



## NEW METHODS FOR COMPARISON OF ERYTHROCYTE SEDIMENTATION CURVES IN ESTIMATION OF AGGREGANT AND ANTIAGGREGANT EFFECTS OF DRUGS

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**Abstract.** Tests of erythrocyte sedimentation provide a measure of the acute phase response to inflammatory diseases, most infections, in cancer, cardiovascular and many other diseases. The term erythrocyte sedimentation rate (ESR) is retained because of traditional usage, although a single measurement after 60 minutes is not a rate. Recommendations of International Council for Standardization in Haematology (ICSH) considers ESR as defined by the height of sedimented column of erythrocytes at a given time (usually one hour). Single parameter characterization of sedimentation of erythrocytes by ESR represents a coarse approach of complex phenomena. The simplicity of the sedimentation rate is only apparent. In practice, a lot of difficulties arises in definition of the "rate" since its estimation requires at least two points on the sedimentation curve. If the sedimentation does not start immediately, a positive lag-time appearing, ESR measured value at one hour is a rate lower than the real slope, estimated from sedimentation curve. If sedimentation is more rapid and curve reach saturation before one hour, the real rate, defined by sedimentation curve is greater than the rate defined by official ESR. A more in depth analysis of data started in the paper from "sedimentation curves" which characterise dynamic evolution in time of phenomena. Comparison of curves arises great difficulties even for mathematicians. Metrics in the spaces of curves implies a more advanced mathematics. Consequently, sedimentation curves were replaced by some of their global parameters such as initial slope or area under curve. Further for comparison of curves were introduced some specific methods from cancer research, biopharmacy and pharmacokinetics. It concerns the rate of sedimentation this was estimated from the initial slope of the curves. In fact the curve could be modelled for a longer time interval using a two-phase linear regression. The slope of the first line could define better the ESR. If we consider evolution of height of sediment as a "survival curve", the Kaplan Meyer method becomes immediately applicable. From dissolution studies in biopharmacy the official metric on the release curves space, based on the sum of squared differences between matched points of the sedimentation curves. A set of methods (linear regression in estimation of the slopes, of the similarity of curves using  $f_2$  metrics, of time-lag, of half-time etc.), as well as comparison of Areas Under Sedimentation Curves, were applied in evaluation of clopidogrel's effect at concentrations between 1 and 8  $\mu\text{g/ml}$ .

**Keywords:** Platelet aggregation, Erythrocytes aggregation, ESR, Clopidogrel, Sedimentation curves metrics

### Introduction

Ex vivo, in absence of entire blood flow, appears a boundary between sedimented and non-sedimented blood, the speed of movement of boundary, defining

the erythrocyte sedimentation rate.

If we consider a single erythrocyte in plasma and calculate its sedimentation velocity by applying the classical Stock law, concerning movement of a sphere in a viscous fluid, it obtains a value of approximately 3 mm/h. Consequently, we have to think that, observed high values of sedimentation in clinical practice, are, in fact, the sedimentation velocities of erythrocytes aggregates. Greater is the number of erythrocytes in aggregate, greater is the sedimentation velocity. Consequently, the sedimentation rate is an indirect estimation of erythrocytes aggregability as well as

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of platelet aggregability.

We also, look further to evaluation of erythrocytes sedimentation as a point of care, screening method for diagnostic of hiperaggregability.

Single parameter characterization[1] of sedimentation of erythrocytes by ESR represents a coarse approach of complex phenomena connected with erythrocyte and platelet aggregation[2,3] in cancer, cardiovascular, inflammatory and many other diseases the result being appropriated only if we look for a roughly screening test.

A more refined approach of the sedimentation phenomena consists first of all in estimation of the slope of sedimentation curve using more than one point. In fact, the curve could be frequently modelled for a longer time interval using a two-phase linear regression. The slope of the first line could define better the sedimentation rate.

Evaluation of aggregant and antiaggregant effects of xenobiotics on ESR requires comparison of curves and more in depth analysis than comparison of single parameters. Metrics in the spaces of curves implies a more advanced mathematics. Some of these metrics are already used in comparison of time course of effects in biopharmacy[4,5] and clinical studies.

Most elaborated in this aim are met in cancer where there are compared survival curves. If we consider the evolution of height of sediment as a "survival curve", the Kaplan Meyer method becomes immediately applicable.

The aim of this paper was to apply a set of mathematical methods[6,7,8] (linear regression in estimation of slopes, comparison of Areas Under Sedimentation Curves, estimation of similarity of curves using  $f_2$  metrics, of time-lags, of half-time etc.) in comparison of sedimentation curves.

It was expected that these advanced tools allow an estimation of clopidogrel concentration – erythrocyte aggregation dependence.

Experiments were performed at clopidogrel concentrations in the therapeutic window of its carboxyl metabolite[9].

Ex vivo methodology for evaluation pharmacokinetic – antiaggregant effect correlation would have as effect the elaboration of therapeutic schedules for new drugs and optimization of schemes for current antiaggregant drugs.

Pharmacokinetic based, improved and personalized antiaggregant treatment for patients resistant to usual antiaggregant drugs will have as effect the avoidance of non-responsiveness appearance after a long period of expensive treatment.

## Experimental Method

The erythrocyte sedimentation was determined at normal volunteers and patients with rheumatic, cancer or coronary artery disease. There were included 46

patients diagnosed with increased ESR (more than 7 mm at 1 h). 0.8 ml blood was collected on 0.1 ml 1% EDTA witch was used instead of citrate due to the limited solubility of Clopidogrel in the presence of citrate. Clopidogrel hydrogensulfate {(S)-(+)-Clopidogrel (S)-(+)-Methyl 2-(4,5,6,7-tetrahydrothieno[3,2-c]pyridin-5-yl)-2-(2chlorophenyl)acetate hydrogensulfate} was purchased from Sigma – Aldrich.

The moving boundary in Westergren tubes purchased from Terumo Medical Corporation was followed at 15, 30 and 60 minutes. Measurements were performed only one hour since patients were selected to have high values of ESR and at the end of one hour interval, erythrocytes were practically completely sedimented.

After measuring the evolution of the height of sedimented column in 0-1 h interval, the sediment was redispersed and determination repeated in order to verify the reproducibility of the method. Then the sediment was again redispersed and was added different volumes of a clopidogrel stock solution, as follows: 25, 50, 75, 100 and 200  $\mu$ l solution of 20  $\mu$ g/ml

The final concentrations were in the range 1-8 $\mu$ g/ml for Clopidogrel.

## Experimental results

### Individual sedimentation curves

Sedimentation curves for a set of representative patients are shown in figure 1 and figure 2.

As it can be seen some patients proved no one sedimentation in two hours interval. These sedimentation curves as well that's of patients with very rapid sedimentation (ESR > 160) were dropped (figure 1, 2).

The effect of imposing the conditions, that all curves start from origin, is a hidden of the fact that aggregation of erythrocytes is preexistent to sedimentation or develops in time, leading to a time-lag in sedimentation.

In fact, the definition of ESR, given by ICSH imply a rate determined by the origin and the value at 1 hour.

### Effect of clopidogrel on sedimentation curves

If we examine the effects on the ESR presented in figures, it appears that for subjects with ESR higher than 60 mm/h the sedimentation is increased and, for that with ESR lower than 40 mm/h, the sedimentation is decreased (figure 3, 4, 5).

A subject appeared to be outlier face to this rule, its ESR value increasing from 30 to 67 mm/h.

The effect of clopidogrel was defined as difference between control mean curve and mean curve in presence of drug. Such an evaluation let to conclusion that clopidogrel has no a statistically significant

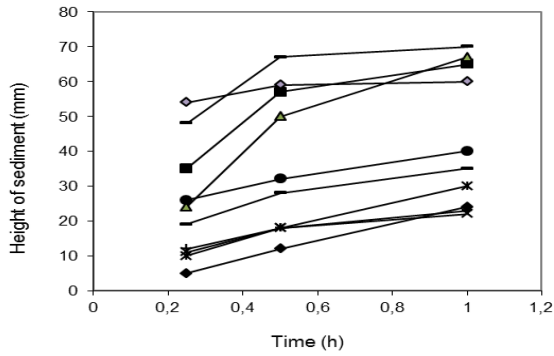


Figure 1. Individual sedimentation curves

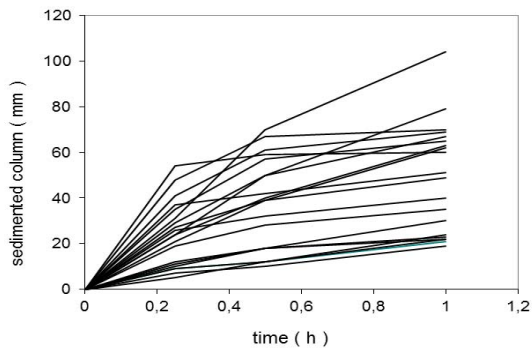


Figure 2. Sedimentation curves started from origin

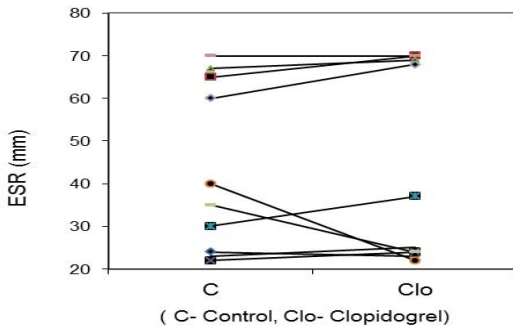


Figure 3. Effect of clopidogrel on ESR

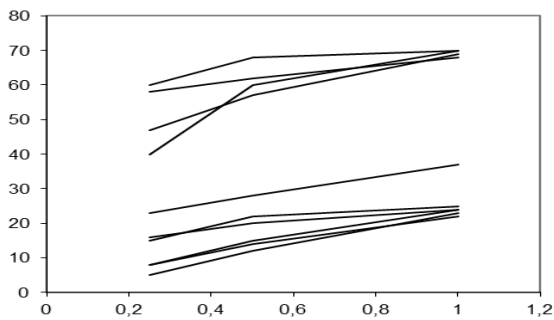


Figure 4. Sedimentation curves in presence of clopidogrel (entire curves)

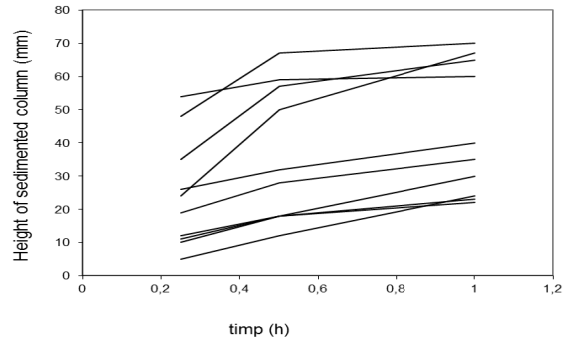


Figure 5. Sedimentation curves in absence of clopidogrel (entire curves)

effect on sedimentation.

Further, evaluation of its effect was made separately, on curves with value at 1 hour greater than 60 mm/h and on curves attaining lower values.

When the effects were considered separately on the two subsets of curves, it was concluded that in case of rapid sedimentation, the effect of clopidogrel is further increasing of the rate. On the contrary, in case of slow sedimentation, the effect is statistically significant decreasing of the rate. Apparent lack of effect obtained in the case of all curves, is a compensation between the two opposed effects.

**Dependence of effect on sedimentation curves on the Clopidogrel concentration.**

The effect of clopidogrel on sedimentation curves is presented in figure 6 and figure 7. Increasing of the concentration of clopidogrel induces a shift of the sedimentation curves: up in case of rapid sedimentation and down in case of slow sedimentation. In other terms, if high ESR means hyperaggregation and low values hypoaggregation, clopidogrel is active as proaggregant of erythrocytes in severe troubles and its effect is beneficial in moderate diseases.

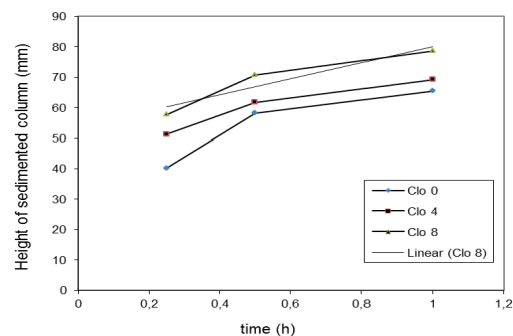


Figure 6. Shift up of sedimentation curves in presence of clopidogrel

Since low aggregation is essentially reversible, clopidogrel appear to be active as antiaggregant in such cases.

It is possible also to be active in the first stage, reversible, of aggregation, called adhesivity. If ag-

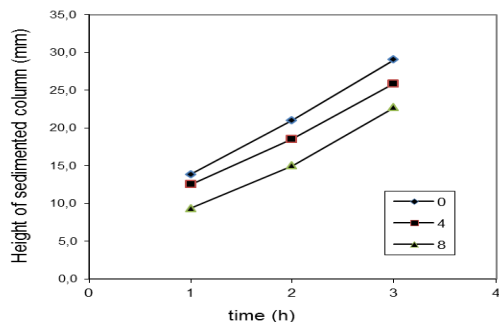


Figure 7. Shift down of sedimentation curves in presence of clopidogrel

gregation passed the threshold toward irreversible aggregation, clopidogrel is no more active.

**Linear dependence of effect on sedimentation curve on Clopidogrel concentration**

Dependence of height of sedimentation curves on the clopidogrel concentration at different measurement times is presented in figure 8 and 9.

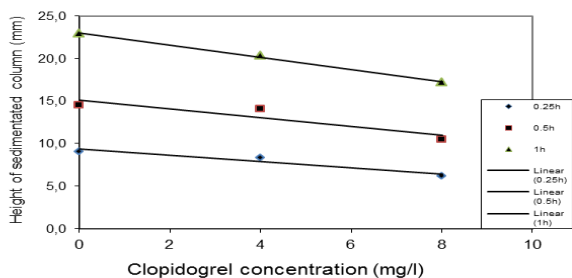


Figure 8. Decrease of average height of sedimented columns on Clopidogrel concentration in case of subset of patients with small ESR

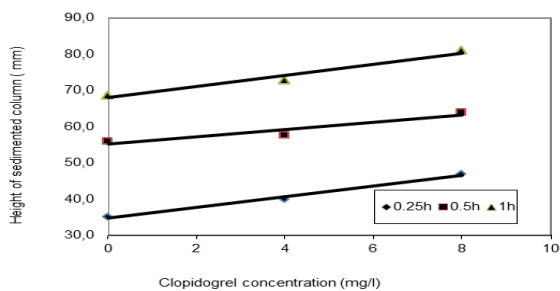


Figure 9. Increase of average height of sedimented columns on Clopidogrel concentration in case of subset of patients with high ESR

Effect of clopidogrel on the average height of sediment column was linear in concentration at all examined times (0.25, 0.5 and 1 hour). Regression lines were more or less parallel, the effect being less dependent on measuring time, at least in case of patients with low ESR values. It is to note the high values of correlation coefficients (between 0.9 and 0.99) which remove doubts concerning a random

effect.

**New methods for comparison of sedimentation curves**

**Limits of the “official endpoint”**

If we define ESR really as a rate, its definition have to be considered as initial slope of the sedimentation curve. If the sedimentation does not start immediately, ESR measured as value found at one hour is an underestimation of initial slope of sedimentation curve.

In this cases, we say that a time-lag appears. Estimation of positive or negative time-lags by linear regression of the initial values of sedimentation is necessary since such instances represent more complex patterns of aggregation and sedimentation which could have physiopathological implications (figure 10, 11, 12).

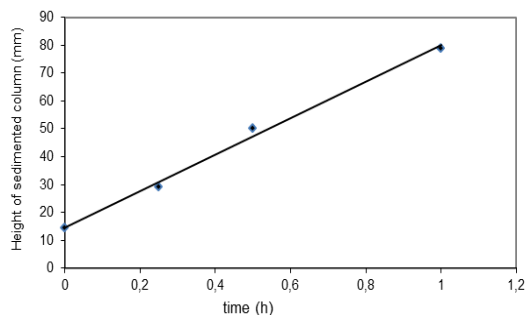


Figure 10. Sedimentation curve with negative lag-time

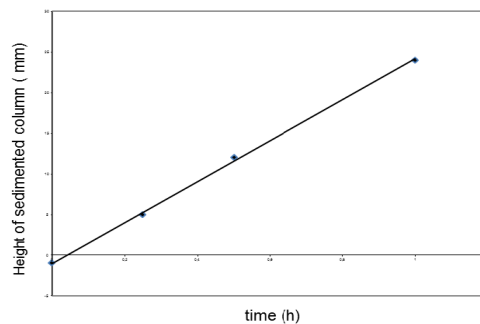


Figure 11. Sedimentation curve with positive lag-time

As can be seen in the figures, some curves associated to patients in our study, really presented such lag-times

**Calculation and comparison of Areas Under Sedimentation Curves (AUC-SC)**

Area under curves is proposed as a global parameter useful first of all in comparison of curves. Area under plasma levels curves of active substances is the most significant parameter in defining bioavailability of a drug. Statistical methods applied in comparison of populations of areas under curves achieved by two

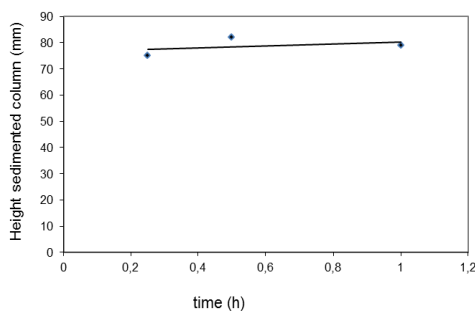


Figure 12. Sedimentation curve saturated before ESR measuring time

drugs evaluate the bioequivalence drugs containing the same active substances. A natural method for calculation of this area is the trapezoid rule:

$$AUC_0^T = \sum_{i=1}^n \frac{f(t_{i-1}) + f(t_i)}{2} * (t_i - t_{i-1})$$

As can be seen in figure 13, decreasing of Area Under Sedimentation Curve on the Clopidogrel concentration is linear in the interval [0 - 3 mg/l] (figure 13).

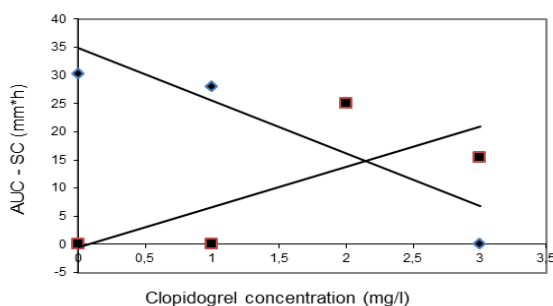


Figure 13. Dependence of the Area under Sedimentation Curves on the Clopidogrel concentration

It seems that evolution of AUC- SCs is biphasic: a linear decrease in the interval [0 – 2 mg/l] with a slope -2.59 followed by a higher rate decrease with slope -9.6 in the [2 – 3 mg/l]. Surely for obtaining a statistical significant result, it would be necessary a greater number of points.

**Estimation of similarity of curves using f2 metrics**

In comparison of dissolution curves in biopharmacy, it is recommended by the Guidance’s (FDA, EMA) the utilisation of the factor of dissimilarity f2: where Ri and Ti, represents the matched points in the reference and tested curves.

If the difference between all points of the curves

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

is 10%, it obtains for f2 the value 50. If curves are identical the calculated value of f2 is 100. Consequently a value of f2 between 50 and 100 indicates similarity of curves. A value of f2 lower than 50, signifies dissimilarity of curve.

In our case, we compared the control curves and curves, obtained after adding Clopidogrel and the formula became:

$$f_2 = 50 * \log \frac{100}{\sqrt{1 + \frac{\sum_{i=1}^n (h_0^i - h_{clo3mg/l}^i)^2}{n}}}$$

We applied the formula for comparison of control curve of one patient with the curve obtained after addition ex vivo of clopidogrel, resulting a concentration of 3 mg/l (table I).

|                   |       |       |       |                 |
|-------------------|-------|-------|-------|-----------------|
| Control           | 22.6  | 34.7  | 46.6  | $x_i$           |
| Clopidogrel 3mg/l | 9.4   | 16.4  | 26.3  | $(x_i - y_i)^2$ |
| $(x_i - y_i)^2$   | 171.9 | 332.0 | 408.9 | $(x_i - y_i)^2$ |
|                   |       | 17.5  |       | $f_2=37.9$      |

Table I. Calculus for comparison of control curve with sedimentation curve in presence of clopidogrel 3 mg/l

**Conclusion:** since f2 was lower than 50, the profiles are not similar and consequently the effect of Clopidogrel in reducing sedimentation was significant.

Comparison between control sedimentation curves and in presence of 2 mg/l Clopidogrel lead to the following result (table II):

|                    |       |      |      |                 |
|--------------------|-------|------|------|-----------------|
| Clopidogrel 0 mg/l | 22.6  | 34.7 | 46.6 | $x_i$           |
| Clopidogrel 2 mg/l | 18,04 | 29.4 | 37,2 | $(x_i - y_i)^2$ |
| $(x_i - y_i)^2$    | 20,8  | 27,3 | 87,1 | $(x_i - y_i)^2$ |
|                    |       | 6,8  |      | $f_2 = 58,4$    |

Table II. Testing of similarity between the control curve and sedimentation curve in presence of clopidogrel 2 mg/l

**Conclusion:** strictly interpretation of f2 value, indicates similarity but more correct is to consider that the curves are at the frontier between similar and not similar, the effect becoming significant starting from Clopidogrel 2 mg/l.

**Comparison between “survival functions”**

Commonly used methods for comparing two survival (curves) functions as are presented in figure 14 the log rank test, testing that logarithms of survival curves are proportional, and Kolmogorov Smirnov test, based on the highest difference of the matched points of survival curves.

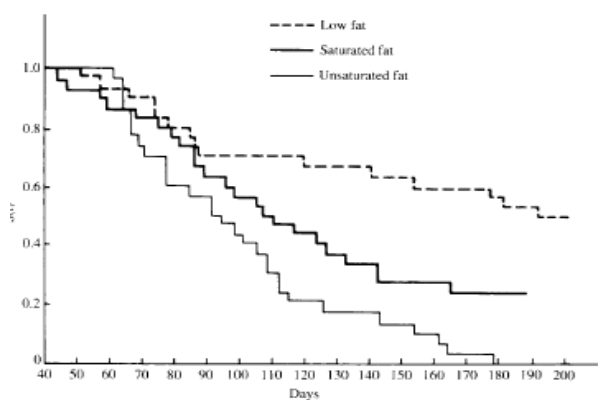


Figure 14. Survival curves

Application: comparison between heights of RBC column presence of Clopidogrel 3 mg/l and control curve in one patient

|      | $n_1$ | $n_2$ | $n=n_1+n_2$ | $d_1$ | $d_2$ | $d=d_1+d_2$ | $e_1$ | $v_1$ |
|------|-------|-------|-------------|-------|-------|-------------|-------|-------|
| 0    | 120   | 120   | 240         | 0     | 0     | 0           | 0     | 0     |
| 0,25 | 97,4  | 111   | 208         | 22,6  | 9,4   | 32          | 15    | 6,8   |
| 0,5  | 85,3  | 104   | 189         | 12,1  | 16,4  | 28,6        | 12,9  | 6     |
| 1    | 73,4  | 93,7  | 167         | 11,9  | 26,3  | 38,2        | 16,8  | 7,3   |
| summ |       |       |             | 46,6  | 52,2  |             | 44,7  | 20,1  |

Tabel III.

$n_1$  - height RBC column (mm) at measuring moment  $t_1$ , corresponding in cancer research to number of patients alive at the moment  $t_1$ ;  
 $d_1$  - height lost in the time interval  $[t_{i-1}, t_i]$  corresponding in cancer research to number of patients dying in the respecting interval;  
 $e_i$  - expected values in the hypothesis that curves differ only by chance and  $v_1$  - pooled dispersion.

$$e_{1k} = \frac{n_{1k}d_k}{n_k} \quad v_{1k} = \frac{n_{1k}n_{2k}d_k(n_k - d_k)}{n_k^2(n_k - 1)} X_{\log rank} = X_{LR} = \frac{\left(\sum_{i=1}^k d_{1i} - \sum_{i=1}^k e_{1i}\right)^2}{\sum_{i=1}^k D(d_{1i})} = \frac{(d_1 - e_1)^2}{v_1} = 0.17$$

**Conclusion:** The obtained value of the test is lower than the 0.95 quintile of Logrank test. Consequently, the test indicates that the difference between control sedimentation curves and curves in presence of 3 mg/l Clopidogrel is not significant. The result is in contradiction with the results obtained using other tests.

**General conclusions**

Single parameter characterization of sedimentation of erythrocytes by ESR represented a coarse approach of evaluation of effect of clopidogrel on sedimentation phenomena, the results being appropriate only if we look for a roughly screening test.

A more refined approach of the sedimentation phenomena in this paper consisted in estimation of the slope of sedimentation curve using more than one point. The curves were modelled for a longer time interval using a two-phase linear regression. The slope of the first line was defined as actual sedimentation rate.

Direct comparison of curves was performed by application of metrics in the spaces of curves, some of its already used in comparison of time course of effects in biopharmacy and clinical studies.

Taking  $n_1$  - height RBC column (mm) at measuring moment  $t_1$ , corresponding in cancer research to number of patients alive at the moment  $t_1$ ;  $d_1$  - height lost in the time interval  $[t_{i-1}, t_i]$  corresponding in cancer research to number of patients dying in the respecting interval, the sedimentation curves were further analysed as survival curves. In this condi-

tions, the Mantel Cox log rank test for comparison of Kaplan Meyer estimators method became immediately applicable.

The official metric  $f_2$  comparing dissolution curves in biopharmacy, based on the sum of squared differences between matched points of the curves, proved to be applicable in establishing similarity or dissimilarity of the time course of sedimentation phenomena. The same conclusion concerns the methods for comparison of areas under curves.

The effect of clopidogrel for subjects with ESR higher than 60 mm/h, the sedimentation was increasing and, for those with ESR lower than 40 mm/h, was decreasing of the sedimentation rate.

Effect of clopidogrel on the average height of sediment column was linear in concentration at all examined times (0.25, 0.5 and 1 hour).

These advanced tools allowed an estimation of clopidogrel concentration – erythrocyte aggregation dependence. All tests indicated that clopidogrel, at concentrations greater than 2 mg/l influences significantly the sedimentation of erythrocytes.

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