocyte suspension (1.14×10^8 cell/ml) in isotonic PBS, pH 7.4, were introduced into the calorimeter titration vessel and kept until the base line was stable. Subsequently, the drug solution was added in a concentration sufficient to induce total lysis (250 μM TFP and 6 mM DBC). DBC addition was divided into two successive steps, 3 mM in each, in order to reduce the heat dilution signal to less than the working scale (30 μW). Drug solutions were introduced at equal flow rates (1 μl/s) into the reaction vessel using a two channel peristaltic pump (model 612 Lund Syringe Pump 2, from Thermometric AB). The heat effect of drug dilution (Q_{dil}) into the same isotonic buffer was measured separately and subtracted from the heat effect obtained in the reaction where cells were present (Q_{react}), in order to obtain the interaction heat (Q_{hem}), which corresponds to the heat effect of drug-induced haemolysis.

3. Results and discussion

3.1. Drug partitioning between membranes and water

Table 1 shows TFP, DBC and PZQ partition coefficients (*P*) between membranes (microsomes, erythrocyte ghosts, liposomes) and water. TFP partition into membranes, in all cases, was higher than those of DBC and PZQ, except in liposomes when TFP and DBC *P* values are similar, revealing that TFP has a greater

Table 1
Partition coefficients[a] for TFP, DBC and PZQ between microsomes (P^{MIC}), erythrocyte ghosts (P^{GHOST}) and liposomes (P^{LIP}) vs. water (PBS)

	P^{MIC}	P^{GHOST}	P^{LIP} [c]
TFP	7172±1229	1380±429	1916±341
DBC[b]	793±119	375±62	2219±436
PZQ	488±179	210±52	[d]–

[a]Each P value represents the mean±SEM of nine experiments; samples in PBS, pH 7.4, incubation time: 10 min, room temperature.
[b]For DBC experiments, pH 6.8, PBS was used.
[c]For P^{LIP}, we used four freeze-thawing cycles which increased the incubation time to nearly 30 min.
[d]P^{LIP} values for PZQ were not obtained due to high scattering that overlaid PZQ absorbance (λ=324).

affinity for the membranes than the other drugs. This affinity difference indicates the specificity of the interaction pathway of drugs into membranes. There is evidence that other phenothiazines bind to erythrocyte membrane protein [27,28]. Ruggiero [29], using ESR, showed that TFP specifically binds to erythrocyte membrane protein and triggers changes in the protein structural arrangement. Moreover, some authors [30–32], using phospholipid vesicles, obtained evidence that DBC interacts in the interface between water and the external monolayer.

The stronger binding of TFP to microsomes may reflect some TFP protein interaction, as the protein content of liver microsomes is higher (ca. 70% of the total membrane weight [33]) than that of ghost membranes (55% [34]). In addition, if we consider the fluidity of the ghost membranes, we realize that the cytoskeleton [29] and the high cholesterol content [35] impose rigidity, restricting TFP partition into them, that can explain the rather small P^{GHOST} value. In fact, P^{GHOST} was slightly lower than P^{LIP}, an unexpected result since liposomes lack proteins. This fact suggests that cytoskeleton rigidity compensates for any TFP/protein interaction in ghost membranes.

3.2. Haemolytic curve

Fig. 1 shows a sigmoidal curve characteristic of the haemolytic effect. No lytic effect was observed below 108 μM TFP, a range in which the membrane incorporates TFP without losing integrity. Beyond 108 μM, lysis increases quickly with TFP concentration due to

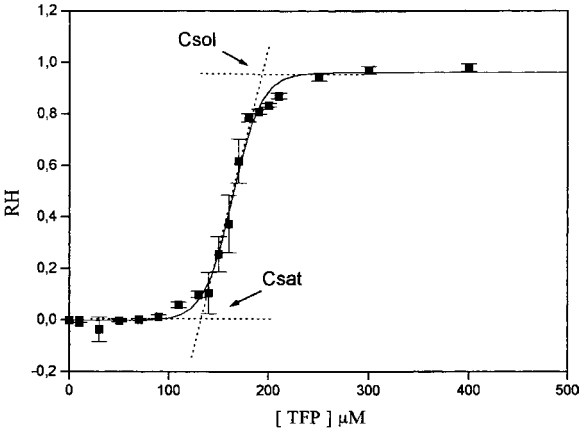

Fig. 1. TFP-induced isotonic haemolyis. Haematocrit=1% in 5 mM PBS, pH 7.4, incubation time=10 min at room temperature. C_{sat} and C_{sol} (see text) are shown.

the cooperative effect of the lipid bilayer. We used haemolytic curves to obtain C_{sat} and C_{sol} values, i.e., the drug concentration for (i) the onset and (ii) complete solubilization of erythrocyte membrane (Fig. 1).

Table 2 presents C_{sat} and C_{sol} of TFP, DBC and PZQ obtained from their respective haemolytic curves (see procedures described in Section 2).

Comparing the haemolytic activity of these three drugs at the 1% haematocrit, we can see that the total lysis induced by TFP (C_{sol}) occurs at a very low concentration (184 μM) with a short incubation time, 10 min, while DBC needs a concentration 20 times greater, 4 mM, and an incubation time equal to 30 min to induce the same effect. For PZQ, the concentration needed for total lysis is 14.2 mM with an incubation time of 90 min.

According to the literature [3], the haemolytic properties are related to the partition properties into

Table 2
Haemolytic concentrations of TFP, DBC and PZQ

	C_{sat} (μM)	C_{sol} (μM)
TFP[a]	108	184
DBC[b]	2300	4000
PZQ[a]	2570	14 200

[a]Samples in PBS, pH 7.4, incubation time of 10 and 90 min for TFP and PZQ, respectively, at room temperature.
[b]For DBC experiments, PBS, pH 6.8, an incubation time of 30 min and room temperature were used.

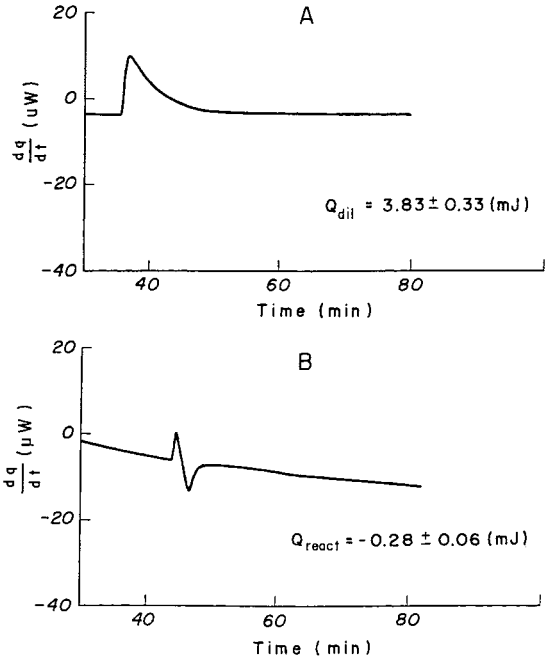

Fig. 2. Heat effect of TFP dilution and of TFP-induced haemolysis: (A) TFP, 250 μM dilution in PBS, pH 7.4; and (B) TFP, 250 μM dilution in erythrocyte suspension (haematocrit= 1%).

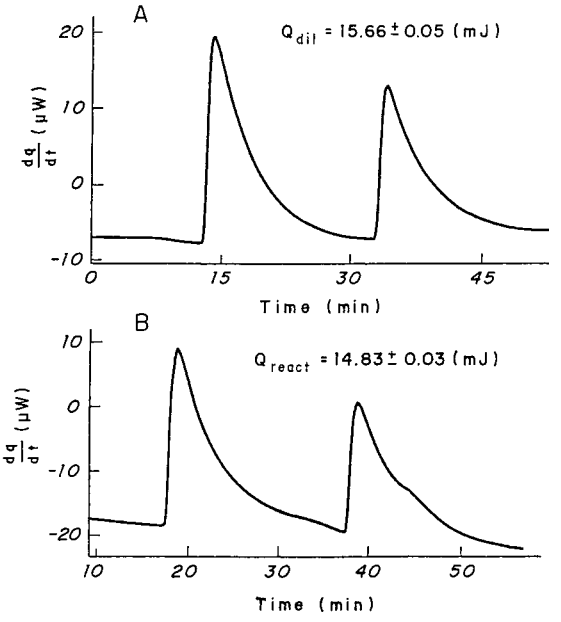

Fig. 3. Heat effect of DBC dilution and of DBC-induced haemolysis: (A) two step 6 mM DBC dilution in PBS, pH 6.8; and (B) two step 6 mM DBC dilution in erythrocyte suspension (haematocrit=1%).

erythrocyte membrane. TFP, the drug that best interacts with erythrocyte membrane has a higher *P* value and is the most effective haemolytic drug, followed by DBC and PZQ.

3.3. Microcalorimetric assay

Fig. 2 (for TFP) and Fig. 3 (for DBC) show the heat effect of drug dilution in the absence of red blood cells (A) and the heat effect of drug-induced haemolysis (B). Table 3 summarizes the heat effect of TFP and DBC dilution (Q_{dil}), the total system heat effect of drug-induced haemolysis (Q_{react}) (without subtraction of Q_{dil}) and the heat effect of drug-induced haemolysis (Q_{hem}). In both cases, drug dilution is endothermic while drug-induced haemolysis culminates in an exothermic process.

Fig. 2 shows that the heat of TFP-induced haemolysis is exothermic (B) while the TFP dilution process is endothermic (A). These results are not in agreement with Aki and Yamamoto [15], who reported the heat effect of TFP-induced haemolysis as an endothermic enthalpy (ΔH=19.6±1.9, in pJ/cell), mistaking the concept and the unit of enthalpy. However, considering the absolute values, the results of the study of heat effect for TFP-induced lysis are similar (in this work, 18.2±0.9 pJ/cell and in [15], 19.6±1.9 pJ/cell). In a later publication [16], the same authors report a large

Table 3
Heat effect[a] of drugs dilution (Q_{dil}), drug erythrocyte total reaction (Q_{react}) and drug-induced haemolysis (Q_{hem})[b]

	Q_{dil} (mJ)	Q_{react} (mJ)	Q_{hem} (mJ)	Q_{hem} (pJ/cell)
TFP	3.83±0.33	−0.28±0.06	−4.1±0.2	−18.2±0.9
DBC	15.66±0.05	14.83±0.03	−0.83±0.04	−3.6±0.01

[a]Each *Q* value represents the mean±SEM of at least four experiments.
[b]Q_{hem} corresponds to $Q_{react}-Q_{dil}$.

negative value of ΔH for TFP-induced haemolysis, contradicting themselves.

The heat of DBC-induced haemolysis (−3.6 pJ/cell) (Table 3) is about four times less than the value for TFP. Even using a much higher DBC concentration to reach complete haemolysis, the heat of DBC-induced lysis was lower indicating that its affinity for the membrane is smaller, in agreement with the P values obtained. This fact indicates that the specific interaction of DBC with the erythrocyte membrane is weak, as previously described [30,31], while TFP shows a stronger interaction (high P values, high haemolytic efficiency and exothermic heat of induced haemolysis). Unfortunately, the very high exothermic dilution heat effect of PZQ has hidden its weak induced lysis thermal activity.

3.4. Critical methodological analysis

There are a few literature references [15,16] concerning microcalorimetric studies of drug-induced haemolysis. The available data for this process has been interpreted as the enthalpy change of the haemoglobin released from one red cell (ΔH_{hemol}) [15]. In another report, however, the same authors consider this process as occurring in two parts: first, an exothermic process, arising from drug binding to erythrocyte, and second, an endothermic one, corresponding to the heat of dilution of haemoglobin released from erythrocyte [16]. The haemolytic phenomena should be considered as a result of a series of events (loss of drug hydration shell, drug-specific membrane interaction, structural membrane rearrangement, haemoglobin and cell content release) that might occur simultaneously or not. Since calorimetry is a non-specific technique, the observed heat effect cannot identify any particular event and shows the overall outcome of these processes.

The experience achieved during this work lead us to avoid the calorimetric titration procedure described by Aki and Yamamoto [16]. Since the drug membrane interaction occurred partially in each drug addition, the weak calorimetric responses of this interaction were always superposed by the much more expressive drug dilution effect. In addition, we noted that erythrocytes sediment during the long period demanded by the titration technique. Due to the above considerations, we present the described protocol, in which the drug is added once, or in the case of DBC, in two portions, which favours membrane interaction sign determination and reduces the total experimental time.

4. Conclusion

The erythrocyte membrane interaction of the drugs TFP, DBC and PZQ studied in this work showed a strong correlation between the drug membrane partition (P) and the disruption of the red blood cell membrane (haemolysis). Since the drug membrane exothermic heat effect of haemolysis (Q_{hem}), which is the outcome of a number of events that produces haemolysis, is directly correlated with the partition coefficients, it suggests that Q_{hem} is mainly reflecting the drug–erythrocyte membrane interaction.

The proposed protocol to study drug–erythrocyte membrane interaction by calorimetry provides precise and confident results.

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