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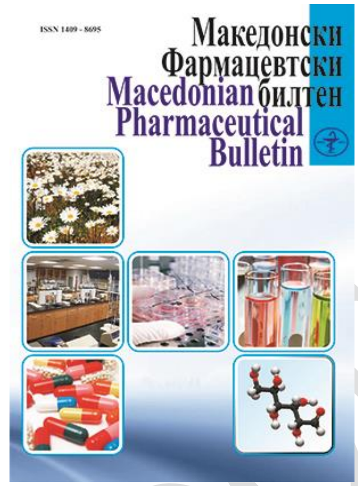

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Bootstrap analysis for dissolution similarity factor f_2 - bringing confidence for borderline results - a case study

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Abstract

In vitro dissolution testing is one of the most important tools for characterizing the performance of oral solid dosage forms. In order to demonstrate similarity between test and reference product, comparative dissolution profiles are performed. Selection of a suitable method for comparison of the dissolution profile of the reference product and the test product is of great importance especially in the case of biowaivers when similarity assessment is based on the *in vitro* data only. Various approaches have been developed for the comparison of dissolution profiles, however the usage of the f_2 similarity factor is widely accepted due to its simplicity. When the conditions for using f_2 similarity factor are not fulfilled due to high variable dissolution profiles, f_2 bootstrap method is then recommended. The focus of this study is to see whether it is benefitial f_2 bootstrap analysis to replace the usage of simple f_2 for comparison of dissolution profiles that fulfill the prerequisites for using the f_2 method in cases when borderline f_2 results are obtained.

Keywords: Bootstrap analysis, comparative dissolution profiles, similarity factor f_2

Introduction

In vitro dissolution testing is one of the most important tools for characterizing the performance of oral solid dosage forms. The rate and extent of drug absorption are determined by its dissolution from the dosage form and it is essential for its bioavailability and therapeutic effectiveness.

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During the development of generic drug products for a selection of the most appropriate candidate formulation that shows similar properties as the reference product before performing the bioequivalence study *in vitro* dissolution profiles are performed. The performance of dissolution profiles during stability testing gives better information for the product quality subjected to various conditions than the dissolution test with one-time point. Dissolution profiles are also used to assess the lot-to-lot quality of a drug product after certain changes are made, like changes in the formulation, the manufacturing process, or scale-up of the manufacturing process (EMA, 2010; FDA 1997).

Dissolution profiles are used as a surrogate of *in vivo* data for Biopharmaceutical classification system (BCS) based biowaivers for highly soluble drugs that belong to BCS class I or III for immediate release products and strength biowaivers (EMA, 2010; FDA, 1997; ICH, 2019). Selection of a suitable method for comparison of the dissolution profile of the reference product and the test product is of great importance especially in the case of biowaivers when similarity assessment is based on the *in vitro* data only.

Various approaches have been developed for the comparison of dissolution profiles. The usage of the f_2 similarity factor is widely accepted due to its simplicity. Even though there are several drawbacks for its use, the f_2 method is recommended for dissolution profile comparison in regulatory guidelines as a method of choice. However, there are some dissimilarities in different regions of the world regarding the conditions that should be fulfilled before using the f_2 factor (Diaz et al., 2016).

According to EMA Guideline on the investigation of bioequivalence, (2010) dissolution similarity using the f_2 statistic can be evaluated with the following equation (Eq.1):

$$f_2 = 50 \cdot \log \left[\frac{100}{\sqrt{1 + \frac{\sum_{t=1}^{t=n} \left[\overline{R}(t) - \overline{T}(t) \right]^2}{n}}} \right] \quad \text{(Eq. 1)}$$

where f_2 is the similarity factor; n is the number of time points; R(t) is the mean percent of the reference drug dissolved at time t after initiation of the study; and T(t) is the mean percent of the test drug dissolved at time t after initiation of the study. For both reference and test formulations, the percent of the active pharmaceutical ingredient dissolved during a specified time point should be determined.

The evaluation of the similarity factor is based on the following conditions:

- a minimum of three-time points (zero excluded),

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- the time points should be the same for the two formulations (reference and test product),
- twelve individual values for every time point for each formulation,
- not more than one mean value of >85% dissolved for any of the formulations,
- the relative standard deviation or the coefficient of variation of any product should be less than 20% for the first time point and less than 10% from second to last time point

An f_2 value between 50 and 100 suggests that the dissolution profiles between the reference and the test product are similar. In cases when some of the above mentioned conditions are not met, for instance the value of f_2 statistic is not suitable (below 50), both EMA and FDA recommend using model-dependent or other model-independent methods for assessing the similarity between the reference and the test product (EMA, 2010; FDA 1997).

In a model-dependent approach, an appropriate mathematical model is selected to describe the dissolution profiles of the two products. The model is then fit to the data and confidence intervals for the model parameters are constructed. These confidence intervals are then compared with the specified similarity region. However, there are some limitations when using the model-dependent approach for assessment of the comparability of the dissolution profiles. As an example, the selection an appropriate model and interpretation of its parameters becomes difficult when the dissolution profiles for the two formulations follow different models (Islam & Begum, 2018). The advantage of the model-independent approaches is that no assumptions on the shape of the dissolution profile curve and fitting of a suitable nonlinear function are necessary.

FDA guidelines prefer using the multivariate statistical difference of which the Mahalanobis distance (MD) is the most common example (Muselik et al., 2021). On the other hand, a document concerning the adequacy of the Mahalanobis distance to assess the comparability of drug dissolution profiles was published by EMA (2018) a few years ago. In this document, EMA does not support the usage of MD as a preferred methodological approach even in situations when the f_2 statistic should not be used. The reason why it is not considered the method of choice is that the usage of MD presents contradictory results. Higher the variability of the results, the MD becomes smaller, indicating similarity of the dissolution profiles and finally leading to false positive results (EMA, 2018).

In this document EMA prefers construction of a confidence interval for f_2 based on the f_2 bootstrap method, where it is stated: "As an additional approach, the bootstrap methodology could be used to derive confidence intervals for f_2 based on quantiles of re-sampling distributions, and this approach could be considered the preferred method over f_2 and MD." (EMA, 2018).

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As stated above, if the f_2 bootstrap can be considered to be the preferred method over f_2 that means that the f_2 bootstrap could be used even though the criteria for using the calculation of f_2 are met. Therefore, the focus of this study is to see whether the f_2 bootstrap analysis could be used instead of simple f_2 for comparison of dissolution profiles, especially in cases when borderline f_2 results are obtained. The comparison is performed between simple f_2 and f_2 bootstrap methods for dissolution profiles with borderline f_2 results that fulfill the prerequisites for using the f_2 method.

Materials and methods

This study compares the results obtained with f_2 and f_2 bootstrap on dissolution profiles of referent and test products near the borderline i.e. f_2 value around the cut-off value of 50. Twenty-five datasets of dissolution profiles of test and corresponding reference products are used for this study (Table 1). Dissolution profiles are mainly performed in the regulatory media at pH 1.2, 4.5 or 6.8 and some of the profiles are performed in bio-relevant media. Samples from 5, 6, or 7-time points are withdrawn for the test and reference products respectively, depending on the solubility of the drug product. F_2 was calculated according to the EMA guideline for the investigation of bioequivalence, while the f_2 bootstrap method was calculated using different software including DD-solver and the open-source software bootf2BCA v1.3 (Mendyk et al., 2013; Zhang et al., 2010).

The number of bootstrapped dissolution profiles was set to 10000. The similarity was assessed using the so-called “worst case scenario”, which consists in comparing the lower limit of a confidence intervals (CI) to the cut-off similarity value of 50. Two dissolution data sets are deemed similar if the lower limit of the 90% CI is equal to or higher than 50. The confidence interval was set to 90%.

All data points until the average dissolution of either reference or test product that reached 85% were used according to EMA recommendations. In the DD-solver software, the cut-off for 85% dissolution was performed manually, while the software Bootf2BCA v.1.3 offers a choice of whether the calculation is desired to be performed with no cut-off, one of the profiles to reach 85% (EMA) or both profiles to reach 85% (FDA). Resampling with replacement of the whole profile was chosen using the bootf2BCA method. Bootf2BCA offers four types of CI including normal approximation CI, Basic CI, Percentile CI, and Bias corrected and accelerated CI. The bias-corrected and accelerated CI approach was chosen for comparison since it produces more precise two-sided confidence intervals for f_2 compared to other methods (Islam & Begum, 2018).

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Results and discussion

The obtained f_2 values from the twenty-five datasets of the reference and test products were found to be between 49.996 and 57.357. These data sets with borderline results were intentionally chosen in order to see whether there is a necessity to use the f_2 bootstrap method instead of the f_2 method for data sets with results around the limit.

The values for the similarity f_2 factor obtained with the conventional approach were above the limit of 50 for 24 out of 25 sets. Only one set (Case 6) had a value below the limit for claiming similarity between dissolution profiles (49.996) (Table 1). The calculations made with the DD-Solver, show that 16 out of 25 results for the f_2-bootstrap are below the cut-off value of 50. Similar results were obtained using the Bootf2BCA method, where 15 out of 25 results for the Bootf2BCA were found to be below the limit of 50 for claiming similarity (Table 1). As it could be seen, the results obtained with the DD solver and the Bootf2BCA differ only for the Case 13 where the result obtained with the DD solver is below the limit of 50 (49.747), while for BootF2BCA it is very slightly above the limit (50.04). Considering that this difference is insignificant (only in decimal units), it could be said that the DD solver and the Bootf2BCA provide very similar results. In general, the f_2 values based on DD-solver and Bootf2BCA were lower compared to the f_2 obtained with the conventional calculation. The lower values for f_2 bootstrap and Bootf2BCA are because of the inclusion of the confidence intervals in the results. Due to the estimation of a confidence interval in the f_2-bootstrap method, false positive results would be obtained using only the f_2 method.

Despite the fact that both programs, DD-solver and Bootf2BCA give similar results, DD-solver for calculation of f_2 bootstrap should be used with precaution and the analyst should make the cut-off for 85% dissolution manually, while Bootf2BCA allows choosing whether to make the calculations according to EMA or FDA guidelines. If no cut-off is used, then the f_2 bootstrap results would be higher or lower depending on the dissolution curves. If at the last points the dissolution curves are approaching each other, then the f_2 bootstrap would give higher results. However, if the dissolution curves at the last points are distant, then the f_2 bootstrap results would be lower. From all datasets only for 3 cases (Case 4, 5 and 6) there is no requirement to make the cut-off since the results in these cases do not show dissolution of more than 85% in the earlier time points. For all other cases, when using DD-solver, the analyst should perform the cut off in order to obtain accurate results. For example, in case 1, 3, 8, 11, 14, 16, 17, 18, 19 and 23 the obtained results with DD-solver are below the acceptable limit of 50 (Table 1). If no cut-off is made before performing the bootstrap analysis in DD-solver by the analyst the acquired results would be:

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51.940, 52.461, 51.992, 51.244, 53.517, 55.283, 55.421, 52.447, 51.052 and 53.409, respectively, meaning that all results are with the acceptable limit for confirmation of similarity leading to false positive results.

In EMA question and answer document concerning the adequacy of the Mahalanobis distance to assess the comparability of drug dissolution profiles, (2018) it is stated that: "when f_2 is considered suitable, i.e. can be used as outlined in Appendix 1 of the CHMP guideline on the investigation of bioequivalence, guideline-compliant evaluation of dissolution similarity does not involve confidence interval estimation to decide upon similarity". Later in the same document, the main drawback of using f_2 is mentioned: "regardless of whether the conditions to adequately apply f_2 in a dissolution experiment are fulfilled or not, the properties of the f_2 sampling distribution do not allow the derivation of exact confidence intervals to adequately quantify the uncertainty of the f_2 estimate".

It is evident that there is no need for using the f_2 bootstrap in cases when the dissolution profiles show high similarity and borderline variability and simple f_2 calculation is sufficient. However, the discussion is whether the f_2 bootstrap method could be useful for the calculation of the similarity around the borderline for f_2 and/or variability close to the upper limit. Is it better to include a confidence interval to quantify the uncertainty around the point estimate? According the obtained results from the datasets (Table 1), it could be said that for dissolution profiles that are used as a surrogate for bio studies like BCS based bio-waivers or strength bio-waivers this approach would be beneficial since only *in vitro* data is used for claiming similarity. Therefore, it can be considered that the usage of f_2 method could be replaced by f_2 bootstrap method.

The CI derivation for f_2 based on bootstrap requires a more profound knowledge of statistics in comparison to the conventional approach (the f_2 calculation), however the use of special software can facilitate the calculation. Calculation of f_2 bootstrap is enabled with the freely available programs for calculation of f_2-bootstrap like DD-solver and Bootf2BCA, where the latter one offers more possibilities and is easier to use.

Conclusion

Even though the f_2 bootstrap method requires profound knowledge for its use, the availability of open-source software offers the opportunity for its facilitated use by non-statisticians. F_2 bootstrap derives confidence intervals to adequately quantify the uncertainty of the f_2 estimate, which is useful for borderline results for f_2 and dissolution profiles with variability near the upper limit. The usage of f_2 bootstrap brings confidence to the obtained results, especially for the comparison of dissolution profiles for strength bio-

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waiver and BCS based bio-waiver where the similarity assessment is based only on the *in vitro* data of the dissolution profiles. Moreover, the decision for candidate bio-batch for bioequivalence would be easier to predict thus saving significant amounts of resources.

References

Diaz, D.A., Colgan, S.T., Langer, C.S., Bandi, N.T., Likar, M.D., Van Alstine, L., 2016. Dissolution similarity requirements: How similar or dissimilar are the global regulatory expectations?. The AAPS Journal 18(1), 15-22. Available at: https://doi.org/10.1208/s12248-015-9830-9

European Medicines Agency, 2010. Guideline on the Investigation of Bioequivalence. CPMP/EWP/QWP/1401/98 Rev. 1/Corr** Available at: https://www.ema.europa.eu/en/documents/scinetific-guideline/guideline-investigation-bioequivalence-rev1_en.pdf

European Medicines Agency, 2018. Question and answer on the adequacy of the Mahalanobis distance to assess the comparability of drug Dissolution Profiles. Avaliable at: https://www.ema.europa.eu/en/documents/scientific guideline/question-answer-adequacy-mahalanobis-distance-assess-comparability-drug-dissolution-profiles_en.pdf

Food and Drug Administration, Center for Drug Evaluation and Research, (FDA, CDER), 1997. Guidance for industry: Dissolution Testing of Immediate Release Solid Oral Dosage Forms. FDA, CDER, Rockville.

ICH Harmonized Tripartite Guideline, 2019. Biopharmaceutics classification system-based biowaivers M9. Available at: M9_Guideline_Step4_2019_1116.pdf (ich.org)

Islam, M.M., Begum M., 2018. Bootstrap confidence intervals for dissolution similarity factor f_2. Biom Biostat. Int. J. 7(5), 397-403. Available at: https://doi/10.15406/bbij.2018.07.00237

Mendyk, A., Paclawski, A., Szlek, J., Jachowicz, R., 2013. PhEq_bootstrap: Open-Source Software for the simulation of f2 Distribution in Cases of large Variability in Dissolution profiles. Dissolutuon Technol, 20, 13-17. Available at: https://doi.org/10.14227/DT200113P13

Muselik, J., Komersova, A., Kubova, K., Matzick, K., Skalicka, B., 2021. A critical overview of FDA and EMA statistical methods to pharmaceutical products. Pharmaceutics 13, 1703. Available at: https://doi.org/10.3390/pharmaceutics 13101703

*Corresponding author email: tachkoska@alkaloid.com.mk

Zhang, Y., Huo, M., Zhou, J., Zou, A., Li, W., Yao, C., Xie, S., 2010. DDSolver: An Add-In Program for Modeling and Comparison of Drug Dissolution Profiles. AAPS J. 12, 263-271. Available at: https://doi.org/10.1208/s12248-010-9185-1

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Резиме

Bootstrap анализа за f_2-фактор на сличност на растворливост- поголема сигурност при гранични резултати- анализа на случај

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Клучни зборови: Bootstrap анализа, компаративни профили на растворливост, f_2 фактор на сличност

In vitro тестирањето на растворливост е една од најважните алатки за карактеризација на перформансите на цврстите дозирани форми за орална примена. Компаративни профили на растворливост се изведуваат со цел да се демонстрира сличност помеѓу тест и референтниот производ. Изборот на соодветен метод за компарација на профилите на растворливост на референтниот и тест производот е од голема важност особено во случаи на biowaiver-и кога проценката на сличност се базира само врз основа на *in vitro* податоците. Иако се развиени различни пристапи за компарација на профилите на растворливост, употребата на f_2 факторот на сличност е широко прифатен поради едноставноста при неговата употреба. Кога условите за употреба на f_2 факторот на сличност не се исполнети поради високо варијабилни профили на растворливост, се препорачува употребата на f_2 bootstrap методот. Фокусот на оваа студија е да се утврди дали е корисно f_2 bootstrap методот да ја замени употребата на f_2 при компарација на профили на растворливост кои ги исполнуваат критериумите за употреба на f_2 методот во случаи каде се добиваат гранични резултати.

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Table 1. Datasets of dissolution profiles of test and reference products

Case No.		Reference product							Test product							Similarity *f2* factor	DD-Solver BootF2	Bootf2BCA bca
		5	10	15	20	30	45	60	5	10	15	20	30	45	60			
Case 1	Average	24,51	54,48	77,29	90,56	97,24	98,22	*	19,02	44,58	65,53	82,00	94,15	97,63	*	51.645	47.711	47,66
	RSD	13,28	9,46	5,19	2,16	0,85	0,38	*	4,97	4,10	3,22	2,93	1,25	1,27	*	56.311	51.051	51,40
Case 2	Average	25,58	55,72	77,56	91,04	97,46	98,29	*	20,77	46,27	67,39	89,15	95,94	98,74	*	53.777	47.138	47,18
	RSD	15,00	9,99	5,91	2,67	1,08	0,73	*	4,68	4,54	4,45	7,63	2,36	0,92	*	51.554	46.734	46,83
Case 3	Average	*	57,40	80,76	94,00	97,96	98,39	*	*	50,31	71,36	85,61	97,40	99,40	*	54.844	53.621	53,68
	RSD	*	6,51	6,63	3,54	0,70	0,78	*	*	13,28	7,54	4,95	1,02	0,75	*	52.721	51.531	51,59
Case 4	Average	*	53,61	63,40	71,30	79,60	85,39	*	*	39,77	53,74	63,38	72,51	79,85	*	54.728	51.255	50,38
	RSD	*	8,29	8,90	4,55	3,81	3,92	*	*	10,94	7,26	6,07	6,19	7,09	*	53.619	48.953	49,08
Case 5	Average	4,47	9,89	13,88	17,30	23,05	29,95	35,58	5,94	12,89	18,62	23,71	32,04	41,15	48,24	56.218	52.159	51,62
	RSD	15,32	8,99	5,63	4,39	2,76	2,34	2,55	15,84	9,71	6,75	4,11	3,55	2,39	2,33	57.357	52.936	52,89
Case 6	Average	4,47	9,89	13,88	17,30	23,05	29,95	35,58	6,38	13,92	19,64	24,34	32,63	42,14	49,54	51.759	47.557	47,32
	RSD	15,32	8,99	5,63	4,39	2,76	2,34	2,55	16,91	9,28	6,10	4,92	3,75	2,61	1,82	49.996	44.056	44,15
Case 7	Average	23,91	52,39	72,74	84,86	90,85	93,33	*	23,94	48,30	63,40	71,52	84,82	91,38	*	52.302	46.425	46,33
	RSD	7,02	5,64	4,44	3,18	1,68	1,40	*	10,28	5,59	3,06	1,66	2,57	2,85	*	51.549	47.513	47,79
Case 8	Average	25,45	53,75	74,75	86,83	93,03	96,12	*	25,45	50,03	65,33	73,41	86,49	92,97	*	50.046	45.149	45,37
	RSD	8,03	6,43	5,33	3,54	1,66	1,53	*	12,23	7,33	3,77	2,77	3,68	3,75	*	51.013	48.005	48,13
Case 9	Average	18,34	44,10	65,43	82,51	94,77	96,90	*	17,65	36,87	55,71	71,41	92,18	96,70	*	51.423	48.051	47,94
	RSD	2,47	3,33	2,54	3,66	0,54	1,20	*	3,70	6,48	4,25	3,08	3,68	0,80	*	53.571	48.821	48,56
Case 10	Average	21,21	46,35	67,03	82,81	94,71	99,01	*	19,39	38,65	57,31	73,24	95,03	97,64	*	52.956	47.332	47,11
	RSD	4,87	5,01	4,13	2,36	2,04	0,98	*	3,23	6,07	4,28	2,96	1,10	0,85	*	56.893	51.571	51,70
Case 11	Average	25,10	54,95	77,44	90,34	97,24	98,28	*	19,38	45,09	66,05	81,58	93,70	94,96	*	55.977	50.084	50,64
	RSD	9,90	8,55	4,49	1,69	0,78	0,26	*	5,47	1,89	1,81	2,00	1,51	1,11	*	53.857	50.131	50,57
Case 12	Average	24,68	54,93	76,48	90,89	97,22	98,18	*	21,01	44,52	64,28	79,68	91,98	95,25	*	51.037	45.977	46,37
	RSD	18,44	10,73	6,53	3,35	1,18	0,71	*	4,30	5,48	6,59	5,56	1,03	0,79	*	54.913	49.584	49,68
Case 13	Average	24,68	54,93	76,48	90,89	97,22	98,18	*	20,50	45,46	65,69	81,08	92,72	94,08	*	55.753	49.749	50,04
	RSD	18,44	10,73	6,53	3,35	1,18	0,71	*	6,60	2,85	2,49	2,74	1,99	1,54	*	51.645	47.711	47,66
Case 14	Average	32,78	64,30	86,97	97,62	97,91	97,96	*	27,96	54,30	75,40	91,74	98,77	98,94	*	56.311	51.051	51,40
	RSD	5,79	4,25	2,64	1,12	0,77	0,57	*	5,47	4,70	3,89	2,97	0,46	0,66	*	53.777	47.138	47,18
Case 15	Average	25,10	54,95	77,44	90,34	97,24	98,28	*	18,78	44,18	65,75	80,24	92,44	94,93	*	51.554	46.734	46,83
	RSD	9,90	8,55	4,49	1,69	0,78	0,26	*	7,08	6,01	5,52	4,81	2,92	1,79	*	54.844	53.621	53,68
Case 16	Average	29,01	61,47	86,65	97,81	97,94	97,81	*	25,48	51,21	74,30	97,14	99,04	99,16	*	52.721	51.531	51,59
	RSD	6,57	4,02	2,98	0,59	0,42	0,54	*	2,10	1,21	1,21	0,83	0,82	0,71	*	54.728	51.255	50,38
Case 17	Average	30,24	63,15	89,22	99,48	99,92	100,75	*	27,34	53,48	76,65	99,23	101,09	100,85	*	53.619	48.953	49,08
	RSD	9,32	4,45	2,66	0,67	0,56	1,33	*	2,88	1,78	1,78	1,43	0,93	0,67	*	56.218	52.159	51,62
Case 18	Average	26,54	59,59	82,22	91,95	95,97	97,86	*	23,17	50,63	71,14	83,62	92,59	97,00	*	57.357	52.936	52,89
	RSD	6,75	4,23	2,44	2,74	1,60	0,83	*	7,06	5,05	4,82	4,43	3,12	0,67	*	51.759	47.557	47,32
Case 19	Average	27,57	60,81	83,57	93,32	97,31	99,39	*	24,67	51,65	72,14	84,51	93,77	98,00	*	49.996	44.056	44,15
	RSD	8,39	5,83	3,28	3,28	1,73	0,37	*	7,04	6,17	5,77	5,05	3,38	0,50	*	52.302	46.425	46,33
Case 20	Average	32,04	63,30	85,96	96,85	97,26	97,24	*	26,05	54,14	79,94	98,03	99,18	99,17	*	51.549	47.513	47,79
	RSD	5,28	4,01	2,61	0,96	0,62	0,58	*	7,63	6,28	3,64	1,10	1,05	1,12	*	50.046	45.149	45,37
Case 21	Average	32,78	64,30	86,97	97,62	97,91	97,96	*	27,30	54,79	79,93	96,96	97,84	98,12	*	51.013	48.005	48,13
	RSD	5,79	4,25	2,64	1,12	0,77	0,57	*	8,16	7,32	5,10	1,31	0,69	0,81	*	51.423	48.051	47,94
Case 22	Average	26,05	54,14	79,94	98,03	99,18	99,17	*	25,46	61,61	92,24	97,99	98,16	98,11	*	53.571	48.821	48,56
	RSD	7,63	6,28	3,64	1,10	1,05	1,12	*	4,69	4,17	1,27	0,70	0,76	0,71	*	52.956	47.332	47,11
Case 23	Average	27,30	54,79	79,93	96,96	97,84	98,12	*	27,61	64,58	93,11	98,14	98,30	98,22	*	56.893	51.571	51,70
	RSD	8,16	7,32	5,10	1,31	0,69	0,81	*	6,15	8,86	1,74	0,83	0,67	1,03	*	55.977	50.084	50,64
Case 24	Average	28,85	63,02	86,04	95,71	97,92	97,86	*	25,11	53,19	77,25	94,27	98,49	100,75	*	53.857	50.131	50,57
	RSD	8,12	6,01	2,92	0,54	0,64	0,67	*	10,17	5,30	2,94	2,89	0,96	0,83	*	51.037	45.977	46,37
Case 25	Average	29,78	63,67	86,36	95,93	98,04	98,37	*	26,67	54,27	77,66	94,55	98,12	100,32	*	54.913	49.584	49,68
	RSD	9,28	7,04	3,33	0,78	0,91	1,03	*	11,19	4,86	2,75	2,14	0,91	0,69	*	55.753	49.749	50,04

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