

PHARMACOKINETIC AND MATHEMATICAL OUTLIERS FOR DRUGS WITH ACTIVE METABOLITES. NOTE I. MODEL INDEPENDENT ANALYSES FOR PENTOXIFYLLINE

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Abstract

The paper presents the difficulties in establishing the bioequivalence of formulations containing pentoxifylline, following a high variability of both absorption and metabolism.

The paper presents the case of two true outliers giving discordant results even after the evaluation of the sum of active substances (i.e parent drug and metabolites).

Examined parameters included C_{max} (maximum concentration), AUC (area under the curve) after single dose and in steady-state after multiple doses, and their ratios. The comparison was made both intra and inter- subjects.

The result was that two subjects were essentially outliers, but only a part of parameters or combinations of parameters showed discordant values.

The conclusion was that the evaluation of bioequivalence would be more efficient or rational starting from the sum of active entities, than separately, as they are required by the current EMEA (European Medicines Agency) Regulations.

Rezumat

Lucrarea descrie dificultățile întâmpinate în stabilirea bioechivalenței formulărilor ce conțin pentoxifilină, ce prezintă o varibilitate mare atât în privința absorbției, cât și a metabolismului.

Lucrarea prezintă două cazuri de discordante ("outliers") ce determină rezultate anormale, chiar și după evaluarea sumei substanțelor active (medicamentul și metabolizii).

Parametrii examinați au fost C_{max} (concentrația maximă), AUC (aria de sub curba) după administrarea în doză unică și în starea de echilibru, precum și rapoartele lor. Comparățiile au fost făcute atât între subiecți, cât și intra individual.

Rezultatul a fost acela că doi subiecți au apărut a fi, în mod evident, outliers, dar numai o parte din parametrii sau combinații ale acestora prezentau valori discordante.

Concluzia a fost că evaluarea bioechivalenței ar putea fi mai eficientă și rațională, pornind de la suma concentrațiilor substanțelor active și mai puțin de la entitățile separate, așa cum este stipulat în Reglementările EMEA (Agenția Europeană a Medicamentului).

Keywords: outliers, pentoxifylline pharmacokinetic, active metabolites.

Introduction

Etymologically, outlier comes from “out of line” signifying some values or set of values differing from the rest of data, without a direct justification for their removal as unreliable values resulting from an infringement of the distribution law of the population data.

Concerning the name, some other terms as “discordant values” or “out-law values” or “non-normal values” or “discrepant values” are in use [1].

General problems to be solved in case of appearance of suspicion of outliers would be:

- decision regarding rejection or omission of suspected values;
- analysis of their distortion effect on statistical inferences of population parameters;
- evaluation of the hypothesis concerning the law or model correlating the experimental data.

Acceptable explanations in order to exclude pharmacokinetic data or to exclude a subject would be protocol violations like vomiting, diarrhoea, analytical failure etc. The search for such explanations must apply to all subjects in all groups independently of the size of the observed pharmacokinetic parameters or its outlying position.

Particularly, in bioequivalence (BE) studies an outlier value becomes a „problem” when its elimination changes the decision of bioequivalence. In this case it appears naturally the suspicion of subjective analysis, oriented by a particular interest of the authors of the study.

It is clear, on the other hand, that only one tablet of reference drug, which doesn't disintegrate, introduces an infinite value in the ratio of areas under the curve (AUC) which is impossible to be compensated by the rest of data.

Recent European proposal [2] for new BE rules specifies concerning the elimination of outliers: “Exclusion of data can never be accepted on the basis of statistical analysis or for pharmacokinetic reasons alone, because it is impossible to distinguish the formulation effects from other effects affecting the pharmacokinetics”.

As on indirect rule, we could understand that correlated pharmacokinetics and statistical arguments would be the only acceptable approach.

The present paper presents the case of two subjects included in a BE study comparing two extended release oral formulations containing pentoxifylline, looking as outliers from the analysis of data concerning both parent drug and metabolites. Since the metabolism is also reversible, a

rational evaluation of bioequivalence would consider both separated and together the parent drug and metabolites since low and high plasma levels of parent drug can be compensated by high or low levels of metabolites.

Since European Regulations require the evaluation separately, for parent drug and metabolites, a considerable part of the subjects could appear as false outliers.

The main goal is the presentation of a multidisciplinary analysis of the information (physicochemical, pharmaceutical, pharmacokinetic, statistic etc.) that has to be correlated in order to decide on the exclusion of data.

Theory:

From the mathematical point of view, the central idea of rejecting some values as outliers is based on the structure of errors in a “normal” distributed population (figure 1).

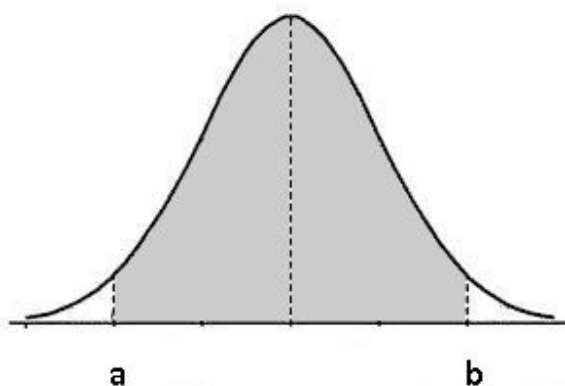


Figure 1

Errors in normal distribution

If values are normally distributed, the most probable is the mean value and probability of other values decreases exponentially with their distance from the mean.

The probability of finding a value in a given interval (a, b) is given by the formula:

$$P(a < X < b) = \frac{1}{\sigma\sqrt{2\pi}} \int_a^b e^{-\frac{(x-\mu)^2}{2\sigma^2}} dx \quad (1)$$

where, σ^2 is the variance and μ is the mean of population.

Values of the integral are found in annexes of all statistical books.

In these conditions, it is easy to verify that a probability of values far no more than three standard deviations from the mean value is very close to 1.

$$P(\mu - 3\sigma < X < \mu + 3\sigma) = P(-3 < \frac{X - \mu}{\sigma} < 3) = \frac{1}{\sqrt{2\pi}} \int_{-3}^3 e^{-\frac{(x)^2}{2}} dx = \quad (2)$$

$$\Phi(3) - \Phi(-3) = 0.997$$

Even in the case of values that are not normally distributed, the probability of the values in the interval $(\mu - 3\sigma, \mu + 3\sigma)$ remains very high.

The classical Tchebashev inequality establishes that whatever the probability distribution of data:

$$P(\mu - 3\sigma < X < \mu + 3\sigma) \geq 0.88 \quad (3)$$

In such case, it seems that it is possible to solve the problem of outliers in the frame of mathematics.

Unfortunately, this is not true. If, for example, the studied drug is metabolized differently by the subjects included in the study, some of them are normal metabolizers, some are poor metabolizers and some rapid metabolizers and the problem becomes infinitely more complex.

A huge value could represent, in fact, a poor metabolizer.

If supplementary, the metabolites are therapeutically active and the authorities require including of data concerning metabolites in the decision concerning BE plasma levels of metabolites, the problem becomes from virtual infinitely complex to actual infinitely complex.

In the following analysis, keeping in mind the reversibility and variability of metabolism, data were compared separately as well as combinations of parent drug and metabolites, after single dose and after multiple doses.

Experimental methods

Clinical method:

Pentoxifylline tablets were administered to healthy subjects in a cross-over two-period, two sequences bioequivalence study.

Tested and reference drugs were administered in two periods of five days, separated by a seven days wash-out period. Plasma levels were measured in the first and the fifth day of each period.

The study was approved by the National Ethics Committee and the National Medicines Agency.

Analytical method:

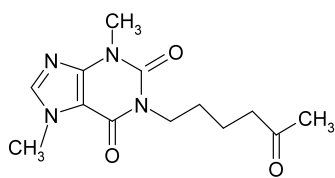
Plasma levels of pentoxifylline and its M1 (1-(5'-hidroxyhexyl)-3,7-dimethylxantine) and M5 (1-(3'-carboxypropyl)-3,7-dimethylxantine) metabolites were determined using a Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC/MS/MS) validated method.

Computerized methods for the estimation of parameters:

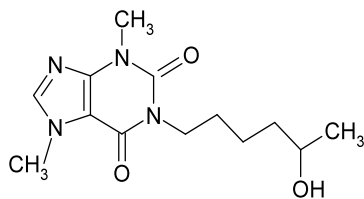
Estimation of parameters was performed using the subroutines of the software KINETICA 3.1 and TOPFIT 2.0 [3].

Results and discussion

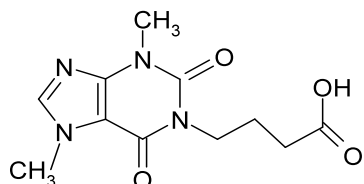
After oral administration, pentoxifylline (3,7-dimethyl-1-(5-oxohexyl)-3,7-dihydro-1H-purin-2,6-dione) is rapidly absorbed and intensively metabolized at the first hepatic passage in two major, active metabolites: 1-(5'-hidroxyhexyl)-3,7-dimethylxantine (M1) and 1-(3'-carboxypropyl)-3,7-dimethylxantine (M5).



Pentoxifylline



Metabolite M1



Metabolite M5

The reduction of the keto group of pentoxifylline to secondary alcohol in M1 takes place in erythrocytes [4] and liver. Conversion to M1 is rapidly reversible and creates a chiral center [5].

Inducers (table I) stimulate the production of the enzyme thus increasing the rate of metabolism causing the substrate drug to clear out of the system faster [6, 7].

Table I
Inducers of the metabolism of pentoxifylline

Inducers of cytochrome P450 isoform 1A2 (CYP1A2)	Inducers of cytochrome P450 isoform 2E1 (CYP2E1)
broccoli brussel sprouts char-grilled meat insulin methyl cholanthrene modafinil nafcillin	ethanol isoniazid eta- naphthoflavone omeprazole tobacco

Consequently, the pharmacokinetics of pentoxifylline and its metabolites M1 and M5 is expected to be highly variable following a high variability in metabolism though in the protocol was stipulated that subjects didn't take any of these inducers.

In these conditions, it was decided to measure both pentoxifylline and its major metabolites M1 and M5. Finally, our data confirmed this hypothesis.

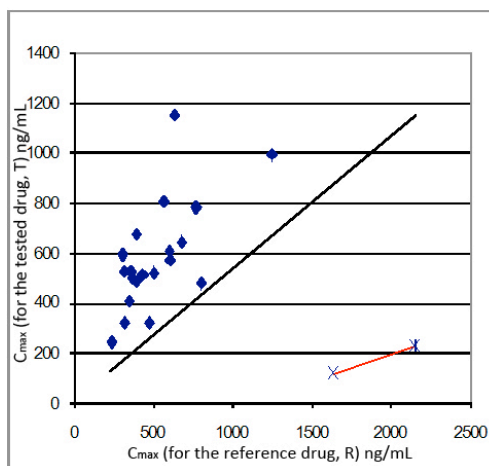
Only intra-variability has an effect on the decision of bioequivalence. Their estimation for the maximum concentration (C_{max}) was measured by Root Mean Square Error from ANOVA and is presented in table II.

Table II
Root Mean Square Error from ANOVA (%)

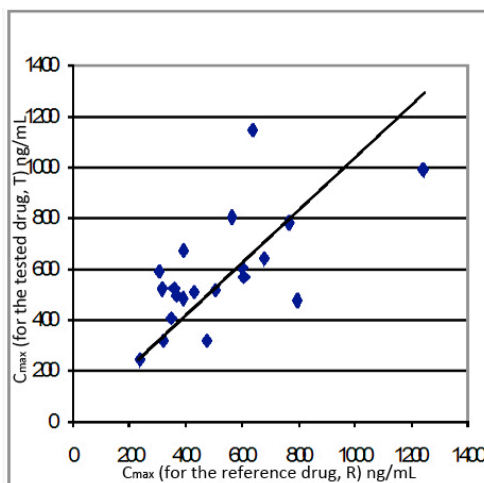
Root Mean Square Error from ANOVA (%) for all subjects			Root Mean Square Error from ANOVA (%) after elimination of N1 and N2 subjects		
Active substance	Single dose	Steady state	Active substance	Single dose	Steady state
Pentoxifylline	42	41	Pentoxifylline	42	35
M1	25	56	M1	25	23
M5	25	26	M5	25	26

It can be seen that a huge variability appeared in the pharmacokinetics of pentoxifylline and M1, mainly at steady-state. Since the values for M1 at single dose and the values for M5 were lower and practically equal, there were examined in detail the pairs of curves for M1 at single dose and multiple doses, in order to estimate the subjects' sources of great variability. There were identified as outlier candidates, two subjects named, in the following, subject N1 and subject N2.

In figure 2 are presented the (R – reference drug, T – tested drug) pairs for maximum concentrations for M1 at steady state. It can be observed that there is a very poor correlation with line $R=T$ (which is normal result), two points, corresponding to Subjects N1 and N2 are „influential points” with a high effect on the estimation of linear regression.

**Figure 2**

(R,T) pairs of C_{max} of M1 at steady-state (22 Subjects)
 where: R – reference drug, T – tested drug

**Figure 3**

(R,T) pairs of C_{max} of M1 at steady-state face to the theoretical line $R=T$
 where: R – reference drug, T – tested drug

Their elimination led to a significant improved alignment of data, as it can be seen in figure 3.

Significant information is to be observed in the representation of all values of C_{max} (figure 2): outliers appear to be the values of maximum concentrations corresponding to subjects N1 and N2, after the administration of the reference drug. In the same time, Root Mean Square Error from ANOVA decreased from 56 to 23, a value close to the other values in table II. The estimation decreased also in the case of the parent

drug from 42 to 35 at steady state, but remained unchanged for the data after single administration.

The effects of the subjects N1 and N2 on the evaluation of bioequivalence were very high, as it can be seen clearly in figure 4 and figure 5, the ratios between T and R being very high in the first period, and extremely low in the second period.

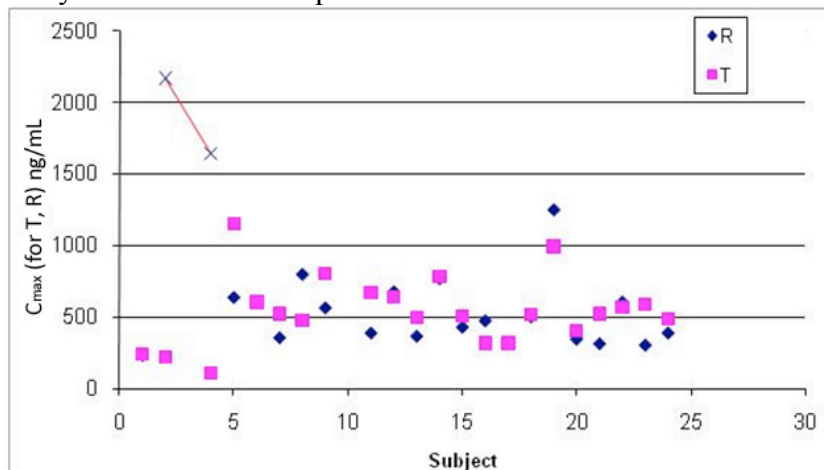


Figure 4

Distribution of C_{max} values of M1 for tested (T) and reference (R) drugs

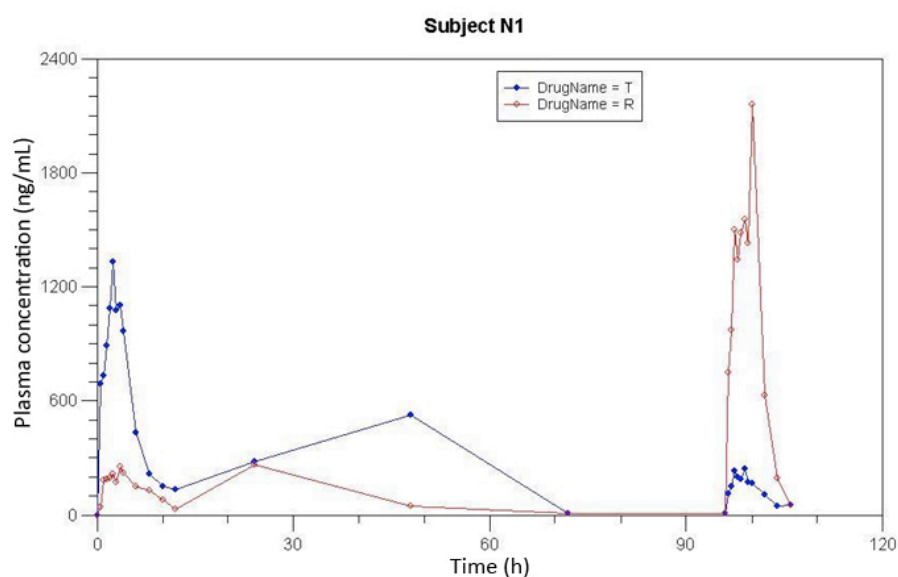


Figure 5

Plasma levels of M1 for subject N1 during five days of experiment

The effects of two outliers on the confidence interval for the ratio of means are presented in Table III. In fact, the decision concerning BE was set up from acceptance to rejection.

Table III

Influence of outliers on the estimation of 90% confidence interval for the ratio of means

Analyzed data	90% Confidence interval for the ratio of means
M1 SGL for AUC _{last} - all subjects	1.00-1.34
M1 SGL for AUC _{last} - after elimination of subjects N1 and N2	0.97-1.21

where: SGL – single dose

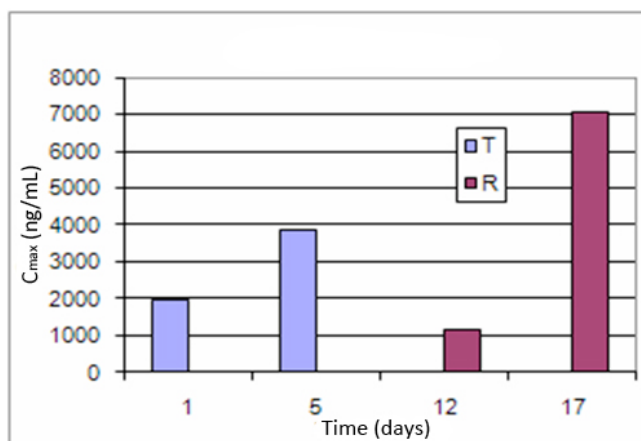
Since it is normal that the disappearance of the parent drug to be followed by increased concentrations of metabolites, it was analyzed the evolution of the sum of the maximum concentrations in the fourth periods of the experiment in the case of subjects N1 and N2: tested drug single dose, tested drug at steady state, reference drug at single dose, reference drug at steady state.

Table IV

Sum of C_{max} values for Pentoxifylline (P),
M1 and M5 for subject N1

C _{max} P+M1+M5, N1 (ng/mL)			
Time (days)	T	R	T/R
1	1941		1.73
5	3839		
2		1124	
7		7067	0.54

where: R – reference drug, T – tested drug, T/R – tested drug : reference drug ratio.

**Figure 6**

Time course of the sum of C_{max} values for Pentoxifylline, M1 and M5 for subject N1

Table V
Sum of $C_{\max}$ values for Pentoxifylline, M1 and M5
for subject N2

$C_{\max}$ P+M1+M5, N2 (ng/mL)			
Time (days)	T	R	T/R
1	2066		1.38
5	945		
12		1500	
17		2408	0.39

where: R – reference drug, T – tested drug, T/R – tested drug : reference drug ratio.

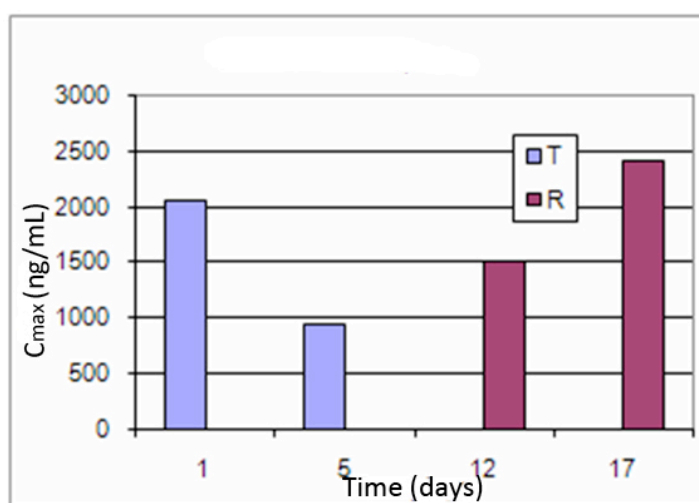


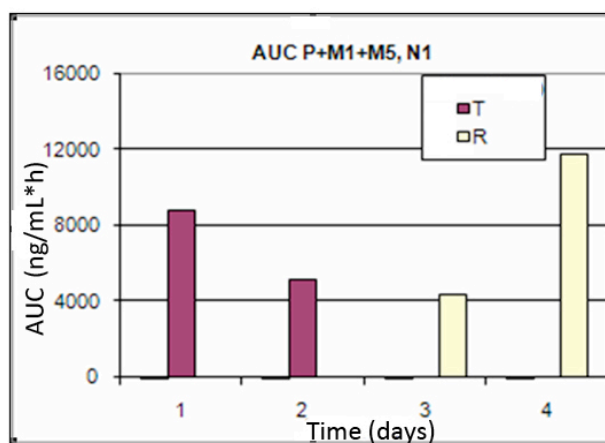
Figure 7

Time course of the sum of $C_{\max}$ values for Pentoxifylline, M1 and M5 for subject N2

Table VI
Sum of AUC values for Pentoxifylline, M1 and M5
for subject N1

AUC P+M1+M5, N1 ng/(mL·h)			
Time (days)	T	R	T/R
1	8801		2.03
5	5151		
12		4337	1.19
17		11757	0.44
$R_{SS}/R_{SGL}=1.34$			

where: R_{SS} – steady state, R_{SGL} – single dose

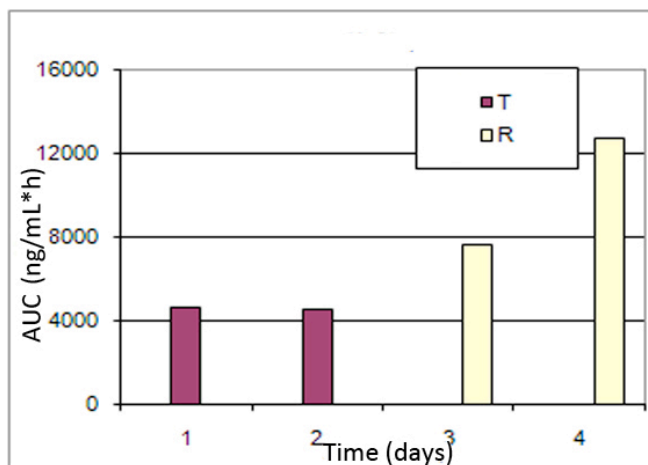
**Figure 8**

Time course of the sum of AUCs values for Pentoxifylline, M1 and M5 for subject N1

Table VII

Sum of AUC values for Pentoxifylline, M1 and M5
for subject N2

AUC P+M1+M5, N2 ng/(mL·h)			
Time (days)	T	R	T/R
1	4624		0.60
5	4520		
12		7643	
17		12734	0.35

**Figure 9**

Time course of the sum of AUCs values for Pentoxifylline, M1 and M5 for subject N2

It can be seen that, if for the tested drug (first two columns in each graphic) it was obtained a great, but somewhat random variability, for the reference drug it was obtained in all cases the same behavior: a great or even huge increase of C_{max} and AUC from single-dose to steady state in the last periods of the experiment.

Calculation of T/R ratio confirms the impression obtained from the examination of the curves. For example, for subject N1 for AUCs, it obtains a 203% greater bioavailability, after the first dose and only 44% of reference at equilibrium.

The C_{max} data in table VIII represent the R, T and ratio T/R values, and in table IX - ascending ordered values for the ratio T/R for all twenty-two subjects included in the study, for the metabolite M1. As can be seen, the T/R ratio's value for the subject N1 is 526.4, so very high, if we compare it with the values of the other subjects.

Table VIII

Cmax values for R, T and T/R ratio

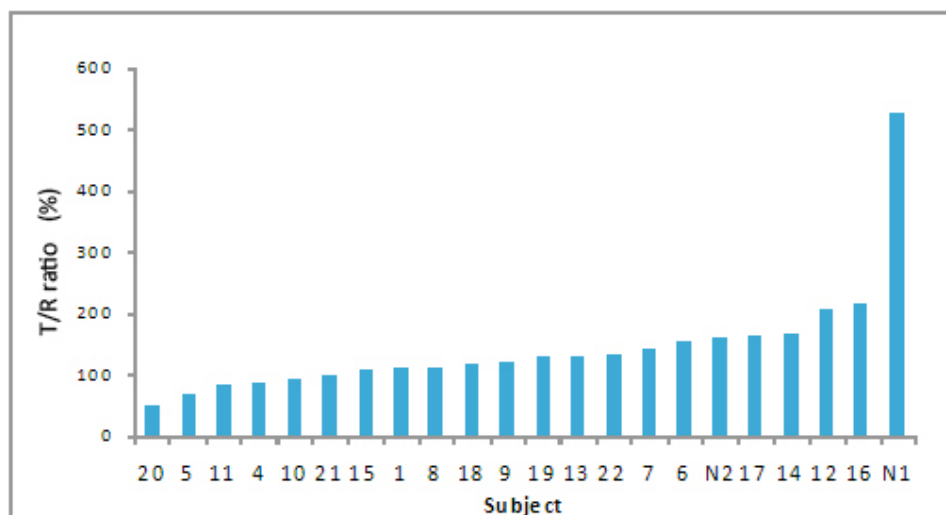
Subject*	R	T	T/R %
1	176.41	196.24	111.2
N1	251.95	1326.23	526.4
N2	1067.76	1714.74	160.6
4	666.33	576.25	86.5
5	650.78	433.35	66.6
6	233.2	359.7	154.2
7	481.68	687.04	142.6
8	398	443.08	111.3
9	299.33	354.55	118.4
10	640.51	589.93	92.1
11	334.32	284.09	85.0
12	366.19	756.21	206.5
13	334.3	437.24	130.8
14	133.15	220.98	166.0
15	249.28	263.97	105.9
16	271.65	585.53	215.5
17	465.8	760.2	163.2
18	313.09	362.85	115.9
19	347.05	447.66	129.0
20	582.77	288.91	49.6
21	296.11	295	99.6
22	354.33	475.15	134.1

Table IX

Ascending ordered values for T/R ratio

Subject	T/R % - ascending ordered
20	49.6
5	66.6
11	85.0
4	86.5
10	92.1
21	99.6
15	105.9
1	111.2
8	111.3
18	115.9
9	118.4
19	129.0
13	130.8
22	134.1
7	142.6
6	154.2
N2	160.6
17	163.2
14	166.0
12	206.5
16	215.5
N1	526.4
r = 0.72	

*The numbers of subjects were assigned randomly

**Figure 10**

Ascending ordered values for the ratio T/R

Though it is clear from the simple examination of the figure 10 that subject N1 is an outlier, we proceeded also to the application of statistical tests.

The Dixon Test was applied [8] for extreme values, in our case, the highest data presented in table V.

$$r = \frac{(X_k - X_{k-2})}{(X_k - X_3)} \quad (4)$$

where, X_k is the highest value, X_l is the opposite extreme.

It was obtained the value $r=0.72$, which means the data is greater than 0.43, the 5 % level of significance.

So, Dixon's criteria for rejecting outliers concluded that subject N1 is an outlier if we consider the data for the metabolite M1.

Another highly recommended test for outliers, the T method, is also calculated as a ratio, designated T_n , as follow [9]:

$$T = \frac{X_n - \bar{X}}{S} \quad (5)$$

where, X_n is the highest/extreme value, $\bar{X}$ is the mean and S is the standard deviation.

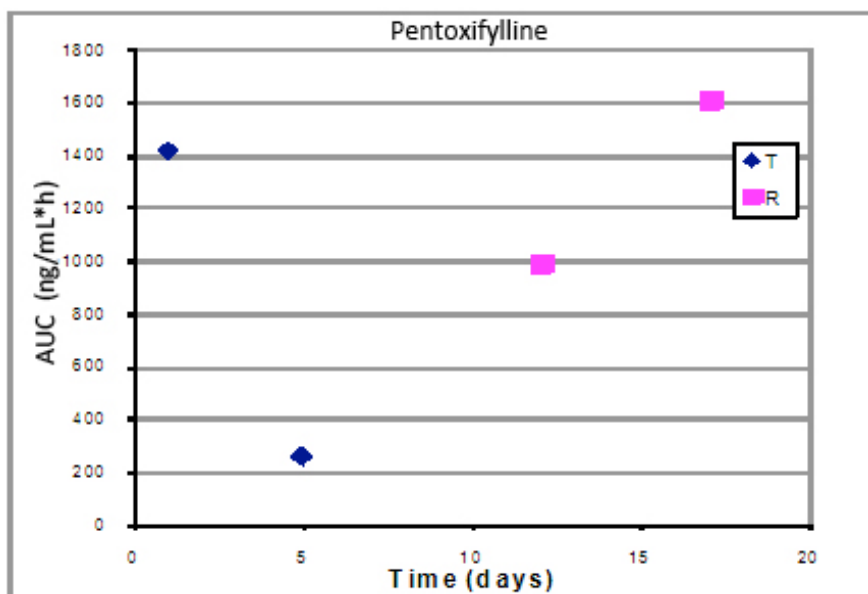
The value obtained for T method for the subject N1, using the data for the metabolite M1 metabolite, was 4.036, so the data is greater than 2.709, the 5% level of significance. We can consider, in this situation too, that the subject N1 as outlier.

Further analyses concerning parent drug and the other metabolite M5, confirmed the conclusion that the subjects N1 and N2 are outliers. For example, for parent drug, it was obtained a huge difference between the geometric means ratio of subject N2 at the first period and the second period, as can be seen in the following table and figure:

Table X

Geometric Means Ratio T/R for AUC, Single dose, Pentoxifylline, Subject N2

Period	Geometric Means Ratio T/R for AUC
First period	144%
Second period	16%

**Figure 11**

AUCs for Pentoxifylline, subject N2

Practically, pharmacokinetic parameters, as well as some of their combinations indicated that the subjects N1 and N2 are outliers.

Conclusions

Subjects N1 and N2 had a completely different behavior not only in comparison with the rest of 20 subjects, but also each of them by himself from one period to another. Their effect on the estimation of intra-variability was dramatic: doubling the values obtained considering the rest of 20 subjects.

The highest differences were obtained in the case of the reference drug, between single dose and steady state evaluations.

Such a phenomenon could be explained by the particularities of the pentoxifylline metabolism (mainly reversible to M1 metabolite, though no special physiological characteristic feature was observed at the two subjects). In these conditions, elimination from the evaluation of bioequivalence of the two subjects is more correct than their abnormal inclusion in the “formulation effects”.

Considering the above results and other additional experience of the authors, we propose the following rule in the analyses of outliers data and subjects in BE studies: first to evaluate the data from the point of view of physiological pharmacokinetics, then apply the statistic tests and finally to evaluate the therapeutic significance.

These conclusions are not in agreement with EMEA General Rules, but in the authors' opinion they represent an approach more correct from the biostatistical point of view and, on the other hand, EMEA Guidelines don't analyze the complex situation of concomitant pharmacokinetics of a parent drug and two active metabolites.

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