

EFFECT OF PLATELETS ANTIAGGREGANT CLOPIDOGREL ON ERYTHROCYTES AGGREGATION AND SEDIMENTATION

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Abstract

Based on theoretical biophysical considerations (Einstein - Stokes and extended Derjaguin, Landau, Verwey and Overbeek theories), it was predicted that platelet antiaggregant clopidogrel has to reduce the aggregation and sedimentation of erythrocytes. Clopidogrel solutions were added to blood samples collected on EDTA for obtaining concentrations within the range 1 - 8 µg/mL, which equals the total plasma concentration of clopidogrel and its metabolites. Sedimentation curves were obtained using the standard Westergren method. For each curve, the starting time, the slope of the linear part and the final height of sediment were evaluated. The comparison of sedimentation curves using f_2 and area under curve (AUC) metrics from bio-pharmacy was an alternative approach to statistical tests, considered more appropriate for clinical interpretation. The dependence between local structure of curves, as well as the area under sedimentation curve (AUSC) global parameter on the concentration of clopidogrel was, as a rule, linear within the range 1 - 4 µg/mL clopidogrel concentration. As a final conclusion, authors consider that results justify the hope that sedimentation of erythrocytes could become an alternative, *ex vivo*, simple method for screening estimation of the extent of clopidogrel therapeutic effect.

Rezumat

Bazată pe teoriile Derjaguin, Landau, Verwey, Overbeek și Einstein - Stokes, s-a emis ipoteza că antiagregantul plachetar clopidogrel trebuie să reducă și agregarea eritrocitelor. Soluții de clopidogrel au fost adăugate la probe de sânge recoltate pe EDTA, pentru a obține concentrații în domeniul 1 - 8 µg/mL, echivalente cu concentrația totală de clopidogrel și metaboliți în sânge, în terapie. Curbele de sedimentare au fost obținute prin metoda Westergren. Pentru fiecare curbă au fost determinate timpul de start, panta părții lineare și înălțimea finală a sedimentului. Compararea curbelor de sedimentare s-a efectuat prin aplicarea metricilor f_2 și aria de sub curbă (AUC) din biofarmacie ca o alternativă la testele statistice. Aceste metrici au fost considerate ca fiind semnificative pentru o interpretare clinică. Dependența între structura locală a curbelor, precum și a parametrului global aria de sub curba sedimentării (AUSC) de concentrația de clopidogrel a fost, de regulă, lineară în domeniul de concentrație 1 - 4 µg/mL. Ca o referință finală, autorii consideră ca rezultatele justifică speranța că evaluarea sedimentării eritrocitelor ar putea deveni o metodă *ex vivo* simplă, pentru *screening*-ul mărimii efectului clopidogrelului în terapie.

Keywords: clopidogrel, erythrocyte sedimentation curves, blood cells aggregation, bioequivalence metrics, Derjaguin, Landau, Verwey and Overbeek theories

Introduction

The problem of clopidogrel therapy is the frequent lack of effect which is known under the name of resistance to treatment [33]. Adjustment of administered doses in order to obtain desired therapeutic efficacy was not very successful [26], the risk of recurrence remaining still high [7].

It is generally accepted that clopidogrel is not active on platelet aggregation [22], the effect being given by its active metabolite: (2Z)-{1-[(1S)-1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfanyl-3-piperidinylidene} acetic acid [21] obtained by biotransformation of the intermediate metabolite 2-oxo-clopidogrel [25].

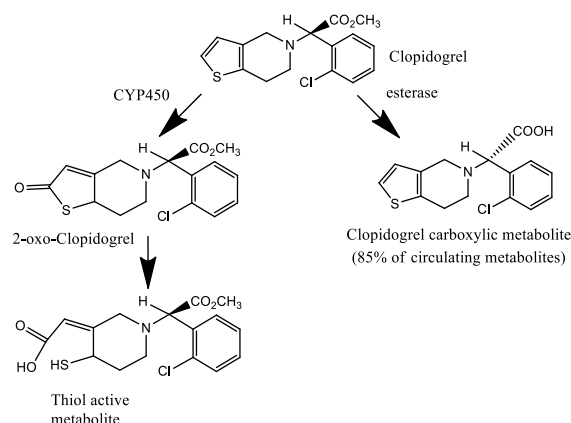


Figure 1.
Schematic representation of clopidogrel metabolism

The lack of clopidogrel activity has been based on the indirect proof obtained by Savi *et al.* [24], who proposed that the *in vivo* activity of clopidogrel is dependent on the hepatic biotransformation to an active metabolite. In these studies, clopidogrel (40 mg/kg bw) was less effective in hepatectomized rats as compared to normal control rats.

Although they concluded that the reduced effect was due to an elimination of the hepatic metabolism, it is likely that hepatectomy also implies many other effects that can lead to an aggregation decrease. Additionally, it is to note that doses used in animal studies were much higher compared with usual humans' doses (75 mg/day). Consequently, other mechanisms of clopidogrel-induced antiaggregation could contribute in humans. Indeed, Weber *et al.* [38] reported that, when 0.01 - 1 $\mu\text{g/mL}$ clopidogrel was incubated with washed platelets within 1 h, a concentration-dependent inhibition of adenosine diphosphate (ADP)-induced platelet aggregation was observed. Inhibition of ADP-induced platelet aggregation by clopidogrel *in vitro* occurred in the absence of measurable effects on the reversal of prostaglandin E1 (PGE1)-stimulated cyclic AMP by ADP.

A more systematic approach to clopidogrel variability dilemma has to take into account the *in vivo* pharmacokinetics of the parent drug and its metabolites. Until recently, only the carboxylic acid metabolite of clopidogrel was possible to be quantified in plasma. Improvement of the HPLC-MS/MS equipment allowed the development and validation of methods for the quantitative evaluation of clopidogrel and three of its metabolites [7, 14]. The application of these methods to pharmacokinetic studies [3, 27-29] enabled a better understanding of the evolution of clopidogrel's plasma concentrations after therapeutic doses.

Since the lack of antiaggregant effect of clopidogrel was not unequivocally proved and also the effect on erythrocyte aggregation, we hypothesized that clopidogrel has an antiaggregant effect both on platelets and erythrocytes that can be tested using the effect on erythrocyte sedimentation. Consequently, we expected that clopidogrel would have an effect of decreasing the erythrocytes sedimentation rate.

Materials and Methods

Materials

Erythrocyte sedimentation. Solutions of clopidogrel were added to blood samples collected on EDTA for obtaining concentrations within the range 1 - 8 $\mu\text{g/mL}$, which equals the total plasma concentration of clopidogrel and its metabolites [15].

Sedimentation curves were obtained using a Becton Dickinson VACUTAINER® and ESR Systems Europe Seditainer®.

After measuring sedimentation within 90 minutes time interval, the samples were gently stirred until complete homogenization. To the reconstituted sample was added clopidogrel and the determination of sedimentation curve was performed for another 90 minutes.

For each curve, the starting time ("time-delay"), the slope of the linear part, as well as the final height of sediment were evaluated. The influence of dilution on the sedimentation was determined using blank phosphate solutions (without clopidogrel).

Results and Discussion

The effect of addition of clopidogrel on the sedimentation curves appeared practically at the concentration 0.5 $\mu\text{g/mL}$. At concentrations of 0.2 and 0.4 $\mu\text{g/mL}$, a small decrease of erythrocyte sedimentation was observed, but the difference was not statistically significant and unlikely clinically relevant.

Effect of clopidogrel 1 $\mu\text{g/mL}$ on sedimentation curves. The comparison of the curves obtained after addition of clopidogrel (Figure 6) with the ones obtained in the absence of clopidogrel suggests a somewhat different effect on the three clusters of the curves. Clopidogrel (1 $\mu\text{g/mL}$) tends to shift down all clusters. It can also be seen that the (60 mm, 60 minutes) point appears as a bifurcation point. After the (60 mm, 60 minutes) point, the curves of the upper cluster are erratically influenced (but mainly decreased), and less affected after this point. The middle cluster (ESR around 60 mm/h) appears to shift down substantially, both with respect to the rate and extent, on the low level curves. The third, lower cluster, becomes lower indeed, practically some of curves could be considered within the "non-sedimentation" region (i.e. erythrocyte sedimentation rate (ESR) < 10 or even zero).

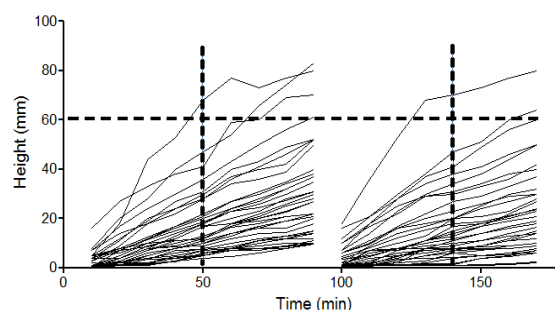


Figure 2.

The sedimentation curves in presence of 1 $\mu\text{g/mL}$ clopidogrel; **a.** control: 0 - 90 minutes; **b.** clopidogrel: 100 - 180 minutes

The comparison of the mean sedimentation curves.

In case of the subjects with low or moderate sedimentation rate, a good linear dependence appeared between the height of sedimented column and the time within first hour after starting the process. Control data and data in the presence of clopidogrel 0.5 µg/mL for patients with lower to moderate erythrocyte sedimentation rate (final height

< 60 mm) are shown in Figure 3. It can be seen that the correlation was excellent ($R^2 > 0.99$).

This is a consequence of the fact that practically all individual curves were approximately straight lines. The hierarchy of the mean curves confirms the global perception regarding the downshift of curves following the addition of clopidogrel. The difference between regression slopes, in the presence of clopidogrel and control curves, was statistically significant ($p < 0.01$).

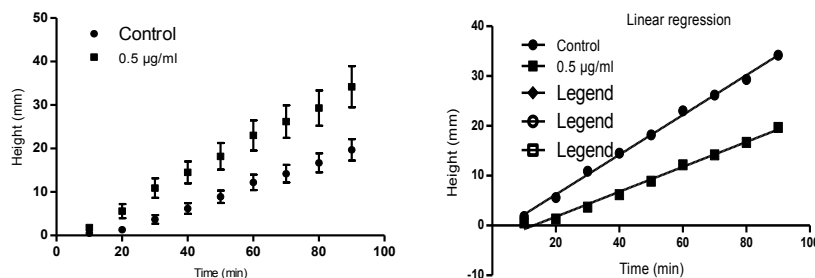


Figure 3.

Comparison and linear regression of mean curves in case of 0.5 µg/mL clopidogrel

The comparison of the initial slopes of sedimentation curves as function of clopidogrel concentration

The slopes of mean sedimentation curves ($n = 25$) are represented in Figure 4. The decrease of slope as function of clopidogrel concentration was fitted with a straight line for which the correlation coefficient was high enough ($R^2 = 0.92$). Maximum effect was of the order of 20 mm decrease in the height of sedimented column, value that can be considered as clinically significant.

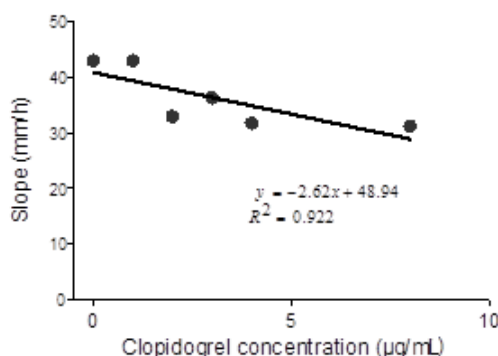


Figure 4.

The dependence of initial slope of mean sedimentation curves on the clopidogrel concentration

The comparison of the effect of clopidogrel at different concentrations on time-lags of mean curves

It was observed that initial linear part of sedimentation curves starts only exceptionally from origin (time zero). This was the reason that, on Figure 2, the curves are represented as a function of

time, starting from 10 minutes. In fact, some of derivatives indicate a “negative” lag-time, maybe due to a positive acceleration at the beginning of processes. The time-lags were calculated for the same mean curves whose slopes were presented on Figure 4. The results, as function of clopidogrel concentration, are shown on Figure 5. It appears that the time-lag is increased by clopidogrel, but the rule of dependence is no clear. Both linear and parabolic regressions proved a poor correlation coefficient. It is to underline that, in case of biological “endpoints”, variability is in all cases high and correlation coefficients are poor. An increased statistical significance is obtained on very large populations, but in such studies, it is possible to test only a reduced number of parameters and hypothesis.

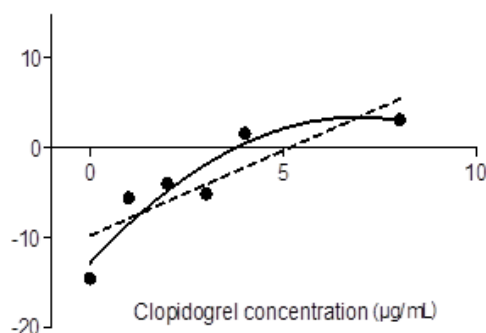


Figure 5.

The dependence of lag-time of mean sedimentation curves on clopidogrel concentration

Once again, the results based on mean curves are to be considered with precautions. Values of time-lags were between -15 minutes and +6 minutes.

In clinical evaluation of erythrocytes sedimentation, the lag-time is ignored. The effect seems to be saturated at 4 µg/mL. The time-lags could be correlated also with slopes. After examination of entire set of curves (Figure 2), it appears that the greater slopes imply, as a rule, a negative time-lags. On the contrary, the low rates imply positive time-lag. The fact that clopidogrel decreases the slope, correlates qualitatively with the effect of time-lags increase.

The effect on standard erythrocyte sedimentation rate (ESR) parameter

The erythrocyte sedimentation rate (ESR) parameter, used in clinical practice is defined as the height of sedimented column after one hour. This parameter represents the simplest approach of aggregation and sedimentation of erythrocytes, but has the advantage of a long time application and a well-established correlation with pathology and therapeutic effects of some drugs.

Figure 6 presents the mean values of this parameter, together with the standard deviations as function of the clopidogrel concentration.

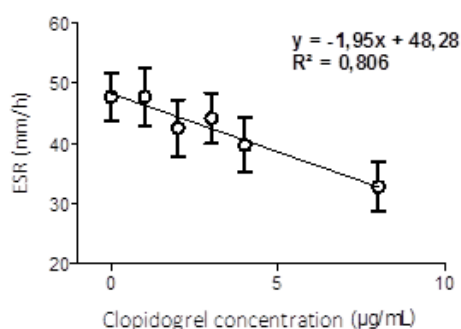


Figure 6.

The ESR as function of clopidogrel concentration
(n = 19)

It is clear that clopidogrel has an effect of decreasing the ESR, most probable following an

effect on aggregation of erythrocytes. Furthermore, dependence is linear with an acceptable correlation coefficient within the entire 0 - 8 µg/mL interval.

Testing the similarity of sedimentation curves using f_2 metric. The analysis of the difference between the two mean curves, above presented in Figure 3, showed that the regression lines were statistically significant different (R^2 , paired Student's t -test and the test for comparison of slopes of the two regression lines). However, the statistical significance does not imply automatically a clinical significance.

In comparative analysis of dissolution curves in biopharmacy the so called f_2 metric, is used in order to establish a "similarity" which assures practically the same performance *in vivo*.

$$f_2 = 50 * \log \frac{100}{\sqrt{1 + \frac{\sum_{i=1}^n (R_i - T_i)^2}{n}}}$$

where R_i and T_i represent the matched points of the percent of released amount in the reference and in the tested dissolution curves.

f_2 value is 100 when the profiles are identical. If one product released instantaneously ($R_i = 100$) and the other release nothing ($T_i = 0$), f_2 is practically zero, but this case is highly improbable.

If the difference between all points of the curves is 10%, a value of 50 is obtained for f_2 . Consequently, a value of f_2 between 50 and 100 is accepted as criterion for similarity. In our case, the control and different clopidogrel curves using the bellow formula were compared:

$$f_2 = 50 * \log \frac{100}{\sqrt{1 + \frac{\sum_{i=1}^n (h_0^i - h_{\text{clopidogrel}}^i)^2}{n}}}$$

where h^i represents the height of the sedimented column.

Similarity was interpreted as lack of a significant effect.

Table I

The evaluation of similarity of the two sedimentation curves using f_2 metric

Time (min.)	20	30	40	50	60	70	80	90
Control	5.6	10.9	14.5	18.2	23	26.2	29.3	34.2
CLO 0.5 µg/mL	1.3	3.7	6.2	8,9	12.2	14.2	16.7	19.7
$(h_0^i - h_{\text{CLO}}^i)^2$	18.5	51.8	68.9	86.5	116.6	144.0	158.8	210.3
$\sum_{i=1}^n (h_0^i - h_{\text{CLO}}^i)^2 = 1349.3$								

$$f_2 = 49.3$$

For example, the value calculated for f_2 in comparison with the mean control curve and the mean curve in presence of clopidogrel 0.5 µg/mL (data shown in Figure 4), as can be seen on Table I, was 49.3, on the border between similarity and

nonsimilarity, although the difference was statistically significant.

Here it has to be remarked that the 10 % value used in establishing the similarity, was conceived based on correlation between *in vitro* dissolution and

plasma, as well as pharmacokinetics of the concerned active substance. To what we have to compare sedimentation curves and which is the appropriate threshold? There is not a single response to this question; this must be established with the particular clinical context, where sedimentation is really important in diagnostic and/or monitoring of the therapeutic schedules.

Area under sedimentation curve (AUSC)

Physiological based pharmacokinetic/pharmacodynamic (PK/PD) - modelling [18, 34], uses as a main parameter the area under plasma levels curves (AUC) as a predictor of the extent of therapeutic effect in pharmacokinetics and bioequivalent studies [19, 20]. This global criterion integrates all parameters of the plasma levels curves – absorption, distribution, metabolism, elimination etc. More recently, methods from bioequivalence studies based on AUC were extended in clinical studies for comparisons of plasma levels or pharmacodynamic effect curves [4, 6, 11, 13, 23].

The results concerning the effect of clopidogrel on AUSCs for the group of subjects with low ESR are shown on Figure 7.

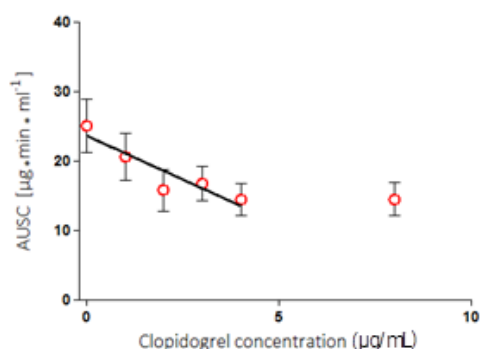


Figure 7.

The effect of clopidogrel on the area under the sedimentation curve in case of patients with low ESR values

The decrease of AUSC over the entire concentration interval was rather exponential than linear, most probable as a consequence of reaching the saturation effect. The effect increased linearly with clopidogrel concentration up to 4 µg/mL. At 8 µg/mL, the result was practically the same as for 4 µg/mL. Lowering of AUSCs is correlated with effects observed on the main size and shape parameters of curves.

A parallel evaluation of all presented effects as function of clopidogrel concentration, suggested, as a global characterization, the conclusion that sedimentation is decreased linearly with approximately 20% maximum effect within the 1 - 4 µg/mL interval.

Sedimentation curves vs. ESR

The Guideline of the International Council for Standardization in Haematology (ICSH), recommends ESR as the height of the sedimented column of erythrocytes at one hour [11]. The term “rate” is not justified since a single point is not defining a rate. Even if we consider the origin as a second point (but not all sedimentation curves start from zero, a lag-time being rather the rule than an exception) the problem is not appropriately solved. For instance, if the sedimentation rate reaches saturation before one hour, the apparent ESR represents an underestimation of the actual ESR (Figure 8). Consequently, it appeared justified in this paper to consider sedimentation curves and their parameters (delay, saturation value and AUSC).

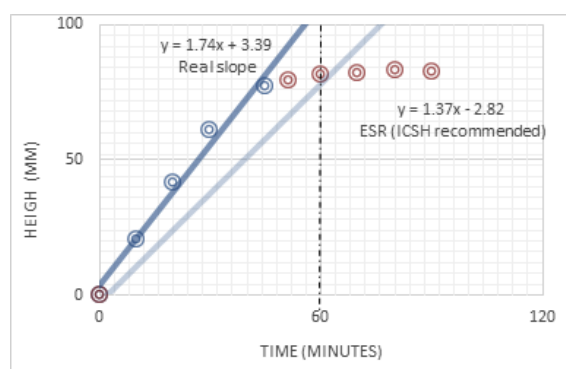


Figure 8.

Underestimation of the rate and area under the curve by the ESR in case of saturation before 60 minutes

The mechanisms of the effect of clopidogrel on erythrocyte aggregation and sedimentation *Erythrocytes “uniform sedimentation”*

Fabry [4] calculated the sedimentation velocity of erythrocytes and their aggregates, considered as spheres moving in plasma as a viscous fluid. However, the values obtained by this method are very low and cannot explain high values observed in clinical practice. A reliable conclusion is that, because the high sedimentation rate values are a consequence of aggregation of erythrocytes and an appearance of a moving boundary is a sign of sedimentation of relatively “uniform” aggregates.

Regarding the time-lag, two situations could appear: (i) aggregates pre-exist in plasma, resulting in an apparent negative time-lag; and (ii) aggregates do not pre-exist but are formed in blood in a given time interval, and the sedimentation starts later, resulting in an apparent positive time-lag.

The biophysical model of interaction between active substances and aggregation

In the circulating blood, the platelets and erythrocytes are normally carried along separately

[1]. In absence of a hydrodynamic stress, the erythrocyte aggregation appears both *in vitro* and *in vivo*.

The population balance equation (PBE), which in fact is a generalization of the von Smoluchowski equation, defines the rate of change of number density of aggregates as:

$$\frac{dn(v,t)}{dt} = \int_{v_0}^{v/2} k_G(w, v-w) n(w,t) n(v-w,t) dw - \int_{v_0}^{\infty} k_G(w, v) n(w,t) n(v,t) dw,$$

where the equation is an integro-differential equation for $n(v, t)$, the number density with respect to particle volume (v) at time (t). $n(w, t) dw$ - represents the number of particles (per unit volume of suspension) with volumes between v and $w + dw$.

Neglecting hydrodynamic interactions, Smoluchowski [30] suggested the following expression for the collision frequency for two spheres of volume w and v :

$$k_G(w, v) = \frac{G}{\pi} \left(\sqrt[3]{w} + \sqrt[3]{v} \right),$$

where G is the shear rate. Huang and Hellums [7-9] have used these equations for modelling the platelet population dynamics, considering that only a part of the collisions $\mathcal{E}$ effectively leads to aggregation.

The probability of aggregation can be considered as deriving from the ratio between van der Waals attraction forces and electrical repulsion, between negatively charged particles or living cells.

Since the aggregation and the sedimentation are concomitant, it would be necessary to elaborate models considering both classes of phenomena. Writing equations is relatively easy, but difficult or impossible to be solved.

Moving boundary sedimentation of erythrocytes with a constant velocity supposes equilibrium: approximately equal size assemblies of rouleaux falling with the same speed. In fact, the actual evolution of the system is slightly different.

The electric charge of red blood cells (RBC) is the main factor determining their repulsion and avoidance of adhesion [18]. Considering the classical DLVO approach, it was obtained that, at all distances up to $d = 50 \text{ \AA}$ the flocculation will appear. Since up to $d = 70 \text{ \AA}$, repulsion is greater than attraction, cells do not aggregate [35, 36].

The proposed mechanisms for the effect of clopidogrel on erythrocyte aggregation and sedimentation

Under physiological conditions the platelet membrane has an overall negative charge [16, 21]. Effective charge is in fact only that appearing beyond the Helmholtz double layer which can be calculated from the zeta potential (ζ), by applying the Gouy Chapman theory [21] and accessible by electrokinetic determinations. ADP, ATP, serotonin (5-HT) and noradrenaline modify the platelet

electrophoretic mobility and there is a correlation between these effects and aggregation of platelets [32].

As a rule, antiplatelet drugs decrease the sedimentation rate of erythrocytes and pro-aggregants increase the sedimentation rate [37]. Consequently, the effect of clopidogrel could likely be due to an increase of ζ , followed by its absorption in the double layer of the erythrocyte membrane. Increasing the repulsion potential, further, leads to decreasing of aggregates size.

Regardless of the presence or absence of a receptor for clopidogrel, due to its amphiphilic character, an accumulation at membrane interfaces, with an effect on the structure of the double layer, is expected.

The structure of the clopidogrel molecule in solution is significantly different from standard representations. Natural Bond Orbital (NBO) analysis showed that the stability of the molecule arises from hyper-conjugation and charge delocalization [31]. The residual negative charge of clopidogrel can increase the electrophoretic potential of the erythrocyte. Consequently, the observed antiaggregant effect may be due to clopidogrel itself, as well as to its metabolites.

Conclusions

The evaluation of the sedimentation of erythrocytes by the sedimentation curve, instead of standard ESR point, allows a more complex analysis, starting from time-delay, slope of linear part and saturation value.

Comparison of the sedimentation curves using f_2 and AUC metrics from biopharmacy is a proposed alternative approach to usual comparison of a single point (one hour), more appropriate for clinical interpretation.

The dependence between local structure, as well as AUC global parameter on the concentration of clopidogrel was, as a rule, linear within the range 1 - 4 $\mu\text{g/mL}$ of clopidogrel concentration. At higher concentrations it seems to appear a saturation effect.

The effect of platelet antiaggregant clopidogrel was in all cases, the decrease of sedimentation. Theoretical considerations based on models of aggregation kinetics and sedimentation of blood as a highly concentrated suspension, suggest that the effect of clopidogrel is exerted at erythrocyte

membrane level, with consequences on aggregation.

The results confirm previously published data and theories concerning biophysical drugs action mechanisms at membrane interfaces. There are data to justify the hope that the sedimentation of erythrocytes could become an alternative, *ex vivo*, simple method for screening estimation of the extent of clopidogrel therapeutic effect.

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