Original Paper

Application of Biopharmaceutical and Clinical Metrics in Analyzing Modification of Platelets Aggregation Curves by Calcium Ion

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ABSTRACT: In context of Evidence Based Medicine concept, Good Clinical Practice rules specify that "data generated should be reliable and robust". Reliability and robustness are further translated in requirements concerning statistical and clinical significance of results. Paper presents main aspects connected with comparison of evolutions of endpoints as function of different parameters like time, administered dose, proportion of active components etc., leading to problems of comparison of curves, with direct application to comparison of platelets aggregation curves in presence of different concentrations of ionic calcium. Theoretical part presents comparison of curves in biopharmacy using f2 metric and area under curve metric, and comparison of survival curves in clinical studies. Platelet aggregation test was performed using Born turbidimetric light transmission method using Helena PACKS-4 Aggregometer. Blood samples were collected from patients in internal medicine ward of Colentina Clinical Hospital. Platelet rich plasma (PRP) was obtained by centrifugation at 200G. Washed platelets where extracted by centrifugation of PRP at 2700 G. The supernatant was replaced with sodium chloride 0.9%. Platelets aggregation was induced by adding different concentrations of calcium gluconate into cuvettes which contained washed platelets. After digitalization, curves were compared using similarity factor f2 and areas under curves. Paper puts in evidence that both type of comparison, after mathematical and statistical evaluation, have to define a clinical threshold for clinical significance. In case of f2, in dissolution studies the threshold is 10%, in case of bioequivalence based on area under curves threshold is 20%. Establishment of the threshold for significant clinical difference in comparison of aggregation curves is not only a problem of statistics. Graphical representation of data suggested significant differences between curves obtained with different concentrations of calcium ion. Application of both f2 method and log-rank test let to conclusion that differences were statistical significant. Representation of aria under curves as function of calcium concentration put in evidence an approximate linear dependence. In spite of apparently objective character of mathematical approach, the problem of comparison of aggregation curves remains practically unsolved since we do not know the threshold between clinical significant and non-significant results.

KEYWORDS: *Biopharmaceutical metrics, Aggregation curves, Survival curves*

Introduction

The recent new regulations of clinical studies [1] define in the first article the main characteristics of clinical trials: "Art. 1 In a clinical trial the rights, safety, dignity and well-being of subjects should be protected and the data generated should be reliable and robust". If the first part concerns more clinicians, the second one refers mainly to mathematicians, but this is only an appearance since reliability and robustness or, in a more general approach "Evidence Based Medicine", addresses equal to mathematicians and clinicians.

One important chapter in the analysis of clinical data concerns comparison of evolutions of endpoints as function of different parameters like time, administered dose, proportion of active components etc., leading to problems of comparison of curves.

Comparison of drug performances in biopharmacy includes comparison of dissolution curves and comparison of plasma levels curves. It concerns in vitro curves it were considered a series of "metrics" and rules for establishing the similarity of dissolution, based on differences or ratios of matched values corresponding to the same measuring time. Some of the methods for comparison are strictly regulated by Food and Drug Administration, European and other country guidance. Further evaluation includes comparison of in vivo curves. Correlations between in vitro and in vivo curves are undertaken in order to obtain models for predicting in vivo pharmacokinetics.

It was recently proposed the extrapolation of application of biopharmaceutical metrics to comparison of erythrocytes sedimentation curves [2]. The gold standard endpoint in case of pharmacokinetics clinical studies is the Area Under Curve (AUC). This was used for evaluation of in vitro-in vivo correlations [3,4], for predicting safety and efficacy of drugs starting from physiological models [5,6], for comparison of administration schedules,

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evaluation of liver-or renal impairment, comparison of special populations, single vs. multiple doses, comparisons in titration studies etc. [7-11].

The area under curve is called "extent of absorption metric" being a measure of total absorbed drug in blood stream and indirectly of the effect. It is accepted that two drugs containing the same active substance which achieves the same area under curve, have the same therapeutic effect.

Blood is a stable, highly concentrated suspension of cells. The theory of Derjaguin, Landau, Verwey, Overbeeck [12,13] extended [14-16] from the theory of aggregation of lyophobic colloids tries to explain both mechanism and the factors implied in physical interactions of all type of living cell suspensions (microorganisms, bacteria, some viruses, yeast and blood) [17,18] and their interactions with different surfaces [19-21]. In agreement with this theory all negatively charged substances decrease aggregation and all positively charged molecules increase aggregation, being potentially thrombogenic. In this context it was expected that calcium ion will increase aggregation.

The aim of this paper is to quantitatively evaluate the calcium influence on platelet aggregation process as function of concentration, by comparison of aggregation curves.

Experimental methods

Platelet aggregation test was performed using Born turbidimetric light transmition method [22-24] using Helena PACKS-4 Aggregometer. Blood samples were collected from patients of internal medicine ward of Colentina Clinical Hospital used for determining erythrocytes sedimentation rate (ESR). After determination of ESR, plasma was separated and processed farther for determination of platelets aggregation.

Plasma as well as whole blood were stored at room temperature (15-30°C). Tests were performed within three hours after sample collection.

Specimen Preparation

Platelet rich plasma (PRP) was prepared by centrifuging samples at 100 x G 10-15 minutes at room temperature. PRP was separated with a plastic pipette and placed into a plastic tube labeled PRP.

Platelet poor plasma (PPP) was prepared by re-centrifuging the remaining blood samples at 2700xG for 10-15 minutes at room temperature. It was removed PPP, placed them in a plastic tube labeled PPP and cover.

Washed platelets where extracted by centrifugation of PRP at 2700G. The supernatant was replaced with sodium chloride 0.9%.

It was selected a lot of 4 probes of 450µl off PRP in which were added 50µl calcium gluconated 94mg/ml solutions with sodium chloride 0.9% at 1:2, 1:2.5, 1:3 and 1:4 ratios resulting the concentrations: 3.13, 2.68, 2.35, and 1.88 mg/ml salt. Since molecular weight of salt is 430 and atomic weight of Ca is 40, final concentrations of calcium ion in samples were 0.29; 0.25; 0.22; 0.17mg/ml.

Theory of comparison of curves in biopharmacy and clinical studies.

Estimation of similarity of dissolution curves using f_2 metrics. In biopharmacy as "surrogate" of bioequivalence studies, in some cases (mainly in the case of poor soluble, highly permeable drugs) is tested the similarity or dissimilarity of dissolution curves [26]. Official parameter in this respect is the f_2 factor:

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

R_i and T_i-represent the experimental percent dissolution values obtained for the reference and tested drug; n-number of measured data.

It concerns the threshold between similarity and non-similarity, this is taken as 10%. If the difference is 10% in all points, it obtains for f_2 the value 50. For this reason, if the f_2 value is greater than 50, the curves are considered similar. If the obtained value is lower than 50 it is accepted the hypothesis of dissimilarity.

Comparison of Areas Under Plasma levels curves (AUC). A tested drug T is called bioequivalent with a reference drug R, if the 90% confidence interval for the ratio of mean AUCs is included in the 0.8-1.25 interval:

$$P\left(0.8 < \frac{\mu_{AUC}^{T}}{\mu_{AUC}^{R}} < 1.25\right) \leq 0.9$$

If drugs are bioequivalent, the regulatory bodies consider the drugs to be therapeutic equivalent and interchangeable on the market.

Definition of bioequivalence if adopted by United States Congress and promulgated as federal law.

We propose the application of these metrics to comparison of aggregation curves. We computed the AUC s under curves and denoted

these as Areas Under Aggregation Curves (AU-AC).

Areas were further calculated using the trapeze rule:

$$AU-AC=\sum_{i=1}^{n}\frac{a(t_{i-1})+a(t_i)}{2}*(t_i-t_{i-1}),\ \text{where}$$

$a(t_{i-1})$ and $a(t_{i-1})$ are % absorbance corresponding to consecutive measurement time points.

The method of comparison of survival curves. The main achievement desired in treatment of cancer is the survival of patients. The distribution in time of the number of patients still alive is called "survival curve". Comparison between treatments includes in all cases comparison of survival curves.

The cumulative distribution function (CDF) for survival time, denoted by *F(t)*, is defined as the probability that a subject fails before or equal to the time t, namely:

$F(t)=P(a\ subject\ \ fails\ before\ or\ at\ time\ t)$, "CDF" is a nondecreasing function of time such that $F(0)=0$ and $F(\infty)=1$

Let $t_1<...<t_k$ be the ordered distinct failure times when events occur and d_k be the number of events at time t_k (Fig. 1). The number of subjects in the risk set just prior to the time t_k, is denoted by n_k.

The Kaplan-Meier nonparametric estimation of the survival function at time t is given by:

$$\hat{S}(t)=\prod_{t_k<t}\left(1-\frac{d_k}{n_k}\right)$$

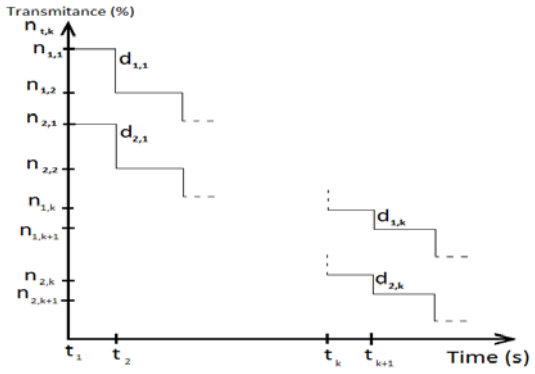

Fig. 1. Graphical representation of two survival curves

It can be observed that, in the time interval $[t_k,t_{k+1}]$ the numbers of surviving patients were reduced by respectively d_{1k} and d_{2k}. Denoting $\mathbf{d_k}=d_{1k}+d_{2k}$ and $\mathbf{n_k}=n_{1k}+n_{2k}$, we can summarize the events as for example in below presented table:

$$\begin{pmatrix} d_{1k} & d_{2k} & \mathbf{d_k} \\ n_{1k}-d_{1k} & n_{2k}-d_{2k} & \dots \\ \mathbf{n_{1k}} & \mathbf{n_{2k}} & \mathbf{n_k} \end{pmatrix}$$

Let us accept the hypothesis: H1: $(d_{1k},n_{1k}-d_{1k})$ *and* $(d_{2k},n_{2k}-d_{2k})$ *are selection from the same population and their differences appeared only by chance* (i.e., the two hazards are the same for treated and control groups).

If such the case, we can estimate the ratio of deaths based on the joined group (control+treatment) as $r_k=\mathbf{d_k}/\mathbf{n}_k$ The expected number of deaths in first group will become:

$$e_{1k}=r_k n_{1k}=\frac{\mathbf{d_k}}{\mathbf{n_k}}n_{1k}$$

Different tests use different estimations of the variance of d_k.

Most usual is the formula

$$v_{1k}=\frac{n_{1k}n_{2k}d_k(n_k-d_k)}{n_k^2(n_k-1)}$$

Using χ^2 test is possible to verify the hypothesis H1, but main interest is connected with comparison of curves as a whole and not at a given time point. Adding a supplementary hypothesis

H2: The hazard ratio HR_k *between treatment and control subjects is the same whatever the time,* and hypothesis $H_3: HR=1$) a method for testing the two survival curves are not different, using the above set of hypothesis, is the log-rank test:

$$X_{\log rank}=X_{LR}=\frac{\left(\sum d_{1k}-\sum e_{1k}\right)^2}{\sum v_k}=\frac{(d_1-e_1)^2}{v_1}$$

, where

$$v_1=\sum v_{1k}=\frac{n_{1k}n_{2k}d_k(n_k-d_k)}{n_k^2(n_k-1)}$$

In fact the test is χ^2 test, the name log-rank coming from one of the methods to build the test.

Results and Discussion

Aggregation curves obtained in case of a patient which suffered some days before the blood sample collection a stroke, in presence calcium gluconate are presented in Fig. 2. In

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order to make a quantitative comparison of curves for estimation of the effect of calcium ion, data were digitalized, and converted in columns in tables in formats easy to apply the proposed calculus. Digitalization is needed since all comparisons are discrete operations.

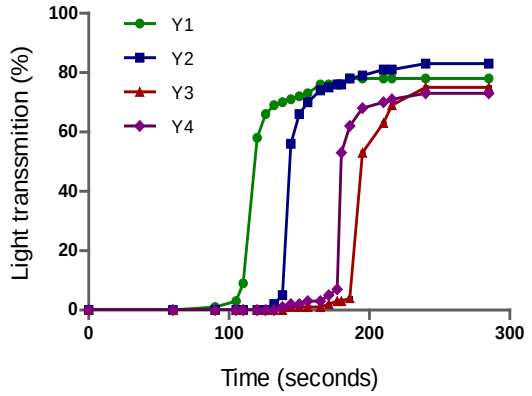

Fig. 2. Platelet aggregation curves in presence of Ca^{2+}

Usual comparison of aggregation curves is based on the maximum percent aggregation response (MPA) to adenosine diphosphate (ADP) and inhibition of platelet aggregation (IPA): Inhibition of aggregation (IPA) is calculated using the formula

$\left[1-(MPA_{(t)}-MPA_{(0)})\right]*100\%$, where $MPA_{(0)}=MPA$ at baseline and $MPA_{(t)}=MPA$ at given time point (t) [25,26].

This approach is based only on the final points of the curves and neglect the fact that curves start at different time points, having different slopes in linear part, different curvature in rest etc. Pass over these parameters means neglection of information connected with initial phase of aggregation, aggregation kinetics, and mean size of aggregates. etc., which phenomena define the time course of pharmacodynamic effect.

Our proposed methods try to improve the comparison by taking into consideration the shape and position of curves in space and, additionally, to consider the assessment of statistical significance of differences between curves.

Evaluation of calcium ions effect as function of concentration by application of biopharmaceutical metric AUC

Curves assigned to the four Ca^{2+} concentrations, represent the transmittance (the percentage of transmitted light) dependence on time. The increase of transmittance indicates the formation of platelet aggregates. In order to make comparison the curves digitalized. Points on curves were selected so that to approximate as well as possible the areas.

Table 1. Calculation of AU-AC in case of the curve Y1

X*(sec)*	**Y_1**	***partial* AU-AC**	***cumulated* AU-AC**
0	0	0	0
60	0	0	0
90	1	15	15
105	3	30	45
110	9	30	75
240	78	1872	9375.5
285	78	3510	**12885.5**

In order to estimate the effect of the of calcium ion on the aggregation, the areas under curves were considered as "primary endpoint". A problem in application of the theory of comparison of aggregation curve is the integration interval in pharmacokinetics it is used the integral from 0 to infinity, $\int_0^{\infty} T(t)dt$ in case of aggregation light transmittance curve attains a saturation value. An adequate approach is to restrict the integral to $\int_0^{T_s}$, where T_s is a time saturation of the curve in case of comparison of more curves, it is naturally to consider the time T_s when all curves attain a saturation value, It was selected the time 285 seconds.

Calculus was undertaken for all four curves using the model presented in Table 1 and represented graphically as function of concentration. The result is presented in Figure 2.

It was obtained a good enough linear model (R^2=0.93) for decrease of AU-AC with increasing Ca^{2+} concentration.

Evaluation of calcium ions effect by application of biopharmaceutical metric f_2

In Table 2 there are presented the transmittence values and the calculus of f_2 undertaken in order to decide concerning similarity of pairs of curves. It was considered that similarity means lack of a calcium effect and non-similarly, the presence of the effect.

Table 2. Calculus of f_2 factor for pairs of curves

X (sec)	Y_1	Y_2	Y_3	Y_4	Y_1 -Y_2	Y_1 -Y_3	Y_1 -Y_4	Y_2 -Y_3
0	0	0	0	0	0	0	0	0
60	0	0	0	0	0	0	0	0
90	1	0	0	0	1	1	1	0
105	3	0	0	0	3	3	3	0
…	…	…	…	…	…	…	…	…
216	78	81	69	71	-3	9	7	12
240	78	83	75	73	-5	3	5	8
285	78	83	75	73	-5	3	5	8
					16869	60781	48073	40104
					4	2	2	2
				f_2 =	28	14	17	18

The results suggest significant differences between all studied pairs of curves, i.e. steps choose for differences in concentrations correspond to significant differences in effect.

The problem to solve in case of platelets aggregation is a comparison between *ex vivo* curves, which can be considered as an intermediary case between *in vitro*, where we accept only a 10 % mean difference as threshold and *in vivo*, where due to a greater variability, we tolerate a 20 % difference. In case of 20% difference, the threshold becomes 35. It can be seen that, in such case also, curves are not similar, i.e. effect of $Ca^{2+}$is statistically significant.

Analysis of data using methods for comparison of survival curves

Turbidimetric method for evaluation of aggregation measure the light transmitted across highly concentrated suspension of platelets. At the beginning, light is practically completely absorbed. Ulterior, following the aggregation of platelets, more and more light is transmitted through the appeared gaps. Finally, at infinite time, when platelets join to a single, giant complex which eventually sediment, light is completed transmitted and absorbed part tends to zero. So that, aggregation curve can be interpreted as "dying" of individual or small complexes of platelets which translates in "dying" of absorbed light quanta.

In this context, the digitalized curves Y_1 and Y_2, appear as can be seen in Fig. 3.

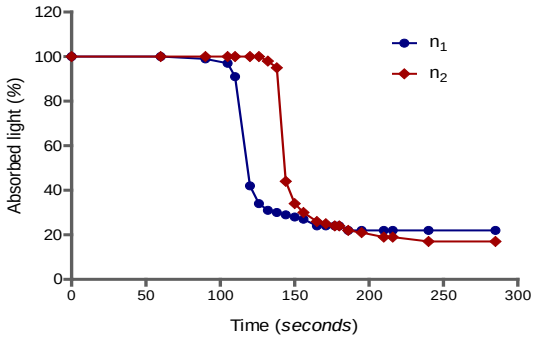

Fig. 3. Aggregation curves as "survival" of platelets and light absorption

χ^2 test was applied in order to establish if the two survival curves are identical or different. A significance difference between the curves can be interpreted as a possible significant effect of calcium as function of concentration, though frequently, there is no a direct correlation between stastistical and clinical significance.

Table 3.

X(sec)	Y_1	Y_2	n_1	n_2	n	d_1	d_2	d	e_1	v_1
0	0	0	100	100	200	0	0	0	0	0
60	0	0	100	100	200	1	0	1	0.5	0.25
90	1	0	99	100	199	2	0	2	0.995	0.497
105	3	0	97	100	197	6	0	6	2.954	1.461
110	9	0	91	100	191	49	0	49	23.35	9.135
120	58	0	42	100	142	8	0	8	2.366	1.584
….	…	…	…	…	…	…	…	…	…	…
240	78	83	22	17	39	0	0	0	0	0
285	78	83	22	17	39	22	17	39	22	0
						100			80.8	26.1

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Since the obtained value (11.17) is greater for example than 3.84 (the threshold $\chi^2_{1;0.95}$) results that, with probability greater than 0.95, the curves are different and increasing of calcium concentration from 0.17mg/ml to 0.22mg/ml led to increase of aggregation.

Conclusions

1. Usual approach of comparison between aggregation curves is based only on the final points of the curves and neglects the fact that curves start at different time points, having different slopes in linear part, different curvature in rest etc. Pass over these parameters means neglection of information connected with initial phase of aggregation, aggregation kinetics, mean size of aggregates etc., which phenomena define the time course of drugs effects on aggregation.

2. F_2 metric can be used to compare pairs of curves and to determine whether they are similar or not starting from a finite number of points selected on the curves. The difference between similar and dissimilar should be a phenomenological criterion to be established correlated with clinical significance.

3. Area under aggregation curves was also a single parameter associated to curve, but this parameter takes into consideration practically all points on curves and proved to be more sensitive to quantitative differences in pro aggregant activity of Ca^{2+} than the final point of curves. The effect of calcium ion on AU-AC depended linear on concentration.

4. The evaluation of statistical significance of curves differences using methods for comparing survival curves in cancer is applicable mainly for the reason that platelet aggregation is an irreversible phenomenon.

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