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Development and metrological characterization of quantitative X-ray diffraction phase analysis for the mixtures of clopidogrel bisulphate polymorphs

Vladimir Uvarov *, Inna Popov

The Hebrew University of Jerusalem, The Faculty of Natural Science, The Center for Nanoscience and Nanotechnology, The Unit for Nanoscopic Characterization, E. Safra Campus, Givat Ram, Jerusalem 91904, Israel

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Abstract

Clopidogrel bisulphate (CLP) is a pharmaceutical compound with a novel mechanism of action for the reduction of atherosclerotic events. Only two crystalline forms (CLP I and CLP II) among the six known polymorphs of CLP have therapeutic activity. The structure of the CLP I polymorph is unknown and the structure of the CLP II polymorph is known only partially. Two techniques of X-ray diffraction quantitative phase analysis have been developed in this work for the quantification of CLP I and CLP II in their mixtures. The first technique is based on use of the whole powder pattern decomposition method (WPDM). WPDM was realized through Powder Cell for Windows v.2.4 (PCW) freeware. The second technique is based on the classical direct method. Metrological characterization and comparison of methods have been performed on the mixtures with known phase composition as well as on the real samples with varying phase content. Quantitative phase analyses of 120 specimens containing mixtures of forms I and II of CLP were performed using both developed techniques. Absolute and relative errors and reproducibility of both methods were found to be very similar. The statistical analysis of obtained results revealed that the WPDM gives higher accuracy. We found that the limit of quantification using both methods is 1.0–1.5 wt.% of phase content in the mix.

Keywords: Clopidogrel; X-ray diffraction; Quantitative phase analysis; Metrological characterization; Whole powder pattern decomposition method; Drug polymorphism

1. Introduction

The quantitative analysis of crystalline phase content in drug materials is becoming more and more important for quality control of medical products. At the same time, the analysis of drug polymorphs can be considered as a specific problem. Polymorphism is a very frequent phenomenon in pharmaceutical formulations. The different polymorphs of a drug usually exhibit different physical and chemical properties. Therefore the various polymorphous forms of a drug can possess different biological influence on recipients [1,2]. In this connection the importance of quantification of polymorphs in pharmaceutical compounds is not in doubt.

CLP is a pharmaceutical compound with a novel mechanism of action for the reduction of atherosclerotic events like myocardial infarction, stroke and death due to vascular causes [3]. The chemical formula of CLP is $C_{16}H_{17}Cl\,N\,O_2S{\cdot}HSO_4$. Six various polymorphs are known for CLP, but only two of them (forms I and II) are used in pharmaceuticals. Crystal structure data for CLP II were reported by Bousquet et al. [4], but without coordinates of hydrogen atoms. The total crystal structure of CLP I is unknown, but Vickers [5] has reported data about its unit cell parameters. Currently, the quantitative phase analysis of mixtures of CLP polymorphs is performed by Fourier transform infrared spectroscopy [6], while XRD technique is used for qualitative analysis only.

However, X-ray powder diffraction analysis is a powerful tool that is widely applied for this purpose and the mixture of polymorphs is a rather favorable object for quantitative XRD analysis (QXRDA). The characteristics of modern QXRDA methods are adduced in reviews [7,8]. There are two principal approaches

* Corresponding author. Tel.: +972 545590770/26584889.
E-mail address: vladimiru@savion.huji.ac.il (V. Uvarov).

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and, accordingly, two groups of methods for QXRDA. In the first approach, the intensity of the single peak and calibration curves constructed using standard mixtures with known weight ratios of analyzable phases are used [9–12]. In the second approach, a whole powder diffraction pattern is analyzed. If structures of all phases in the specimen are known, the Rietveld method can be directly applied for phase quantification [13–15]. Combinations of Rietveld and whole powder pattern decomposition method (WPDM) have been proposed for the cases when structural information is missing for at least one of the phases in the mixture [16–18]. This approach was realized in Powder Cell for Windows v.2.4 (PCW) [19], QUANTO [18], MAUD [20] and SIROQUANT [21]. Only PCW automatically realizes a phase quantification procedure using WPDM, while others perform QXRDA in step-by-step mode. Methods of the second group are less sensitive to such interfering factors as peaks overlapping, preferred orientation of the particles, and the crystallite sizes. It is obvious that for realization of the second group of these methods it is necessary to have specialized software (able to extract structural information from the experimental XRD pattern and to perform back rescaling-fitting procedure at which weight fraction values are obtained), and a powerful PC (not available around 20 years ago, when this technique was first proposed).

In the present work we describe two techniques we developed for QXRDA of CLP polymorphs in the mixtures. The first technique is based on the direct method, while the second one exploits WPDM. We present here the metrological characteristics of both developed techniques. Within the frame of the present study we also estimate the applicability PCW for the quantitative analysis of a mixture of the polymorphs in the case when the structure of one of them is unknown.

2. Materials and methods

Pure CLP I and CLP II and their mixtures have been obtained from the Casali Institute for Applied Chemistry of the Hebrew University of Jerusalem. CLP I has monoclinic unit cell with $a = 12.63$ Å, $b = 15.22$ Å, $c = 10.43$ Å, $\beta = 113.5^\circ$ and space group $P2_1$. CLP II belongs to the orthorhombic system and its unit cell parameters are $a = 10.32$ Å, $b = 20.12$ Å, $c = 9.19$ Å. The symmetry of this phase corresponds to the space group $P2_12_12_1$. Mixtures of CLP I in CLP II at proportions of 10, 20 30, 40, 50, 60, 70 and 80% were prepared for determination of calibration coefficients and metrological characterization of the developed methods.

X-ray powder diffraction measurements were performed on the D8 Advance diffractometer (Bruker AXS, Karlsruhe, Germany) with a goniometer radius 217.5 mm, Göbel Mirror parallel-beam optics, 2° Sollers slits and 0.2 mm receiving slit. Standard sample holders were carefully filled with the powder samples. The specimen weight was 0.5 g approximately. XRD patterns within the range 6° to 36° 2θ were recorded at room temperature using Cu Kα radiation ($\lambda = 1.5418$ Å) with following measurement conditions: tube voltage of 40 kV, tube current of 40 mA, step-scan mode with a step size of 0.02° 2θ and counting time of 1 s/step.

Quantification of phase content was carried out with the classical direct method and the WPDM routine. In the classical direct method, the percentage of each phase according to [22] was calculated as

$$c_i = I_i \rho_i k_i \mu^* = I_i \rho_i k_i \Sigma c_i \mu_i^* \quad (1)$$

where c_i is the percentage of i-th phase, ρ_i the density of i-th phase, k_i the calibration coefficient for i-th phase, μ^* the mass absorption coefficient of the specimen and I_i is the intensity of analytical peak of i-th phase.

The density and mass absorption coefficient do not change from sample to sample at the analysis of a mixture of polymorphs. Therefore we can rewrite Eq. (1) as $c_i = k_i I_i$. The value of calibration coefficient k_i has a dimension of percentage/count. We calculated the percentage value using several diffraction peaks by the following equation:

$$c_i = \frac{\sum_{j=1}^{n} I_{i,j} k_{i,j}}{n} = \frac{\sum_{j=1}^{n} c_{i,j}}{n} \quad (2)$$

where c_i is the percentage of i-th phase, the index j concerns to j-th diffraction peak of i-th phase, n is number of diffraction peaks that were used for percentage calculation. Thus for reliability the percentage of each phase was averaged over several diffraction peaks. Such approach allows diminishing an effect of sample preparation quality on the quantification result and therefore increases a reliability of the method. The values for calibration coefficients k_i were derived from plot I_i versus C_i. The value of peak intensity was manually measured for each diffraction peak as its height above a background line. After normalization procedure was applied, we finally calculated C_i as

$$c_{n,i} = 100 \times \frac{c_i}{\sum_{i=1}^{n} c_i} \quad (3)$$

where $c_{n,i}$ is normalized percentage of the i-th phase and n is number of the phases.

The WPDM is realized in the "decomposition + refinement" subroutine of PCW software that utilizes the Le Bail fitting [23] for extraction of structural factors of the phase with unknown structure. Simultaneously the scale factors for all presenting phases are calculated and their values are used for the percentage determination. The scaling factor represents a proportional number for the fit of an experimental pattern. The ratio of the scaling factors is used to determine the partial content of each phase within a multiphase sample. When all the phases of a mixture are polymorphs of the same compound, they have the same value absorption coefficient that allows receiving correct quantitative results even if the structure of one of phases (polymorphs) is unknown. The routine requires inputting the structural data for each phase expected in a mixture, acquisition conditions for XRD (radiation, geometry of diffractometer, 2θ range). For a phase with unknown structure space group number and unit cell parameters are entered. The profile function should be selected from a proposed list before the routine is started. We found that Pseudo-Voigt function provides the best fitting for the experimental diffraction peaks shape.

Altogether 120 samples have been analyzed. An assessment of the accuracy of QXRDA methods was carried out using the

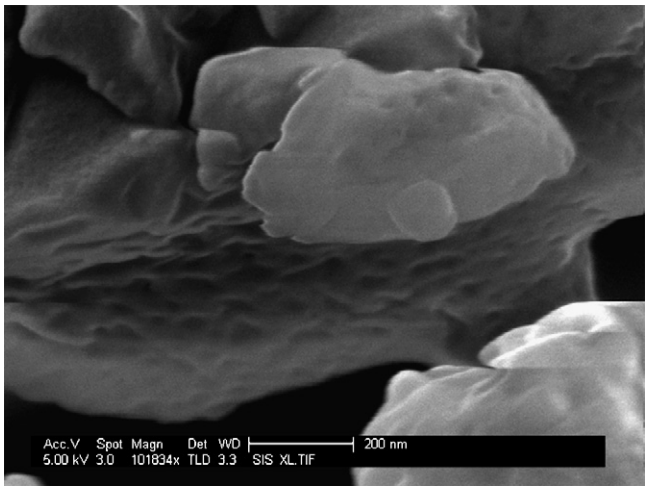

Fig. 1. HR secondary electrons image of CLP powder.

samples with known phase content. The statistical treatment of the obtained results and their comparative estimation were performed according to the recommendations of Tobias and Croarkin [24].

The value of crystallite size was determined from the experimental XRD data through Scherrer equation. The routine was performed with PCW using all the observable peaks. Pseudo-Voigt function was chosen for profile fitting. The instrumental broadening was determined using LaB_6 powder (NIST SRM 660). The average value of crystallite size was found to be in the range of 50–60 nm for each tested sample. Microstructural study of the CLP powders was performed with high-resolution scanning electron microscope (HR SEM) Sirion (FEI Company, Netherland). HR topographic image of CLP powder in secondary electrons is presented in Fig. 1. It is seen that the fracture surface of a submicron powder particle is inhomogeneous at the scale of tens of nanometers and closely resembles typical pattern of intergranular cleavage. The grains identified within the fracture surface are all in the range of 45–60 nm size that fits well the crystallite size as-evaluated from the XRD data.

3. Results and discussion

Fig. 2 shows the XRD patterns from CLP I (a), CLP II (b) and their mixture (c). It is seen that only a few intensive peaks do not overlap and, hence, can be used for the quantification by the direct method. These peaks (with d-spacing 8.13 Å, 5.98 Å and 4.32 Å for CLP I and with d-spacing 7.24 Å and 6.88 Å for CLP II) were chosen for analysis. The patterns used for calculation of calibration coefficients were acquired from mixtures in repeated manner three to five times. The background was subtracted using EVA software (Bruker AXS). The plots I_i versus C_i were built for each chosen peak. Fig. 3 shows one of such plots for peak with d-spacing 8.13 Å. The value of calibration coefficient was taken equal to that in the linear regression equation, which was calculated by considering the zero intercept and taking into account all experimental data ($k = 0.2131$). From the mathematical point of view a little bit higher value of correlation coefficient R^2 (0.9881 vs. 0.988) could be achieved if $y = ax + b$ will be used as approximation function instead of $y = ax$ applied on Fig. 3. But in this case the approximated line ($y = 0.21x + 0.6505$) intersects y-axis at non-zero value at $x = 0$, that means positive phase content at zero peak intensity. Therefore for physical meaning we have chosen function $y = ax$ for definition of calibration coefficients. The following values of the calibration coefficients have been received: $k(8.13) = 0.2131$, $k(5.98) = 0.2240$, $k(4.32) = 0.1858$, $k(7.24) = 0.2871$, $k(6.88) = 0.1378$. In the further analysis these values were used for calculation of the phase content by Eq. (2).

The metrological estimation of the applied techniques was carried out in two steps. First, the reproducibility and correctness

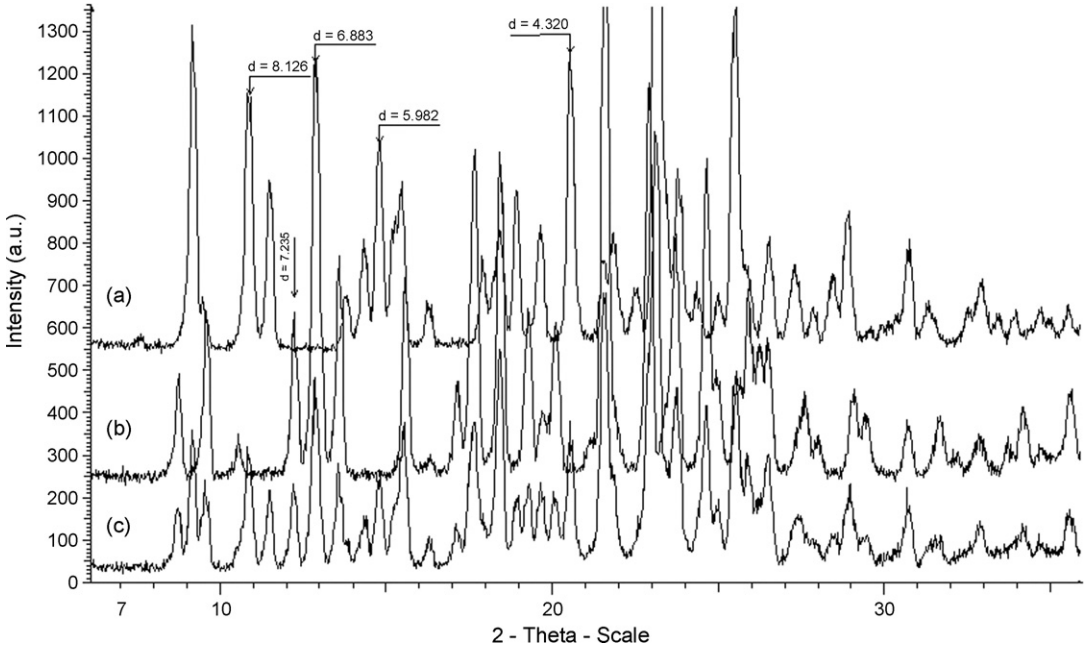

Fig. 2. X-ray diffraction patterns acquired from pure CLP I (a), pure CLP II (b) and their mixture (c).

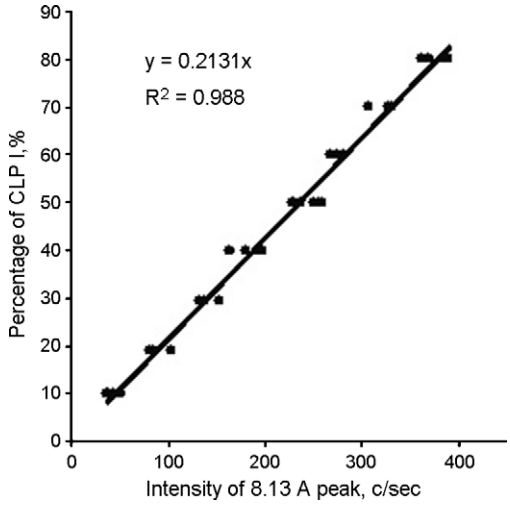

Fig. 3. Plot of intensity of 8.13 Å peak (after background subtraction) vs. actual CLP I percentage.

of both methods have been estimated by the analysis of artificial mixtures with known percentage of CLP I and CLP II. Altogether six artificial mixtures have been analyzed for this purpose. Each sample has been analyzed three to four times with re-filling of the sample holder. This procedure allows eliminating of acquisition errors caused by the sample preparation and instability of the equipment. The results of quantitative analysis of these mixtures are presented on Fig. 4 (for clarity, only results for CLP I are shown). As is seen from Fig. 4, we obtained sufficiently correct results with each used method. Results of the statistical treatment of these data with using the simple Student's *t*-test are presented in Table 1. The absolute error D, relative error v_c, variance V_c, standard deviation s_c and *t*-coefficient were calculated, respectively, as

$$D = \bar{c} - c_0 \tag{4}$$

$$v_c = 100 \times \frac{|D|}{c_0} \tag{5}$$

$$V_c = s_c^2 = \frac{\sum_{i=1}^{n}(c_i - \bar{c})^2}{n-1} \tag{6}$$

$$t = \left| \frac{(\bar{c} - c_0) \times \sqrt{n}}{s_c} \right| \tag{7}$$

where $\bar{c}$ and c_0 are average and true percentage of CLP I in mixture, respectively, and n is number of observations.

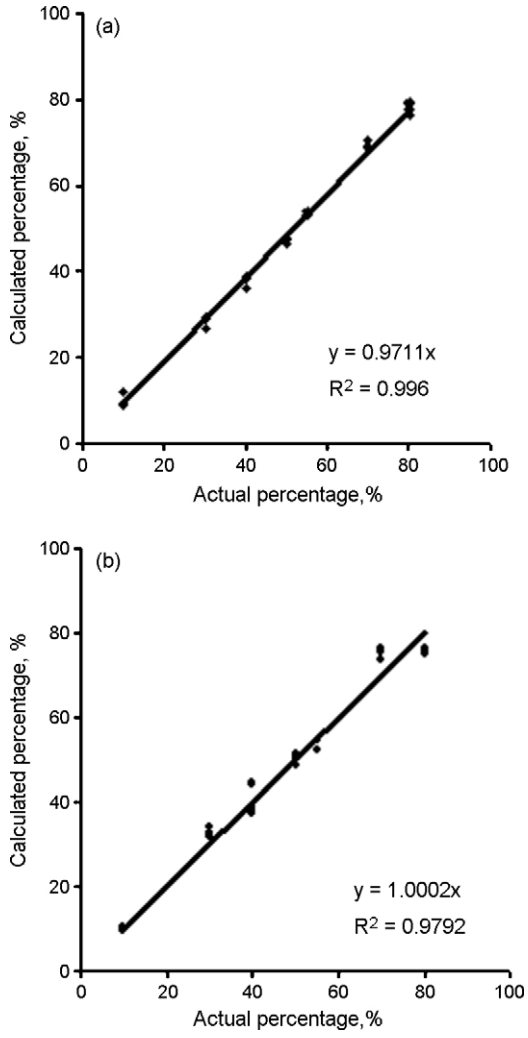

Fig. 4. Plot of actual CLP I percentage vs. calculated percentage: (a) whole powder pattern decomposition method and (b) direct method.

The critical value of t_{tab} for the 5% significance level and a number of observations (n) 3 is equal to 4.3. As follows from Table 1, in most cases both methods give statistically insignificant error. For WPDM the errors are statistically insignificant for all intervals of phase contents. However for direct method the error is statistically significant at the percentage above 70%.

We used the modified Student's *t*-test for an estimation of the statistical significance of a divergence between the average results of both methods (see Table 2). The average standard

Table 1
Statistical treatment of the results of the analysis of control samples

Actual percentage of CLP I (%)	Mean (%)	Absolute error	Relative error	Variance	Student's coefficient
10	9.7/10.2	−0.3/0.2	−3.0/2.0	0.66/0.52	0.79/0.67
30	28.4/32.9	−1.6/2.9	−5.3/9.7	1.38/1.67	1.26/5.7
40	37.8/40.2	−2.2/0.2	−5.5/0.5	1.48/3.67	2.57/0.10
50	48.2/50.2	−1.8/0.2	−3.6/0.4	0.77/1.03	4.04/0.39
70	69.6/75.4	−0.4/5.4	−0.6/7.7	0.92/1.27	0.75/7.36
80	78.1/75.9	−1.9/−4.1	−2.4/−5.1	1.42/0.43	2.67/19.1

Numerator is data for PCW; denominator is data for the direct method.

Table 2
An estimation of the statistical significance of a divergence between mean results of both methods

Actual percentage of CLP I (%)	$\bar{c}_{PCW} - \bar{c}_{DM}$	$\bar{s}$	t
10	−0.5	0.594	1.03
30	−4.5	1.532	3.60
40	−2.4	2.798	1.05
50	−2.0	0.909	2.69
70	−5.8	1.089	6.52
80	2.2	1.049	2.57

deviation $\bar{S}$ and the Student's factor t were calculated as

$$\bar{s} = \sqrt{\frac{\bar{s}^2_{PCW} + \bar{s}^2_{DM}}{2}} \quad (8)$$

$$t = \frac{|\bar{c}_{PCW} - \bar{c}_{DM}|}{\bar{s}} \times \sqrt{\frac{n}{2}} \quad (9)$$

Indexes "PCW" and "DM" relate to the WPDM and direct method accordingly.

In this case the critical value of t_{tab} for the 5% significance level and a number of observations (n) 3 is equal to 2.78. It is evident that divergences between average values are sometimes statistically significant. Comparing the data presented in Tables 1 and 2 we conclude that WPDM yields more correct results than the direct method at the percentage higher than 70%. However, we should note that as the number of repeated analyses was not large, it is difficult to assess the received results unambiguously. Therefore, at the second step, the statistical significance of differences between the results of both methods has been estimated at the analysis of real samples.

The comparative plot of CLP I percentage calculated by PCW and direct method is shown on Fig. 5. It is obvious, that both compared sets are in good agreement. For statistical treatment we divided these results into five groups as follows: range of less than 10%, 10–30%, 30–50%, 50–70% and range of more than 70%.

To ascertain the homogeneity of the variances in each group we applied Fisher's test. The results of calculations of average values, variances and standard deviation for all intervals and both applied methods are presented in Table 3. The sample variances V_c were calculated by Eq. (6). The relative standard deviations $s_r(c)$ were calculated as

$$s_r(c) = \frac{s(c)}{\bar{c}} \quad (10)$$

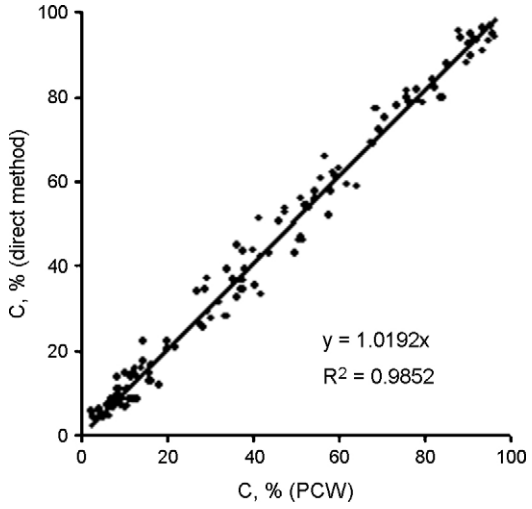

Fig. 5. Plot of percentage CLP I calculated by PCW vs. that calculated by direct method.

According to Fisher's test the variances are similar if the calculated value of Fisher's criterion ξ is less than a tabulated value, i.e.

$$\xi = \frac{s_1^2}{s_2^2} = \frac{V_1}{V_2} \leq F(P, f1, f2) \quad (11)$$

where $F(P, f1, f2)$ is tabulated value of Fisher's coefficient, $P = 0.95$ is confidence probability and $f1$, $f2$ are number of degrees of freedom (i.e. number of the specimens in each group). Value of $F(P, f1, f2)$ is 2.12 for $f \approx 25$. The Fisher's test reveals that variances are practically homogeneous for all size groups.

As is clearly seen in Table 3, within each group the average values of CLP I percentage obtained by both methods are very close. So, till this point we did not get any reliable information on the advantage or accuracy of each tested method. In such a situation we can only estimate the statistical significance of the observed differences. Student's t-test for correlated samples has been applied to ascertain the statistical significance of the differences between the results calculated by both methods. Since we are interested only in the differences between the methods, we should consider only one variable, $D_i = c_{a,i} - c_{b,i}$ ($c_{a,i}$ and $c_{b,i}$ are CLP I percentage that were calculated by different methods). The standard deviation σ_{M_D} of the sampling distribution M_D and t-value for the Student's t-test were calculated as

$$M_D = \frac{\sum D_i}{n} \quad (12)$$

Table 3
Results of the preliminary statistical treatment of the experimental data

Class of percentage (%)	n	Mean percentage (%)	Sampling variances	Relative standard deviation	Fisher's coefficient
<10	26	6.1/6.8	4.8/6.3	0.36/0.37	1.31
10–30	26	16.6/18.5	46.4/71.3	0.41/0.46	1.54
30–50	22	39.1/39.6	28.4/63.4	0.14/0.20	2.23
50–70	20	58.6/60.3	43.8/93.5	0.11/0.16	2.13
>70	26	86.0/87.4	65.6/60.0	0.09/0.09	1.09

Numerator is data for PCW; denominator is data for the direct method.

Table 4
Statistical estimation of the divergence of the CLP I percentage obtained by various QXRDA methods

Class of percentage, %	Number observations	M_D	σ_{M_D}	$t = \left\lvert \frac{M_D}{\sigma_{M_D}} \right\rvert$
<10	26	−0.75	0.370	2.03
10–30	26	−0.79	0.548	1.44
30–50	22	−0.45	1.220	0.37
50–70	20	−1.65	1.310	1.26
>70	26	−1.40	0.618	2.26

$$\sigma_{M_D} = \sqrt{\frac{\sum D_i^2 - \left(\sum D_i\right)^2/n}{n \times (n-1)}} \quad (13)$$

$$t_{cal} = \left| \frac{M_D}{\sigma_{M_D}} \right| \quad (14)$$

The results of these calculations are summarized in Table 4.

According to Student's t-test the difference between the methods is significant if $t_{cal} > t_{tab}$. The critical value of t_{tab} for the 5% significance level and a number of observations (n) about 25 is equal to 2.06. As is clearly seen, the difference between two methods is statistically significant only for one class of the percentage—more than 70%. However, the average absolute difference between methods does not exceed 1.7% (see Table 3). Comparing the data presented in Tables 1 and 4 we find that average results of CLP I percentage as-determined by the direct method practically always is slightly higher that that for WPDM. It is true both for the analysis of artificial mixes, and for the analysis of real samples. In view of this fact it is possible to assert that both methods give uniformly precise results at CLP percentage of up to 70%, while WPDM should be favored when percentage of one component is more than 70%.

We have also estimated the limit of detection (LD) achievable by application of both methods. For WPDM we found that PCW routine gives non-zero content of the second phase even when pure single-phase powder of one polymorph is analyzed. We supposed that this confusing result originates from unknown structure of one of polymorphs used for the test and the peak overlapping. Therefore, we also analyzed the phase content and LD within two artificial mixtures with low content (2 wt.%) of CLP I and CLP II, respectively. WPDM provided correct percentage of the phases for both mixtures. That is why we conclude that most probably 1.0–1.5% is the real LD value for both phases for WPDM analysis.

The LD for a direct method was calculated as

$$c_{min} = 3 s_b k \quad (15)$$

where c_{min} is the limit of detection, s_b the standard deviation of the background signal and k is the calibration coefficient. The s_b value has been calculated by Eq. (6) with replacement of the percentage by background intensity. The average s_b has been determined on three background intervals and equaled to 3.62. Thus the LD values were found to be 2 and 1.5% for CLP I and CLP II (for 4.32 Å and 6.88 Å peaks, respectively). Since we did not find published data on application of QXRDA methods for study of CLP polymorphs, we compare the received LD values with those obtained for other polymorphs in pharmaceutical formulations. At the analysis of monnitol polymorphs LD changed from 0.3% up to 2.1% [12], while LD was found to be about 0.75% at the analysis of cefepime polymorphs [25] and 3.4% at QXRDA of ranitidine–HCl polymorphs [26]. According to [27] the LD value varies from 2% to 4% at the QXRDA of various polymorphs. Therefore the limit of detection achieved at the present study is of the same order of magnitude as the reported data. We wish to stress here that the LD value for CLP could not be reduced more, because the most intensive diffraction peaks could not be used for quantification.

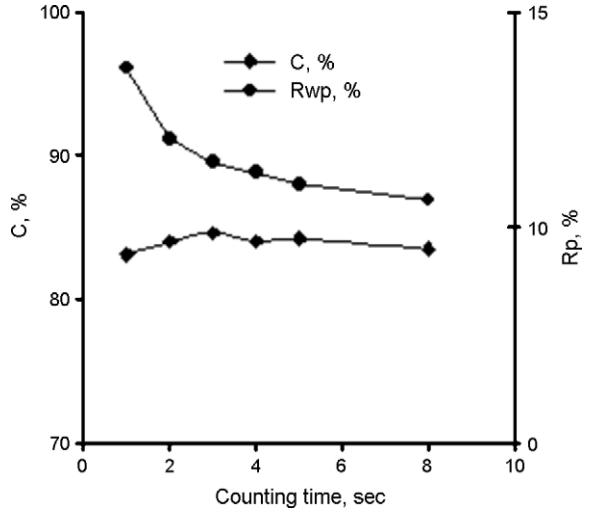

Fig. 6. Values of CLP I percentage and R_{wp} factor, calculated with different counting time (PCW software).

To estimate the effect of counting time and fitting quality on the results of quantitative analysis, the XRD patterns were recorded at different counting times. Usually fitting quality for the whole diffraction pattern is estimated by the value of R_{wp} factor [28] that is calculated by the following equation:

$$R_{wp} = 100 \sqrt{\frac{\sum_{i-1,n} w_i |y_i - y_{c,i}|^2}{\sum_{i=1,n} w_i y_i^2}} \quad (16)$$

where y_i and $y_{c,i}$ are measured and calculated profile intensities, w_i the weight of the observation, $w_i = 1/\boldsymbol{\sigma}_i^2$ and $\boldsymbol{\sigma}_i^2$ is the variance of the profile intensity y_i. For all the XRD patterns acquired as a single scan with 1 s/step counting time we typically obtained R_{wp} values of 13–14%. As is seen in Fig. 6, accumulating raw data over a longer counting time really resulted in decreasing the value of R_{wp} factor to 10–11% for 8 s, i.e. fitting quality was improved. At the same time, the calculated value of percentage remained practically the same for each counting time. The similar phenomenon was observed earlier and was reported in Ref. [29]. For practical considerations, this result confirms that the percentage calculated from the data acquired at the time limiting conditions (i.e. 1 s/step counting time) could be used.

4. Conclusions

Two techniques of X-ray diffraction quantitative phase analysis have been developed in this work for the quantification of phase content in the mixtures of polymorphs of pharmaceutical formulation clopidogrel bisulphate. It is proved that application of QXRDA to analysis of mixtures of polymorphs provides correct results even in a difficult situation when structural information about the co-existing phases in not complete and significant peak overlapping is unavoidable.

It is shown for the first time that WPDM method realized through PCW software being directly applied to the analysis CLP polymorphs mixture performs good profile fitting and calculates the percentage of components correctly. We also developed the scheme for QXRDA through the classical direct method and found the values of calibration coefficients required for calculation of phase content through the intensity of diffraction peaks. These values could be used any time quantitative phase information about CLP-based phases is extracted from XRD data acquired on another powder diffractometer. Both QXRDA tested methods give very similar results. Absolute and relative errors of both methods are comparable. The reproducibility of the results of both methods is identical. The statistical analysis of results indicates that the WPDM gives more correct results since its reproducibility is better. In addition the WPDM yields more reliable results at low percentages of defined phases. The inferior limit of detection for the WPDM is found to be 1.0–1.5%, while for the direct method it is around 1.5–2%. We found that increasing the counting time from 1 to 8 s/step does not affect the final result of QXRDA, although it does improve the fitting quality of the whole diffraction pattern. We believe the results presented here could serve as practical recommendations for application of XRD technique for quantitative analysis of polymorph mixtures.

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