D. A. Leontiev et al /J. Pharm. Sci. & Res. Vol. 10(11), 2018, 2709-2714

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

# Characterization of a Reference Standard for Qualification of Differential Scanning Calorimetry Intended for Purity Determination in Certification of Pharmaceutical Reference Standards

**D. A. Leontiev*[1], N. V. Volovyk[1], O. V. Bevz[2], O. V. Vashchenko[3], O. I. Gryzodub[1]**

[1] *Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines, 33 Astronomichna St., Kharkiv, Ukraine, 61085*

[2] *National University of Pharmacy, 53, Pushkinska St., Kharkiv, Ukraine, 61002*

[3] *Institute for Scintillation Materials of NAS of Ukraine, 60 Science Ave., 60, Kharkiv, Ukraine, 61001*

**Abstract**

**Aim:** To select and characterize a reference standard for equipment qualification suitable for performance verification in the certification of pharmaceutical reference standards by differential scanning calorimetry (DSC) based on the metrological criteria of the State Pharmacopoeia of Ukraine.

**Material and Methods:** The purity analyses were performed in accordance with a standard procedure by DSC. For the selected candidate, the results of purity determination were compared with those obtained by gas chromatography.

**Results and Discussion:** The metrological criteria for results of purity determination in the certification of pharmaceutical reference standards by DSC used as an alternative method were formulated. A candidate material of cholesterol for performance verification of DSC met the established requirements. For cholesterol, the characteristics of purity, melting point and enthalpy of fusion were certified simultaneously. The further study of intra- and inter-laboratory variation of the results is required to characterize the uncertainty of the purity determination.

**Conclusions:** a candidate material of cholesterol can be used as a reference standard for equipment qualification for purity determination by DSC for characterization of pharmaceutical reference standards.

***Keywords*:** cholesterol, differential scanning calorimetry, equipment qualification, homogeneity, pharmaceutical reference standards, purity.

## INTRODUCTION

An assigned value ($X_{RS}$), or purity, of the primary pharmaceutical reference standard (phRS) that is an individual substance of high purity intended for quantitative determination is generally established by subtracting total impurities from 100% (the mass balance method), the correctness of which should then be confirmed by an independent method, for instance, by differential scanning calorimetry (DSC).

In the pharmaceutical sector, the standard requirements for specifications for the quality control (QC) of medicines are set in advance, and the specificity of a particular medicine is taken into account only in exceptional cases [1]. This allowed the State Pharmacopoeia of Ukraine (SPhU) to formulate the requirements for the maximum permissible uncertainty of the assigned value of the reference standards (RS) intended for quantitative pharmaceutical tests ($max\Delta_{RS}$), see *5.12N Reference Standards* [2]. Hereinafter under uncertainty, we understand a one-sided confidence interval for the 95% reliability level. In accordance with the SPhU approach, which is consistent with the ones of the United States Pharmacopoeia (USP) and the European Pharmacopoeia (Ph. Eur.), $max\Delta_{RS}$ = 0.5% is the most stringent requirement for RS used for quantitative determinations [3].

Assessment of purity and homogeneity are amongst the tasks to be solved when certifying RS. For purity assessment, the mean value is found, whereas for homogeneity, if destructive methods are used, the assessment is done from a single determination, the result of which is largely dependent on a test portion in case of significant inhomogeneity. While for purity assessment the required value of uncertainty can be achieved by increasing the number of analyses, for homogeneity assessment, the possibility of using an analysis method can be determined by the capabilities of an analytical instrument or the method itself. Suitability of DSC for the certification of phRS based on metrological requirements for them, to the best of our knowledge, has not been carried out yet.

Certification of RS imposes high metrological requirements for the analysis results, which are often extreme for the analysis method to fulfil. Therefore, when carrying out the analyses, it is crucial to monitor the state of the analytical system (the state of measuring equipment, the correctness of its calibration, the accuracy of the analyst's work, etc.) concerning the analysis task, see *<1058> Analytical Instrument Qualification* [4]. RS for equipment qualification ($RS_{EQ}$) is an effective tool that allows performance monitoring of the entire analytical system. For the tasks of certification of phRS and QC of medicines, it is convenient to use the $RS_{EQ}$ for which such properties as purity, melting point and enthalpy have been certified. Furthermore, these parameters are recommended to report when determining purity by the method of DSC by the ASTM recommendations [5].

There is a number of certified reference materials (CRM) for verification of purity by the method of DSC (NIST Standard Reference Material 1514 [6], LGC2013 [7]), for which only one characteristic – purity has been certified with an indication of its uncertainty. There is also a representative set of CRM, for which enthalpy of fusion and melting point have been certified (for example, LGC2603 Naphthalene - DSC calibration standard, LGC2604 Benzil - DSC calibration standard, LGC2605 Acetanilide - DSC calibration standard, etc.) [**Error! Bookmark not defined.**]. However, to the best of our knowledge, there is no $RS_{EQ}$ for which the various characteristics that can be of interest in phRS certification have been certified.

It should be noted that the simultaneous standardization of purity, enthalpy of fusion and melting point has an internal contradiction. The purer the substance is, the more correctly and precisely the enthalpy of fusion and melting temperature are determined. However, an $RS_{EQ}$ should have a sufficiently large content of impurities to be of practical interest in the certification of phRS. The compromise could potentially be achieved by applying the requirements for $max\Delta_{RS}$ for phRS.

To date, the European Pharmacopoeia (Ph.Eur.) has certified $RS_{EQ}$, including those for the thermal analysis method – thermogravimetry (TGA), for the determination of volatile impurities (EP CRS "Kit for equipment qualification") [8]. For the RS of aminosalicylate and amoxicillin, the approximate *RSD* values for replicate determinations to be obtained when qualifying the equipment in another laboratory for the specified analysis conditions are indicated. Besides, the analysis conditions and $RS_{EQ}$ simulate the conditions of the analysis of QC of medicines.

A laboratory can set its criteria. The presence of $RS_{EQ}$ with the provided recommended characteristics for the equipment is extremely valuable for the laboratory since it allows monitoring over the equipment performance for the laboratory's tasks. However, we do not know any $RS_{EQ}$ for the DSC method for which the repeatability of the results is specified.

There are publications in which $X_{RS}$ established by the mass balance method is compared with the purity value obtained by the DSC method ($X_{Alt}$). However, the majority of studied phRS are unsuitable to be used as $RS_{EQ}$ for being very pure substances [9, 10], safety concerns [11] or other reasons. In work [12], the $X_{RS}$ and $X_{Alt}$ of sixteen phRS obtained by the DSC method have been compared. The authors show that some of the phRS melt with decomposition and, therefore, are inapplicable for the DSC method. Some of the phRS have been certified as secondary RS, and the authors argue that a great difference between $X_{Alt}$ values may be due to the high value of $\Delta_{RS}$ for $X_{RS}$. For some phRS, the value for $X_{RS}$ specified by the authors is lesser than the minimum purity for similar commercially available substances. Therefore, the selection of a suitable candidate for $RS_{EQ}$ is an urgent task.

The aim of our work is to select a $RS_{EQ}$ suitable for the tasks of certification of phRS by the method of DSC based on the metrological criteria of the SPhU.

## Materials And Methods

The suitability for equipment qualification of the following pharmaceutical substances and their impurities was studied: chlorquinaldol, clopidogrel hydrosulfate, cholesterol, phenylephrine hydrochloride, paracetamol, and the impurities synthesized in the laboratory (Metamizole impurity A (4-formylamino-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one), Metamizole impurity B (4-aminoantipyrine), (4-amino-1,5-dimethyl-2-phenyl-1,2-dihydro-3H-pyrazol-3-one),

Diclofenac impurity A (1-(2,6-dichlorophenyl)-1,3-dihydro-2H-indol-2-one.

The following studies were conducted using a sample of cholesterol, which was a crystalline powder of a white or almost white colour (CAS 57-88-5, formula: $C_{27}H_{46}O$; M.m. 386.65 g/mol) (Sigma, lot SLBK9675V), with purity of (≥99%).

DSC was performed using a Mettler Toledo DSC 1/700. The overall performance of the apparatus, including the heat flow, temperature and enthalpy, was calibrated monthly using the indium NIST traceable CRM and the In Check programmed method stored in the STARe software in accordance with the user's manual. The purity analysis was performed according to a standard procedure, see, e.g. [5, 13, 14].

In the study, an Agilent 7890A gas chromatograph with an ASL 7693 autosampler; an Agilent HP-1 capillary quartz column, 30m x 0.32mm, ID 0.25μm, part. No 19091Z-413; "Sartorius" MC 210 S balance; the ISO volumetric glassware of class A were used.

## Results And Discussion

**The rationale for the requirements for metrological characteristics of the analysis result in the phRS certification**

Based on the fact that an $RS_{EQ}$ practically simulates the results to be obtained when certifying phRS, we formulated metrological requirements for the analysis results obtained using an $RS_{EQ}$. Hereinafter, the value of 0.5% is used as the $max\Delta_{RS}$.

***Task 1: determination of phRS purity***. The results of $X_{RS}$ established by subtracting the impurity content from 100% and by an alternative method ($X_{Alt}$) may differ due to the uncertainty inherent in both analysis results. However, if the difference between $X_{RS}$ and $X_{Alt}$ exceeds $max\Delta_{RS}$, it indicates a lack of knowledge about the impurities and questions the correctness of $X_{RS}$. Therefore, it is reasonable to impose the following requirements for the results of determining the purity of phRS by the alternative method:

$$\Delta_{RS\_Alt} \le max\Delta_{RS} \quad (1)$$

$$|X_{Alt} - X_{RS}| \le max\Delta_{RS} \quad (2)$$

where $\Delta_{RS\_Alt}$ is the uncertainty of the assessment of purity of RS by the alternative method.

If the requirement (1) is not observed, it is incorrect to compare $X_{Alt}$ and $X_{RS}$. By increasing the number of replicate determinations, $\Delta_{RS\_Alt}$ can be reduced. Failure to comply with the requirement (2) may indicate either the lack of knowledge of the composition of impurities or the fact that DSC is not applicable to the object of analysis. If the former is the case, the result of $X_{RS}$ or $X_{Alt}$ may need to be corrected. If the latter is the case, it is necessary to look for another alternative method for determination of purity.

Typically, 3 to 10 replicate measurements are used to obtain the average analysis result. In the simplest case, the confidence interval can be used as an uncertainty assessment for $\Delta_{RS\_Alt}$:

$$\Delta_{RS\_Alt} = RSD \times t_{one\text{-}sided} / \sqrt{n} \quad (3)$$

where *RSD* is the relative standard deviation for the results of determination of $RS_{EQ}$ purity by the method of DSC; $t_{one\text{-}sided}$ is the one-sided Student's coefficient for the confidence level of 95% and the number of degrees of freedom of *n-1*; *n* is the number of replicate determinations used for averaging.

It should be noted that we use a one-sided Student's coefficient as the use of the two-sided one corresponds to the reliability level of 97.5%, [15]. Setting the groundlessly high reliability level can lead to non-compliance with the acceptance criteria since, in the phRS certification, the extreme metrological requirements are often applied to the analysis results.

The approach used is correct when other components of uncertainty (e.g. the instrument calibration uncertainty) are insignificant in relation to $\Delta_{RS\_Alt}$. According to the SPhU approach, the component of uncertainty ($\Delta_1$) is negligible in relation to the total uncertainty ($\Delta_2$) at the 95% reliability level when fulfilling the following ratio (the principle of insignificance):

$$\Delta_1 \le 0.32 \times \Delta_2 \quad (4)$$

There may be the case that it is necessary to specify the conclusions made on the assumption of insignificance more precisely. However, we argue that the approach is suitable for the initial assessments of applicability of DSC for the phRS certification and selection of $RS_{EQ}$.

Then, for the DSC method with the use of the $RS_{EQ}$ candidate according to (3), the *RSD* is acceptable if it is within the following limits:

The maximum requirement:

For $n = 3$,

$$maxRSD = max\Delta_{RS} / t_{one\text{-}sided,\ n=3} \times \sqrt{3} = 0.30 \quad (5)$$

The minimum requirement:

For $n = 10$,

$$maxRSD = max\Delta_{RS} / t_{one\text{-}sided,\ n=10} \times \sqrt{10} = 0.86 \quad (6)$$

***Task 2: the study of phRS homogeneity***. The specificity of the DSC method is that due to the poor thermal conductivity of organic substances, the correct results of determining the purity can be obtained only for very small test portions of the test sample. Under the ASTM recommendations [5], the test portion should be 1 to 3 mg. However, in the procedures for QC of medicines, the test portions of 5 to 200 mg of phRS are used. The increase of the test portion of RS used in the procedure for QC of medicines by 2 times is equivalent to the averaging of two independent results of the analysis obtained with the use of the initial test portion of RS [16], i.e. theoretically, the variation caused by inhomogeneity of RS decreases by √2 times. Therefore, in case of detection of significant heterogeneity of phRS by the DSC method, it is necessary to evaluate its impact for the test portion of phRS used in the procedure for QC of medicines.

As discussed above, phRS required for QC of medicines are not always very pure substances. When the actual content of impurities determined by the DSC method (primarily, related impurities) are at the level of 0.5% ($max\Delta_{RS}$), the confirmation of homogeneity is of practical interest for the phRS certification. Moreover, by the ISO Guide 17034 recommendations [17], the homogeneity study should always be conducted as the procedure for the quality assurance of RS even if the material for phRS certification is potentially homogeneous.

The homogeneity studies may be carried out using any suitable method of analysis. If the object of the homogeneity study is the variation in the content of the related impurities that can be correctly determined by DSC, the use of the DSC method alone for studying the homogeneity of phRS is considered suitable and sufficient. ISO Guide [18] recommends assessing homogeneity of RS by variation within and between the RS package (within- and between-unit homogeneity, respectively). The problem is that the majority of phRS are dispensed in quantities sufficient for performing a single analysis only. For such RS, the within-unit homogeneity study makes no sense. Moreover, the between-unit variability reflects the within-unit variability. Therefore, we consider only the homogeneity between separate units.

Homogeneity is assessed as a standard deviation or the corresponding confidence interval of a single value of phRS purity determination [**Error! Bookmark not defined.**, 18, 19]:

$$\Delta_{Homog} = RSD \times t_{two\text{-}sided} \quad (7)$$

where *RSD* is the relative standard deviation for results of determination of $RS_{EQ}$ purity by the method of DSC;

$t_{two\text{-}sided}$ is the two-sided confidence interval for the confidence level of 95% and the number of degrees of freedom of *n-1*.

When studying homogeneity, ISO proposes to use the analysis of variance (ANOVA) to determine the variation caused by the actual heterogeneity of RS from the total variation of the results of the analysis. But ANOVA cannot be used for destructive analysis methods since it is impossible to re-analyse the same test portion of RS. In this case, the requirements for homogeneity can be applied directly to the analysis results (without subtracting the analytical variation). While the direct method gives the most correct assessment of homogeneity of RS, it may also overestimate heterogeneity of RS [20].

Several successive melting and solidification cycles of the test sample can be carried out in DSC when using the substances that are thermally stable in the melting range [11]. This is done, for example, to eliminate the influence of polymorphism on the result of purity determination. However, this approach has limitations. Therefore, we will proceed from the fact that each sample is subjected to analysis only once as for the destructive analysis method.

ISO recommends the use of at least 10 samples to study homogeneity of RS. Therefore, further we assume that 10 individual analysis results from 10 independent samples will be used to study homogeneity.

Following the SPhU approach, the heterogeneity of phRS is acceptable when the following ratio is met:

$max\Delta_{Homog} \le max\Delta_{RS}$, or $max\Delta_{Homog} \le 0.5\%$;

$$RSD \times t_{two\text{-}sided} \le 0.5\%; \text{ or } RSD \le 0.5 / 2.2622; RSD \le 0.22 \quad (8)$$

The same approach is recommended in the ISO guides [18, 19].

Ideally, $\Delta_{Anal}$ should be negligible in relation to $max\Delta_{Homog}$. However, this condition may not always be feasible. Therefore, in practice, a compromise is possible. On the other hand, the destructive analysis method is not suitable for studying the homogeneity of RS if $\Delta_{Anal} > max\Delta_{RS}$ (i.e. > 0.5%). This enables us to formulate the maximum and minimum requirements for the repeatability of the results of purity determination by the DSC method for the phRS homogeneity assessment:

The maximum requirement:

$$\Delta_{Homog} \le 0.32 \times max\Delta_{RS};\ maxRSD = 0.32 \times 0.5/2.2622 = 0.071 \quad (9)$$

The minimum requirement:

$$\Delta_{Homog} \le max\Delta_{RS};\ maxRSD = 0.5/2.2622 = 0.22 \quad (10)$$

Our task is to select a sufficiently homogeneous $RS_{EQ}$ so that its heterogeneity is insignificant compared to the variation of the analysis results. However, in practice, it is time-consuming to prove the absence of heterogeneity. Therefore, there is an only requirement at the first stage: the results of purity determination obtained with the use of $RS_{EQ}$ should meet the requirements of (9) or (10).

***Task 3: determination of enthalpy of fusion and melting point***. The melting point and enthalpy of fusion are indicators that are specific to a particular substance, which allows their use for identification. The article published in the Pharmacopoeial Forum proposes to include heat of fusion values of the USP melting point standards to better standardize the results provided by the industry [21]. At the same time, it is quite difficult to formulate scientifically based criteria for accuracy and precision of determining the melting point and enthalpy of fusion based on the tasks of the phRS certification or QC of medicines. In this regard, it is logical to impose the following requirements: the repeatability of the melting point and enthalpy of fusion of $RS_{EQ}$ should not be worse than those of RS intended for the determination of these parameters.

**Requirements for $RS_{EQ}$ for determination of purity by the method of DSC**

It should be mentioned that the requirements for $RS_{EQ}$ are not limited to the following ones.

***General requirements***. The $RS_{EQ}$ candidate should be:

- non-toxic, environmentally friendly, stable, inexpensive, easily available;
- convenient in operation, in particular, not too hygroscopic, i.e. should not require special protection from the atmospheric moisture.

***The functional properties concerning the DSC method.*** To be optimal for the application of the DSC method, the $RS_{EQ}$ candidate should possess the following properties:

- melt without decomposition;
- contain a single crystal modification;
- the melting point should be high enough so that the present volatile impurities do not interfere with the determination of purity associated with the content of nonvolatile impurities;
- form a eutectic mixture with impurities.

Preferably, the impurities to be determined by the DSC method should be related to the active substance and having a similar molecular weight.

***The functional properties concerning the problem to be solved***. To be optimal for solving the task set, the $RS_{EQ}$ candidate should possess the following properties:

- be an organic substance like most phRS, i.e. have a similar thermal conductivity;
- allow standardizing a variety of properties that are determined by the DSC method and can be important in the certification of phRS and QC of medicines;
- have an acceptable level of impurities and similarity in determining the content of impurities with other methods of analysis.

The uncertainty of determining the purity for the DSC method is improved by decreasing the content of impurities. The method is considered unsuitable for the quantitative assessment of purity if the content of impurities is greater than 2.5 mol %, see [**Error! Bookmark not defined.**], <891> *Thermal Analysis* [4], *2.2.34 Thermal analysis* [22]. Therefore, when choosing a candidate for $RS_{EQ}$, the total content of impurities to be determined by the DSC method is important.

Determination of the impurity content in $RS_{EQ}$ at a level that is insignificant compared to $max\Delta_{RS}$, in other words when the minimum content of impurities (*minC*) is 0.16% (*minC* =

0.32×0.5% = 0.16%), is uninformative for the task of phRS certification. Taking into account that $max\Delta_{RS}$ = 0.5% for phRS, it is desirable that $minC \approx 0.5\%$. How large the impurity content can be for $RS_{EQ}$ is determined by the uncertainty of $X_{Alt}$; the requirement $\Delta_{RS\_Alt} \leq 0.5\%$ should be observed.

The result of the determination of the content of impurities by the DSC method should be similar to those obtained by other methods to verify the correctness of knowledge about impurities. In the optimum case, the discrepancy should be insignificant with respect to $max\Delta_{RS}$, i.e. 0.5%×0.32 = 0.16%. However, such a small discrepancy may not be achievable in practice. The discrepancy that is equal to $max\Delta_{RS}$, i.e. to 0.5%, is maximum permissible. Thus, the following requirements can be imposed:

The maximum requirement:

$$|X_{RS} - X_{Alt}| \leq 0.32 \times max\Delta_{RS}; |X_{RS} - X_{Alt}| \leq 0.16 \quad (11)$$

The minimum requirement:

$$|X_{RS} - X_{Alt}| \leq max\Delta_{RS}; |X_{RS} - X_{Alt}| \leq 0.5 \quad (12)$$

**Experimental results**

All studied $RS_{EQ}$ candidates were organic substances, which melted without decomposition. The melting points of the $RS_{EQ}$ were above 100 °C, and the content of related impurities in them was of approximately 0.5% (according to the results of determination by chromatographic methods).

**Table 1. The results of determination of the melting point, enthalpy of fusion and purity of cholesterol**

| The sample No. | The test portion, mg | The melting point, °C | Enthalpy of fusion, kJ/mol | Purity, % | The melting point of a pure substance, °C | The content of volatile impurities, % |
|---|---|---|---|---|---|---|
| 1. | 2.92 | 149.113 | 28.09 | 99.75 | 149.12 | 1.8 |
| 2. | 2.22 | 149.097 | 28.04 | 99.78 | 149.10 | 2.6 |
| 3. | 2.70 | 149.092 | 27.24 | 99.53 | 149.15 | 2.2 |
| 4. | 2.72 | 149.047 | 27.77 | 99.62 | 149.09 | 3.0 |
| 5. | 2.32 | 149.046 | 27.97 | 99.63 | 149.10 | 2.4 |
| 6. | 2.55 | 149.110 | 27.39 | 99.59 | 149.18 | 4.8 |
| 7. | 2.52 | 149.105 | 27.06 | 99.57 | 149.16 | 2.5 |
| 8. | 2.50 | 149.097 | 27.96 | 99.63 | 149.13 | 2.1 |
| 9. | 2.38 | 149.045 | 27.95 | 99.83 | 149.06 | 2.3 |
| 10. | 2.63 | 149.096 | 27.27 | 99.84 | 149.08 | 3.1 |
| *mean* | 2.546 | 149.08 | 27.7 | 99.68 | 149.12 | 2.7 |
| *SD* | | 0.028 | 0.39 | 0.11 | 0.038 | 0.84 |
| *RSD* | | 0.018 | 1.4 | 0.11 | 0.026 | 31 |
| *t* = 1.8331* | | | | | | |
| *C.I.** | | 0.051 | 0.72 | 0.21 | 0.070 | 1.5 |
| *C.I.%** | | 0.034 | 2.6 | 0.21 | 0.047 | 58 |
| *SDmean** | | 0.0087 | 0.12 | 0.036 | 0.012 | 0.27 |
| *RSDmean** | | 0.0058 | 0.45 | 0.036 | 0.0081 | 9.9 |
| *C.I.mean** | | 0.016 | 0.23 | 0.065 | 0.022 | 0.49 |
| *C.I.mean%** | | 0.011 | 0.82 | 0.066 | 0.015 | 18 |

**t* is the one-sided Student's coefficient for the confidence level of 95% and the number of degrees of freedom of 9; *C.I.* = *SD* × *t*; *C.I.%* = *RSD* × *t*; *SDmean* = *SD*/√10; *RSDmean* = *RSD*/√10; *C.I.mean* = *SD* × *t*/√10; *C.I.mean%* = *RSD* × *t*/√10.

We tested the substances listed in Section 2 and found out that in spite of having suitable melting points and impurity contents, all of them, except for cholesterol, were unsuitable for further research as they exhibited polymorphism. Therefore, the following studies were conducted on a sample of cholesterol.

The typical DSC scan and purity analysis for cholesterol is shown in Fig. 1. The purity of cholesterol was determined by the melting point peak of 10 replicate measurements in scanning in the temperature range of 50 ÷ 200 °C with the rate of 1°C/min. The peak was sharp with a regular shape; the baseline was linear.

The test portions, results of determination for 10 independent measurements of the melting point, enthalpy of fusion and purity of cholesterol are shown in Tab. 1.

It should be noted that for each sample, the mass after melting did not coincide with the initial mass. We assume that the volatile impurities were not anything other than the adsorption water that was removed at the beginning of heating the sample in the DSC experiment (at 130 ÷ 132° C). The removal of volatile impurities occurred at a lower temperature than melting of cholesterol, which, therefore, did not interfere with the determination of purity.

The results obtained are in good agreement with the literature data (www.sigmaaldrich.com; https://pubchem.ncbi.nlm.nih.gov), see Tab. 2.

**Table 2. Comparison of the experimental results of determination of the melting point and enthalpy of fusion of cholesterol with the literature data**

| | Literature data | Experimental data |
|---|---|---|
| Melting point, °C | 147.0-150.0 | 149.12 |
| Enthalpy, kJ/mol | 26.50-28.50 | 27.70 |

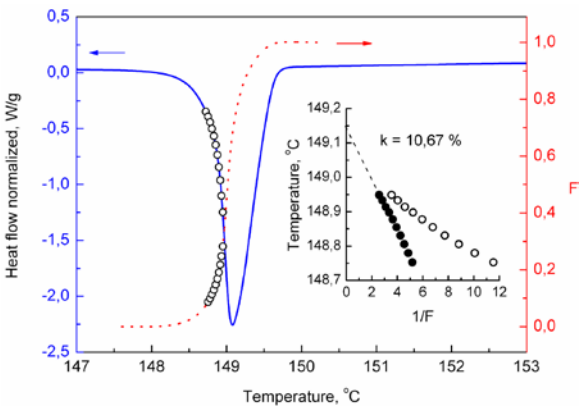


**Fig. 1. Details of purity analysis for cholesterol: DSC scan and completeness of fusion (F).**

The used experimental data are marked with circles. Inset: Van't Hoff plot (open symbols) and its linearization (solid symbols); *k* is the corrected value for linearization (data for sample No. 3 are shown; see Tab. 1).

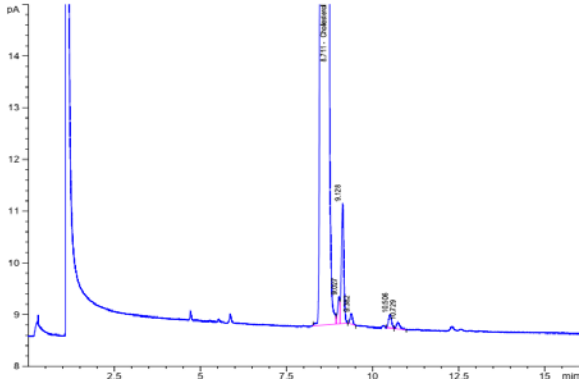

**Fig. 2. The typical chromatogram of cholesterol obtained in the conditions specified in the monograph *Cholesterol* (Assay), Ph.Eur.**

The cholesterol purity was independently determined by gas chromatography in accordance with the monograph of the Ph.Eur. (Assay) [22], see Fig. 2.

Since there was no information about the nature of impurities, we assumed that those were related impurities, and for them, the response was close to that of cholesterol. The content of impurities was determined by the method of internal normalization. According to the results of the analysis of 3 independent test solutions with 2 replicate chromatograms for each, the purity of cholesterol was 99.40%. The difference between the values determined by the DSC method was 0.28%, which is less than the critical value of 0.5%, and, therefore, comply with the requirements (11) and (12) specified for $RS_{EQ}$.

Interestingly that the NIST CRM 1514 designed to verify the purity determination obtained by the DSC method also gives a result biased towards lesser values compared to that obtained by liquid chromatography; the difference is 0.2 to 0.4 molar % [**Error! Bookmark not defined.**]. Authors assume that impurities are in the form of a solid solution, and, therefore, are not detected by the DSC method. However, they conclude that the presence of impurities in such an amount does not much interfere with the intended use of the CRM.

For EP CRS (voriconazole CRS 2, triamcinolone acetonide CRS 6, simvastatine CRS 5) intended for quantitative determination, the differences in the certified values established by the mass balance method and by the DSC reach 0.5% of the mass (www.edqm.eu/en/reference-standards-training-resources). This is in good agreement with the criterion (12): the maximum allowable difference of the purity assessment obtained by orthogonal methods of analysis is 0.5%.

From the Tab. 1, we can see that for cholesterol, the uncertainty assessment based on the repeatability of the analysis results (*C.I.mean* = 0.066), is close to the reported values of uncertainty for NIST CRM 1514 (the content of impurities is 0.69 ± 0.07 molar %) and LGC2013 (the content of impurities is 0.1 ± 0.1 molar %). The melting point for the average value has significant figures in the hundredths of $^{o}$C just like for the RS proposed for calibration by the melting temperature [**Error! Bookmark not defined.**]. Enthalpy of fusion, too, has significant figures in the hundredths just like other RS for which this parameter was certified.

To sum up, the suitability of the studied sample of cholesterol for the use as a candidate for $RS_{EQ}$ is as follows:

<u>- for the task of determining the phRS purity</u>. The average value of purity is different from the one found by gas chromatography by 0.28%. As expected, the requirements for the insignificance of $|X_{RS} - X_{Alt}|$ were not met (11), whereas the requirements for the maximum allowable difference (12) and the maximum requirements for the repeatability of the results (5) were fulfilled (*maxRSD* = 0.30%; actual *RSD* = 0.11%).

<u>- for the task of the phRS homogeneity study</u>. The maximum requirements for insignificance of $\Delta_{An}$ (9) were not met (*maxRSD* = 0.071), whereas the minimum requirements (10) (*maxRSD* = 0.22) were fulfilled.

We can assess how $\Delta_{An}$ distorts the assessment of homogeneity. For maximum permissible variation of the analysis results:

$\Delta_{Homog}$ = 0.5%. $\Delta_{An}$ = *RSD* × *t*(*n*-1; two-side; 95%) = 0.113 × 2.2622 = 0.226%;

$$\Delta_{Homog} = \sqrt{\Delta_{An}^2 + \Delta_{RS\_H}^2};$$

$$\Delta_{RS\_H} = \sqrt{\Delta_{Homog}^2 - \Delta_{An}^2} = \sqrt{0.5^2 - 0.226^2} = 0.43\%;$$

$$\left(\Delta_{Homog} - \Delta_{RS\_H}\right)/\Delta_{Homog} \times 100\% = (0.5 - 0.43)/0.5 \times 100\% = 14\%$$

.

where $\Delta_{RS\_H}$ is the heterogeneity caused by phRS itself.

When using DSC with the obtained metrological characteristics, the requirements for the actual value of the maximum heterogeneity of phRS were tightened by 14% only, which is acceptable from the practical standpoint. Thus, $\Delta_{Homog}$ is decreased from 0.5% to 0.43%. Therefore, the studied sample of cholesterol meets the requirements for $RS_{EQ}$ for the determination of purity by the DSC method. The sample also allows us to simultaneously test the accuracy and precision of determining the melting point and the enthalpy of fusion.

This $RS_{EQ}$ candidate can be used for its purpose as follows. In the certificate for $RS_{EQ}$, the conditions of the analysis and the actual values of the results of the analysis obtained in this work should are reported. The laboratory performing the equipment qualification obtains the results of the analysis under the conditions specified in the certificate and compares them. This approach is used by the Ph.Eur. for EP CRS "Kit for equipment qualification" [**Error! Bookmark not defined.**].

We propose to include the following information in the certificate for $RS_{EQ}$ of cholesterol:

"Purity by DSC determined on 2–3 mg of the substance applying the below temperature programme. Heating programme: hold for 10 min at 135 °C, then heat to 160 °C with the heating rate of 1°C/min. Container: about 40 µl. Without purging (air atmosphere). n = 10.

Purity (amount fraction): 99.7%. SD = 0.11.

Enthalpy of fusion: 27.7 kJ/mol. SD = 0.39.

Temperature of the melting peak maximum: 149.12 $^{o}$C. SD = 0.038".

When using such an RS, the laboratory should make a decision concerning the state of the analytical system independently, which may create difficulties. Therefore, it may be useful to indicate the uncertainty of the certified value and the limits within which the results of the analysis should be in the certificate, as it does Ph.Eur. for non-thermal methods of impurity determination. Such a characterization of an RS is possible when studying the intra-laboratory and inter-laboratory variation of the results.

In this regard, further, we will study the intra- (robustness and intermediate precision) and inter-laboratory variation of the results to determine the uncertainty typical for the normal laboratory practice using the candidate material of cholesterol.

## CONCLUSIONS

1. The criteria for metrological characteristics of the results of purity determination for the establishment of the certified value and homogeneity study in the certification of pharmaceutical reference standards by DSC was formulated based on the requirements of the State Pharmacopoeia of Ukraine.
2. The sample of cholesterol was selected as a reference standard candidate for equipment qualification for purity tests by DSC.
3. For the selected sample of cholesterol, the melting point and enthalpy of fusion can be simultaneously certified. The metrological characteristics obtained are as good as those for the available RS – pure substances intended for these purposes.
4. The further study of intra- and inter-laboratory variation of the results obtained with the use of the selected sample of cholesterol is necessary to characterize the uncertainty of the purity determination results.

## REFERENCES

1. *ICH Specifications and Control Tests on the Finished Product (Topic 3AQ11a)*. London: EMEA, 1991, 83-94.
2. *The State Pharmacopoeia of Ukraine*, 2nd ed., Vol. 1, Ukrainian Scientific Pharmacopoeial Center for Quality of Medicines, Kharkiv 2015.

3. Manning, R. G., Lane, S. et al., *Pharm. Forum 2007, 33(6),* 1300-1310.
4. *The United States Pharmacopoeia*, 41 - NF 36, The United States Pharmacopeial Convention, Rockville 2018.
5. *ASTM E 928-08(2014). Standard test method for purity by differential scanning calorimetry* 2014.
6. *NIST Standard Reference Material 1514.*
7. *LGC Reference materials for physical properties* 2008/2009.
8. *EP CRS. Kit for equipment qualification*.
9. Yang, D., Wang, F., Zhang, L., Gong, N., Lv Y. *Acta Pharm Sin B* 2015, 5 (3), 231-237.
10. Nogueira, R., Rocha, W. F., Silva et al., *J. Braz. Chem. Soc. 2012*, 23 (3), 435-444.
11. Kestens, V., Roebben, G., Linsinger, T., *Accred. Qual. Assur*. 2010, 15(5), 269-281.
12. Mathkar, S., Kumar, S., Bystol, A. et al., *J. Pharm. Biomed. Anal.* 2009, 49 (3), 627-631.
13. Brown, M. E., *J. Chem. Educ. 1979*, 56 (5), 310-313
14. Souza Araújo, A. A., dos Santos, B. M., et al. *Braz. J. Pharm. Sci. 2010, 46 (1),* 37-43.
15. Bettencourt da Silva, R., Williams, A. *Eurachem/CITAC Guide: Setting and Using Target Uncertainty in Chemical Measurement. 1st ed. 2015*.
16. Rossbach, M., Grobecker, K. H., Accred. Qua.l Assur. 1999, 4, 56-63.
17. *ISO Guide 17034:2016*.
18. *ISO Guide 35:2017*.
19. *ISO Guide 13528:2015*.
20. Adriaan, M.H. *Accred. Qual. Assur*. 2001, 6, 26–30.
21. Guillermo A Casay, Osomwonken J Igbinosun, Kristina Lilova et al. 2018, *Pharm. Forum* 43(5).
22. *The European Pharmacopoeia,* 9th ed., Vol. 6, European Directorate for the Quality of Medicines & HealthCare of the Council of Europe, Strasbourg 2018.