Benefits of harmonisation of monographs and international reference standards

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Objective

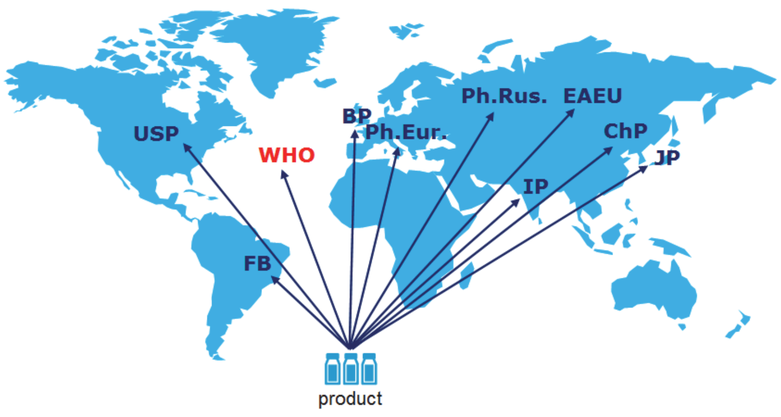
The consequences of availability of international reference standards for patients and industry are outlined. The scenarios discussed are:

1. No monograph and international reference standard is available.
2. Several monographs and international reference standards that are not harmonised are available.
3. One harmonised monograph with corresponding international reference standard is available.

Conclusions and recommendation

- **Patient dosage might be impacted by lack of harmonised monographs and harmonised reference standard.**
- **Harmonisation will ensure consistent patient dosage and support the industry goal of delivering products of excellent quality to all regions of the world.**
- **History has shown that harmonisation is possible.**
- **If harmonisation is not possible it is recommended to**
  - **calibrate pharmacopoeia standards against one "golden" reference standard, or**
  - **mutually recognise already implemented international reference standards.**

Current global situation on monographs and reference standards. Which one to follow?



Introduction

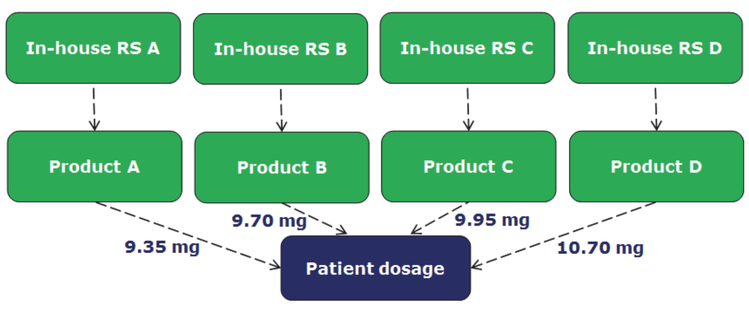
Products must comply with the Pharmacopoeial monographs, i.e. comply with the limits in the monograph when analysed with the analytical method and reference standard stated. Currently, several monographs and international reference standards are available for many products.

The question is which standard should the industry follow?

Some monographs and international reference standards have been harmonised which means that international reference standards from the different pharmacopoeias (and NIBSC) are the same batch and have the same certified value across regions. One example is the Ph.Eur./USP/JP insulin aspart reference standard with the same certified value (7.62 mg per vial).

Three scenarios regarding monographs and reference standards are outlined and discussed.

Scenario 1 - No monograph and international reference standard is available



Scenario 1: A-D illustrate different manufacturers. RS: Reference standard.

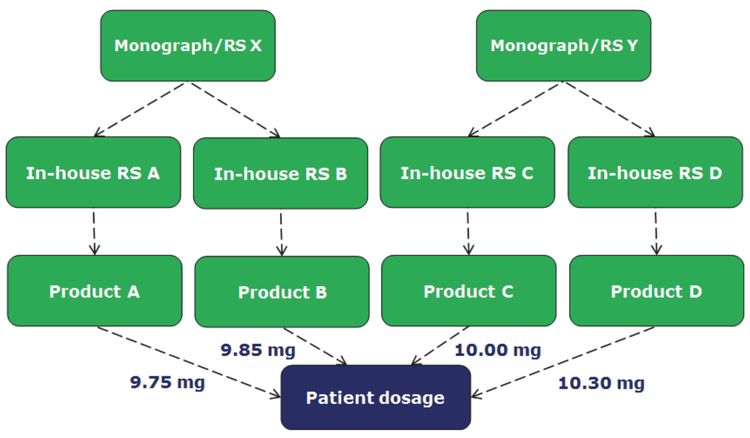
In **Scenario 1** each manufacturer has to define and control their own analytical method and in-house reference standard as illustrated. Hence manufacturers may use different analytical principles to certify their in-house reference standard and to analyse their products.

The different in-house reference standards will have different true content/potency level, which is not an issue. However the difference between the true value and the certified value of an reference standard may vary significantly as illustrated in the **box**. This is primarily due to the use of different methods/analytical principles. Depending on the size of difference and whether it is positive or negative, the difference might influence the analytical results for products significantly.

Hence the products produced by different manufacturers may be assigned significant different content or potency values. **This may have a direct significant impact on patient dosage** if patients shift between manufacturers.

An example of this is Somatropin, where Japan discovered a difference of up to 25% in effect for products from different manufacturers when administering the same dose. The reason for this was use of different bio-assays with very high variation. Eventually this was solved by replacing the bio-assays with a harmonised monograph content method and establishing a mutual recognised WHO standard.

Scenario 2 - Several monographs and international reference standards not harmonised



Scenario 2: A-D illustrate different manufacturers. X-Y illustrate different monographs and corresponding reference standards. RS: Reference standard.

In **Scenario 2** the certified value of the individual reference standards is set by each pharmacopoeia and manufacturers might follow different monographs with the corresponding international reference standard as illustrated.

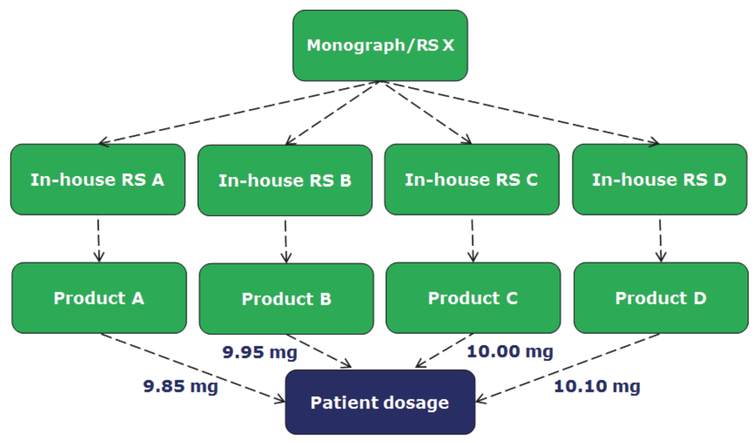
As in scenario 1 the different reference standards will have different certified values for content/potency, which is not an issue, but the difference between the true value and the certified value of an reference standard may vary as illustrated in the **box**. In scenario 2 the difference is due to the use of different pharmacopoeial method and the nature of the corresponding reference standard used as well as the uncertainty at establishment.

The size of difference in scenario 2 is expected to be smaller than in scenario 1 because methods are defined by the pharmacopoeias. Furthermore the difference is even smaller for manufactures using the same monograph and corresponding reference standard.

However the products produced by different manufacturers may still be assigned different content or potency values. **This may have a direct impact on patient dosage** if patients shift between manufacturers. However the impact is not as pronounced as in scenario 1.

Another disadvantage of scenario 2 is risk of no availability of products in different countries as products may not be able to comply with all pharmacopoeias.

Scenario 3 - One harmonised mono-graph with corresponding international reference standard



Scenario 3: A-D illustrate different manufacturers. X is the harmonised monograph and corresponding reference standard. RS: Reference standard.

In **Scenario 3** the analytical method and the corresponding international reference standard used to establish in-house reference standard is the same for all manufacturers as illustrated.

Use of same monograph ensures that products are tested under the same conditions which enhances similar product quality irrespectively of manufacturers as well as support consistent dosage for patients.

Hence **consistent dosage for patients** irrespective of manufacturer and country **is expected to be significantly improved** compared to scenario 1 and 2.

From a patient perspective as well as an industrial perspective it is of great advantage to have harmonised monographs and corresponding international reference standards.

Recommendations in case of no harmonisation

If harmonisation is not possible it is recommended that new international reference standards are certified against one "golden" reference standard or that mutual recognition of reference standards is applied.

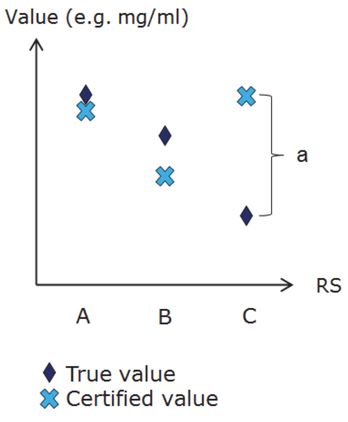
It is also recommended that the equivalent reference standards from the major pharmacopoeias are included in the collaborative studies. This approach could provide data for manufacturers in order to get knowledge about content/potency of their products analysed according to different monographs and corresponding international reference standards.

Box info

The level of the true value of different RS do not influence on patient dosage.

However the difference (a) between the true value and the certified value of a RS varies due to:

- method used
- uncertainty at establishment
- the nature of the RS used for calibration



A-C illustrate different RS. RS: Reference standard.

Conflict of interest disclosure

AR, LVL, BEBH, LEG, TL, KB, PH and GBW are employees of Novo Nordisk A/S, Denmark.

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The approach of the State Pharmacopoeia of Ukraine to the homogeneity study of pharmacopoeial reference standards

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ABSTRACT

Pharmacopoeial reference standards are primarily used in pharmaceutical analyses to ensure adequate quality control of medicines. However, only when the uncertainty for the property value assigned to a reference standard is known can the consistency and comparability of measurement results both over time and between laboratories be guaranteed.

By ISO Guide 17034, the combined standard uncertainty for the assigned value of the reference standard should take into consideration all relevant uncertainty sources, including the ones associated with the possible heterogeneity of the reference standard. An assessment of the homogeneity of reference standards in final packaged forms has to be carried out by their producers. Yet, for most pharmaceutical substances used as candidate reference materials are very pure, the homogeneity assessment of pharmacopoeial reference standards is often underestimated. As there is a number of the pharmaceutical reference standards containing large amounts of impurities (mainly, crystal hydrates), the issues concerning the homogeneity assessment should be addressed.

Conventionally, characterisation of a pharmacopoeial reference standard is the assignment of the property value to a batch of the pharmaceutical substance packaged in vials (units) in an amount sufficient to perform a single measurement or run of the experiment. This means that the laboratory will be assessing the quality of the medicine using only one unit of the reference standard from the batch. Consequently, any unit of the reference standard should be representative of the whole batch. The standard analytical practice of taking only one test portion from the unit for the measurement compounds the problem of ensuring the correctness of the measurement results; the smaller the test portion, the higher the measurement uncertainty.

Considering the above, it is essential for the reference standard producer to ensure the acceptable between-unit heterogeneity of the reference standard and, if necessary, establish the minimum test portion that provides sufficient homogeneity for the intended use. Still, pharmacopoeias do not provide recommendations on conducting homogeneity assessment of reference standards. This leads to using ISO guidelines not specifically targeted at and, accordingly, have limitations for the use in the pharmaceutical sector.

We propose the acceptance criteria and procedure to control the homogeneity of pharmacopoeial reference standards based on their intended use.

INTRODUCTION

ISO Guide 17034:2016

- Requires the assessment of the homogeneity of any reference material to ensure its fitness for purpose

ISO Guide 35:2017

- Provides guidance for homogeneity testing and the establishment of the minimum sample size

- considers the within- and between-unit heterogeneity
- proposes to use the standard deviation (*SD*) as a heterogeneity marker
- if the standard uncertainty associated with heterogeneity (u_{hom}) is significant, recommends its inclusion to the uncertainty for a certified value of a reference standard (u_{rs})
- suggests using ANOVA to separate estimation of within-run, between-run and between-unit variances
- allows increasing the contribution of u_{hom} to the u_{rs} in case of insufficient precision of the measurement procedure used for homogeneity studies
- if u_{hom} is extremely large, allows omitting it from the uncertainty budget in special circumstances

AIM

To establish acceptance criteria to control homogeneity of the pharmaceutical reference standards based on their intended use

The approach to the homogeneity assessment of pharmaceutical reference standards should rest on the ISO approach and take into account the specifics of the pharmaceutical sector

RESULTS AND DISCUSSION

Rationale

- Specifications for the quality control of medicines are set in advance
- For medicines with the content limits of API of ± 5%, no further justification is required
- ANOVA requires conducting $n>1$ replicate measurements, which makes it unsuitable for destructive methods of testing ($n=1$), which is often the case in the characterisation of pharmaceutical reference standards (titration, thermal methods of analysis)
- u_{hom} can make a major contribution to u_{rs}.
- By the State Pharmacopoeia of Ukraine,
  - for medicines, the target uncertainty of measurement results (U_{target}) should be insignificant compared to the specification limits
  - the maximum permissible expanded uncertainty for the value assigned to a reference standard ($max\Delta_{rs}$) should be negligible compared to the target uncertainty of measurement results (U_{target})

Consequently, for pharmacopoeial reference standards intended for assays in medicines with the content limits of ± 5%, the maximum permissible expanded uncertainty for the value assigned to a reference standard

$$max\Delta_{rs} = 0.5\%.$$

- The value of 0.5% is often the lowest extreme value for analysis methods used for characterisation of pharmaceutical reference standards at which it may be impossible to separate the heterogeneity contribution (insufficient precision)
- Therefore, for pharmaceutical reference standards, we consider inclusion of the separate assessment of the heterogeneity contribution to the uncertainty budget unreasonable unless proven otherwise.

RESULTS AND DISCUSSION (continued)

The proposed approach

- The expanded uncertainty associated with u_{hom} (U_{hom}) should be negligible compared to the target measurement uncertainty (U_{target}).
- For any test portion, the property value of the pharmaceutical reference standard should lie within $max\Delta_{rs}$.

However, when a single criterion based on the requirements for the *SD* or confidence interval (CI) is used, it is possible that the requirements are satisfied even though the property values obtained for some test portions fall outside $max\Delta_{rs}$ and, as a result, the pharmaceutical reference standard is not rejected.

The suggested procedure

- determine the property values for n test portions analysed (X_i) for the degrees of freedom greater than or equal to 9;
- for X_i, calculate individual content deviations (d_i) and a two-sided 95% CI (U_{hom}).

Acceptance criteria

We consider pharmacopoeial reference standards meet the requirements for homogeneity when the two following criteria are both fulfilled:

Criterion 1. U_{hom} should be less than or equal to $max\Delta_{rs}$.

Criterion 2. None of the individual content deviations (d_i) should exceed the 99% CI derived from $max\Delta_{rs}$ ($maxd$).

We consider $max\Delta_{rs}$ as a 95% two-sided CI for the population standard deviation

$$max\Delta_{rs} = Z_p \times \sigma$$

where

σ Is the population standard deviation,

Z_p is the normal deviate.

Then, for 99% CI,

$$maxd = (2.59/1.96) \times 0.5\% = 0.66\%,$$

where 2.59 and 1.96 are the normal deviates for 95% and 99% CI, respectively.

The approach is close to the one used to confirm the uniformity of dosage units.

The approach has been tested in the certification of reference standards of the State Pharmacopoeia of Ukraine

Water present in a large amount in the pharmacopoeial reference standard may serve as a marker of its homogeneity.

Since the uncertainty for the property value of the pharmacopoeial reference standard (X) is estimated, the *SD* values obtained for the water content (SD_W) can be converted to *RSD* for the property value (RSD_X):

$$\mathrm{RSD_X} = \frac{SD_W \times 100\%}{100\% - \bar{W}}$$

Deviations in the water content can be assessed as follows:

$$\mathrm{d} = \frac{W_i - \bar{W}}{100\% - \bar{W}} \times 100\%$$

Examples

Atropine sulfate

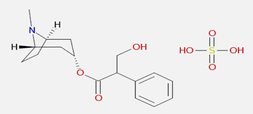

Table 1. The results of the homogeneity assessment of atropine sulfate; test portion: 50 mg

#	W_i %	d_i
1	3.88	0.19
2	3.59	-0.11
3	3.62	-0.08
4	3.60	-0.10
5	3.67	-0.03
6	3.58	-0.12
7	3.63	-0.07
8	3.65	-0.05
9	3.58	-0.12
10	3.75	0.05
$\bar{W}$	**3.66**	-
SD	0.094	-
RSD_X	0.098	-
U_{Hom}	**0.22 < 0.50**	**0.19 < 0.66**
Conformity	Yes	Yes

Calcium oxalate monohydrate

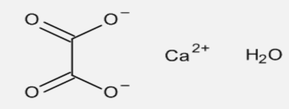

Table 2. The results of the homogeneity assessment of calcium oxalate monohydrate; test portion: 20 mg

#	W_i %	d_i
1	11.4	-0.50
2	11.9	0.07
3	11.8	-0.05
4	11.8	-0.05
5	11.8	-0.05
6	11.8	-0.05
7	11.7	-0.16
8	11.7	-0.16
9	11.8	-0.05
10	11.9	0.07
$\bar{W}$	**11.8**	-
SD	0.143	-
RSD_X	0.162	-
U_{Hom}	**0.37 < 0.50**	**|-0.50| < 0.66**
Conformity	Yes	Yes

Ouabain

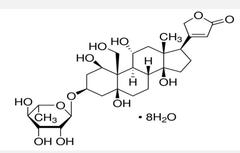

Table 3. The results of the homogeneity assessment of ouabain

#	Test portion					
	40 mg		80 mg		120 mg	
	W_i %	d_i	W_i %	d_i	W_i %	d_i
1	19.87	-0.11	19.20	-0.06	19.13	0.26
2	19.48	0.37	18.90	0.31	19.23	0.14
3	19.12	0.82	19.00	0.19	19.32	0.02
4	19.26	0.65	19.03	0.15	19.28	0.07
5	19.13	0.81	19.02	0.16	19.53	-0.24
6	20.75	-1.21	19.62	-0.58	19.70	-0.45
7	20.62	-1.05	19.17	-0.02	19.26	0.10
8	19.57	0.26	19.16	-0.01	19.28	0.07
9	20.34	-0.70	19.15	0.00	19.36	-0.02
10	19.69	0.11	19.29	-0.17	19.35	0.26
$\bar{W}$	**19.78**	-	**19.15**	-	**19.34**	-
SD	0.60	-	**0.20**	-	**0.16**	-
RSD_X	0.75	-	**0.25**	-	**0.20**	-
U_{Hom}	**1.69 >**	**1,21 > 0.66**	**0.56 >**	**0.58 < 0.66**	**0.45 < 0.50**	**0.45 < 0.66**
	0.50		**0.50**			
Conformity	No	No	No	Yes	Yes	Yes

- Examples are given for the homogeneity assessment of the reference standards intended for assays in medicines.
- The test portions specified in the user's procedures were used as a starting point.
- $U_{Hom} = t_{95} \times RSD_X = 2.262 \times RSD_X$

The approach does not aim at finding the value of the uncertainty associated with the heterogeneity of the reference standard, but at confirming that it does not exceed the critical value, which simplifies the homogeneity study.

CONCLUSION

Acceptance criteria for homogeneity assessment of pharmaceutical reference standards have been established. The criteria are based on estimates of:

1) the confidence interval for variability arising from heterogeneity;
2) the range for individual deviations.

13th INTERNATIONAL SYMPOSIUM ON PHARMACEUTICAL REFERENCE STANDARDS

Joint EDQM-USP Symposium organised by the European Directorate for the Quality of Medicines & HealthCare (EDQM), Council of Europe

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The approach adopted in Ukraine to the certification of working reference standards for assays of medicines

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INTRODUCTION

Working (secondary) laboratory reference standards (WRS) are widely used for routine quality control of medicines. Only if the risks of incorrect decisions on compliance related to the use of WRS are managed by the medicine manufacturer is the use of WRS correct. The acceptable level of the risk can be assessed using the uncertainty concept. The metrological control should cover the entire chain of measurements starting from the certification of the pharmacopoeial (primary) reference standards (PhRS) to the WRS certification to the decision on compliance of medicines with the specifications.

The pharmaceutical sector has the following specifics:

- a lack of information about the use of the uncertainty concept by pharmacopoeias;
- the uncertainty concept can only be applied based on the standardization rules adopted in the pharmaceutical sector;
- even though WRS are calibrated in a measurement laboratory, the approach required for a calibration laboratory should be used.

Given the above, the State Pharmacopoeia of Ukraine (SPhU) developed the uncertainty concept based on which the principles for the PhRS and WRS certification were established. The system of the WRS certification that rests on the approach adopted by SPhU is being implemented into the laboratory practice of Ukrainian pharmaceutical companies.

RESULTS AND DISCUSSION

Metrological aspects introduced into the State Pharmacopoeia of Ukraine

By the State Pharmacopoeia of Ukraine,

- The requirements for the target measurement uncertainty (U_{target}) has been established for the basic pharmacopoeial tests based on the pharmacopoeial decision-making rule (*SPhU 5.3.N.2^N Validation of analytical procedures and tests*)

Table 1. Recommendations for the target uncertainty of a measurement result (U_{target})

TEST	RECOMMENDATIONS
ASSAY (two-sided specification limits) for some **APIs***:	$U_{target} = B_{Upper} - 100\ \%$
for **medicines** (for limits symmetrical around 100 %):	$U_{target} = 0.32 \times (B_{Upper} - B_{Lower})/2$
ASSAY (one-sided specification limits) for **APIs** and **medicines** (typically for Herbals):	$U_{target} = 6.4\ \%$
UNIFORMITY of DOSAGE UNITS, DISSOLUTION:	$U_{target} = 3\ \%$
RELATED SUBSTANCES **Limit tests**: **Quantitative tests**:	 $U_{target} = 16\ \%$ $U_{target} = 5\ \%$

* in the case when the assigned limit is caused only by analytical variability

B_{Lower} – the lower content limit, B_{Upper} – the upper content limit

- The maximum permissible expanded uncertainty for the value assigned to a reference standard ($max\Delta_{rs}$) should be negligible compared to the target uncertainty of measurement results (U_{target}) (*SPhU 5.12^N Reference Standards*)

$$max\Delta_{rs} = 0.32 \times U_{target.}$$

Apparently, for pharmacopoeial reference standards intended for assays in medicines with the content limits (± *B*) of ± 5%, $max\Delta_{rs}$ = 0.5%.

- For any metrological characteristic of SPhU RS or WRS (Δ_{char}, Δ_{hom}, Δ_{stab}), the requirement set out for $max\Delta_{rs}$ is applied. This is due to the fact that the requirement comprises 1/10 of the content limits for assay in medicines (±*B)*; therefore, all sources of uncertainty for both SPhU RS and WRS do not affect the reliability of the decision on compliance. At that, the uncertainty of 0.5% is extremely achievable for most analytical methods used for the PhRS certification, which makes finding the uncertainty budget for $max\Delta_{rs}$ for both PhRS and WRS impossible.

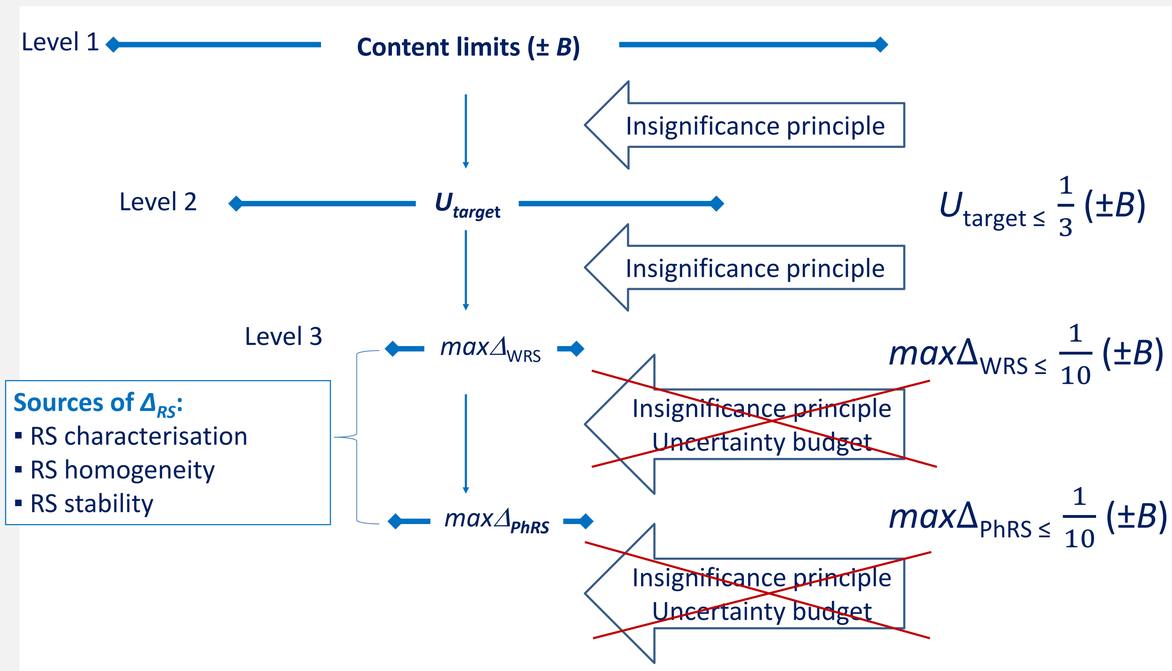

Fig. 1. The SPhU approach to the requirements for uncertainty sources

- $max\Delta_{rs}$ is specified in the certificate for SPhU RS; it remains the same when the batch of SPhU RS is changed.
- The shelf life for SPhU RS is established in the certificate, which allows using the same shelf life for WRS.

The WRS certification system adopted in Ukraine

- WRS are calibrated against SPhU RS by the procedure in which the WRS are used for assays in medicines.
- At least 2 solutions of WRS and SPhU RS are used.
- The sample preparation of the solutions and the number of replicate measurements are optimized so that the predicted uncertainty (*SPhU 5.3.N.1^N Statistical analysis of results of chemical assays and tests*) for the WRS calibration result does not exceed $max\Delta_{rs}$. The gravimetric method is typically used for the preparation of solutions.
- For the results obtained, the actual uncertainty for the WRS calibration is controlled.
- On-site training of personnel that certifies WRS is provided.

By SPhU *5.3.N.2^N*, for comparative analysis methods, the procedure fits its purpose if any of the systematic components of the uncertainty is insignificant (does not exceed 0.5% for *B* = 5%). Therefore, the procedure for assay validated by the SPhU approach is considered suitable for the WRS calibration.

An example of the WRS calibration

Manufacturer's procedure for assay of nicergoline in tablets (content limits: ± 5%; $max\Delta_{rs}$ = 0.5%)	The procedure for WRS certification of nicergoline
Reference solution. Dissolve 50 mg of the SPhU RS of nicergoline in a diluent and bring the solution to a volume of 50 mL. 2 ml of the resulting solution dilute to 100 mL.	Prepare two solutions of PhRS and two solutions of WRS. Dissolve 100 mg of PhRS or WRS of nicergoline (m_1) in a diluent and bring the solution to a volume of 100 mL. Determine the mass of the contents of the flask (M_1). Dilute 2 mL of an accurately weighted aliquot (m_2) to 100 mL. Determine the mass of the contents of the flask (M_2).
Measure the optical density (*A*) on a spectrophotometer (2.2.25) at a wavelength of 288 nm.	
Calculate concentrations of the solutions (*C*): $C = \frac{m_1 \times m_2}{M_1 \times M_2}$ and the normalized responses (*R*): $R = \bar{A}/C$ for the solution of WRS or PhRS.	
Calculate the average of the *R* obtained for the solutions of WRS and PhRS ($\bar{R}_{WRS}$ and $\bar{R}_{PhRS}$, respectively).	
Calculate the certified value of WRS (X_{WRS}): $X_{WRS} = \frac{X_{PhRS} \times \bar{R}_{WRS}}{\bar{R}_{PhRS}}$	

Table 2. Experimental results of the WRS certification of nicergoline

	WRS_1	WRS_2	$PhRS_1$	$PhRS_2$
m_1	0.09961	0.10036	0.10018	0.10006
M_1	83.354	83.301	83.217	83.304
m_2	1.6343	1.6332	1.6388	1.6397
M_2	83.334	83.281	83.253	83.262
C	2.3436E-05	2.3626E-05	2.3697E-05	2.3655E-05
A_1 (288nm)	0.365	0.371	0.371	0.370
A_2 (288nm)	0.367	0.370	0.371	0.370
A_3 (288nm)	0.366	0.371	0.371	0.369
A_4 (288nm)	0.369	0.373	0.371	0.369
A_5 (288nm)	0.370	0.374	0.371	0.369
A_6 (288nm)	0.370	0.375	0.372	0.370
A_7 (288nm)	0.370	0.376	0.372	0.370
$\bar{A}$	0.36814	0.37286	0.37129	0.36957
RSD	0.575	0.608	0.1314	0.1446
RSD_{pooled}		0.43		
R	15.708	15.782	15.668	15.623
D, %	-0.47		0.29	
$\bar{R}$	15.745		15.646	
X	X_{WRS} = 100.5 %		X_{PhRS} = 99.9 %	

Quality control of the WRS calibration results

1. Check for outliers
2. Test of the variances equality
3. The difference in *R* between two solutions of WRS or PhRS (D, %) should not exceed $max\Delta_{rs}$.
4. Estimation of the uncertainty of the certified value due to the characterisation of WRS (Δ_{WRS_char}):

$$\Delta_{WRS\_char} = \sqrt{\Delta^2_{SP} + \Delta^2_{RP}} = \sqrt{0.2^2 + 0.278^2} = 0.34\ (\%)$$

where Δ_{SP} and Δ_{RP} is the uncertainty due to the sample preparation and replicate measurement, respectively;

Δ_{SP} = 0.2mg/100 mg ×100% = 0.2%;

$\Delta_{RP} = RSD_{pooled} \times t_{95;\ one\text{-}sided;\ fp=24} / \sqrt{7} = 0.43\% \times 1.7109 / 2.646 = 0.278\%$.

CONCLUSION

The WRS system has been implemented in more than 20 laboratories and operating for more than 15 years in Ukraine.

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Medicines & Healthcare products Regulatory Agency

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Implemented by the Council of Europe

NIBSC Confidence in Biological Medicines

The development and establishment of an erythropoietin CRS with defined dimer content for SEC system suitability qualification

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INTRODUCTION

Erythropoietin (EPO) is a biotherapeutic medicine widely used to treat anaemia resulting, for example, from chemotherapy. Requirements of the European Pharmacopoeia (2) stipulate that the level of EPO-dimer must be quantified in EPO Active Pharmaceutical Ingredients (with a limit of 2%) using SEC HPLC. However, quantification is hampered by the lack of reference preparations containing stable measurable levels of EPO-dimer (2)which would allow verification of the suitability of the analytical system used. We describe here the development and establishment of a lyophilised, chemically cross-linked EPO preparation as a system suitability reference material for the size-exclusion chromatographic separation of EPO (Project BSP 137).

MATERIALS AND METHODS

Trial Preparation: Glutaraldehyde is widely used to cross-link proteins (3). A highly dimerised EPO was made following optimisation of the glutaraldehyde to protein ratio, incubation period and temperature and this was then diluted into monomeric EPO to give approximately 2% dimer. EPO (total content 100µg) formulated in 3% trehalose, 0.3% arginine, 0.01% Tween20, 0.45% NaCl, 20mM NaP buffer pH 7.4 was dispensed and successfully freeze dried, without changing the dimer content (4).

Candidate Chemical Reference Substance (cCRS) Preparation: Following the success of the small scale study, EDQM supplied sufficient amounts of EPO (a mixture of α and β forms) to fill and freeze dry a cCRS batch at NIBSC with a target of approximately 2% cross-linked dimer. This yielded a total of 5,318 vials at 100µg EPO per vial in the same formulation, with a dry mass of 0.412g/vial, a CV of fill of 0.59%, a residual moisture content of 1.59% w/w (by coulometric Karl Fischer titration) with an inert headspace of nitrogen gas (oxygen content 1.22%).

Collaborative Study: The preparation was evaluated in six laboratories in 5 countries using the Ph Eur method for SEC HPLC described (1). Dimer content and resolution of the dimer from the monomer peak was determined (Table 1, Fig 1). Stability post reconstitution was studied and thermal stressing was undertaken at NIBSC with storage at elevated temperature to assess the impact on the lyophilised material.

RESULTS

Lab	Oligomer		Dimer				Monomer	
	Rel Ret	%	Rel Ret	%	Resolution	P/V ratio	Retention (min)	%
1	0.83	0.16	0.89	3.33	1.72	6.73	18.1	96.5
2	0.81	0.1	0.87	3.38	1.87	10.8	31.8	96.5
3	0.78	0.03	0.89	3.83	1.50	4.93	14.3	96.1
4	0.84	0.17	0.90	3.60	1.73	5.71	18.0	96.2
5	0.79	0.02	0.90	3.00	1.39	3.99	27.7	97.0
6	0.82	0.17	0.88	3.41	1.88	18.63	36.3	96.4
Mean	0.81	0.11	0.89	3.42	1.68	8.46	n.a.	96.5

Table 1: SEC HPLC evaluation of cCRS dimerised EPO lyophilised preparation—each laboratory result is a mean of 4 determinations.

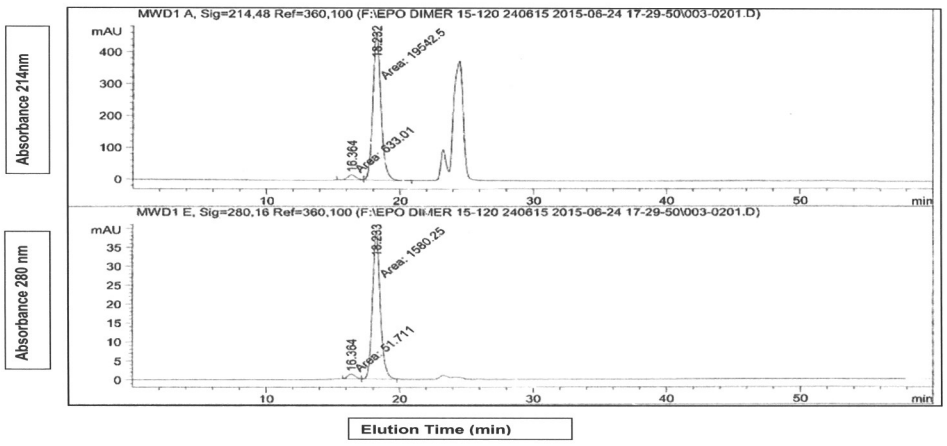

Fig 1: SEC HPLC of dimerised EPO preparation showing absorbance at 214 and 280nm (peaks after monomer are excipient-related)

All six laboratories were able to resolve the EPO dimer from the monomer with a relative retention of 0.87 to 0.90 (dimer/monomer) and a resolution of 1.4 to 1.9. With laboratory 5 excluded, the overall mean dimer content was 3.51% (with CV = 6.0%).
Five laboratories undertook stability testing on the reconstituted cCRS material. A satisfactory stability under refrigerated conditions (+2-8°C) could be established for up to 3 days after reconstitution of the cCRS.
The accelerated and real time stability studies undertaken indicate that though some aggregate forms over time the material remains suitable for purpose.

CONCLUSIONS

Based on the study results, the cCRS was adopted as Ph. Eur. EPO for SEC system suitability CRS batch 1 by the Ph. Eur. Commission at its 159th session in November 2017. The revisions proposed were captured in the Ph. Eur. 9.6 (in force on 1 January 2019).

The CRS is available from the EDQM under catalogue number Y0002009. An example chromatogram is provided with the CRS.

Given its good stability, the CRS may be shipped at ambient temperature to users and should be stored at -20°C upon receipt and until use. The CRS will be monitored at regular intervals

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We thank Standards Processing Division (NIBSC) for the preparation of the definitive fill and Dr Garinot, ANSM France, Dr Portela, Inframed IP, Portugal, Dr Mulugeta , MPA, Sweden and Dr Jorajuria at the EDQM for participating in the collaborative evaluation.

Particular thanks go to Roche Diagnostic GmbH, Germany, and Janssen-Cilag, Switzerland, for the supply of the starting materials.

Establishment of the first Ph. Eur. Biological Reference Preparation for Hepatitis E virus RNA for NAT testing

Council of Europe, European Directorate for the Quality of Medicines & HealthCare, Biological Standardisation Programme

The Biological Standardisation Program (BSP) exists since 1994 and is co-funded by the Council of Europe (EDQM) and the EU Commission. The BSP focuses on the standardisation of the methods and tools for the quality control of biologicals by establishing reference standards and validating new methods with particular focus on reducing, refining and replacing the use of animals (3Rs initiative). The scope of the studies includes biotech products, vaccines and sera for human use, vaccines and sera for veterinary use, blood products and contaminants and allergens. These activities are supervised by the BSP Steering Committee which is composed of the chairs of Ph. Eur. Groups of Experts 6 (Biological and biotechnological substances), 6B (Human plasma and plasma products), 15 (Human vaccines and sera), 15V (Veterinary vaccines and sera) as well as co-opted experts and delegates from the EU Commission, EMA, BWP, IWP and WHO and the EDQM Director. Here we present the results of a study to establish the first Ph. Eur. Biological Reference Preparation (BRP) for Hepatitis E virus RNA for NAT testing. The publication of the study report is openly accessible [1].

Introduction

Hepatitis E virus (HEV) is a single stranded RNA virus belonging to the Hepeviridae family. HEV is a major cause of acute hepatitis where genotypes 1 & 2 HEV are transmitted by the faecal-oral route and is associated with contamination of drinking water. In industrialised areas with safer water sanitation, autochthonous cases of HEV occur and infection with HEV appears more prevalent than originally believed. These cases are mainly caused by genotype 3 HEV originating from undercooked animal meat.

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Since the WHO IS availability is limited, a new Ph. Eur. Biological Reference Preparation (BRP) had to be established further to the decision to include nucleic acid testing (NAT) for the detection of HEV RNA in the Ph. Eur. texts.

To this purpose, an international collaborative study (coded BSP127) was launched in the framework of the Biological Standardisation Programme (BSP) of the European Directorate for the Quality of Medicines & HealthCare (EDQM, Council of Europe) and the Commission of the European Union (EU).

Participants

24 laboratories from14 countries worldwide (Europe, Asia, Americas) enrolled in the study; 23 laboratories reported data.

Materials

The candidate BRP was prepared from a human plasma positive for genotype 3f HEV kindly donated by Dr Cornelia Adhoch (Robert-Koch Institut, Berlin, Germany).

The HEV positive plasma was diluted in pooled citrated human plasma tested negative for HBV, HCV, HIV-1/2, HEV RNA, anti-HEV IgM and anti-HEV IgG and freeze-dried in screw-cap glass vials (0.5 mL/vial).

The fill volume CV was 1.0% (n=30). The residual moisture was determined by Karl Fischer analysis on vials filled with 0.5 mL of plasma diluent and distributed throughout the freeze-drier. The mean residual moisture of the diluent plasma was 0.6% (n=10).

The homogeneity of the filling/freeze-drying was assessed using HEV RT-PCR. The crossing threshold or cycle threshold (C_T) values were determined for 25 vials of the candidate BRP; the mean C_T value was 28.9, with a CV of 0.79%, indicating that the filling was of acceptable homogeneity.

The candidate BRP was stored at -20°C.

Accelerated thermal degradation stability study showed that the candidate BRP is stable at low temperature; a drop of 0.3 log10 IU/mL in titre (relative to the WHO IS) was observed after storage at +20°C for 6 months. It was difficult to compare stability at higher temperatures with the WHO IS because of problems encountered in reconstituting vials of the IS after prolonged incubation.

Temp. (°C)	Time (months)	Titre ($\log_{10}$ IU/mL)
-20	3	4.55
	6	4.52
+4	3	4.53
	6	4.43
+20	3	4.43
	6	4.19
+37	3	3.91
	6	3.50
+45	3	3.73
	6	2.74

Methods and statistical analyses

All participants were asked to perform 3 assay runs using their routine method for HEV RNA. For each run, a new set of samples had to be used. All dilutions had to be performed using HEV negative plasma.

- For quantitative tests, participants were requested to use the WHO 1st IS for HEV RNA (6329/10) to create a standard curve and samples reported directly in IU/mL.
- For laboratories performing qualitative assays, participants were requested to assay the candidate BRP alongside the 1st IS for HEV RNA (6329/10) by a series of one $\log_{10}$ dilution steps to obtain an initial estimate of an end-point. They were requested to perform 3 subsequent assays, with half-$\log_{10}$ dilutions around the end-point estimated in the first assay.

Standard electronic data reporting sheets and a method reporting form were provided to all participants.

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Qualitative assay results were reported as positive or negative, i.e. HEV RNA detected or negative; C_T, S/Co or RLU values were also requested to be reported if real-time PCR or transcription-mediated assays were used.

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- For assays reporting C_T values, these were evaluated for both qualitative and quantitative methods (relative to WHO IS) using a parallel line model for each laboratory and assay run, as well as combined for all evaluable (i.e. valid) assay runs.
- Parallel line and sigmoid evaluation model as well as the combination of assays were performed according to methods as described in the Ph. Eur. general chapter 5.3. "Statistical analysis of results of biological assays and tests". The statistical analysis was performed with SAS®/STAT software and CombiStats software [3].

Results

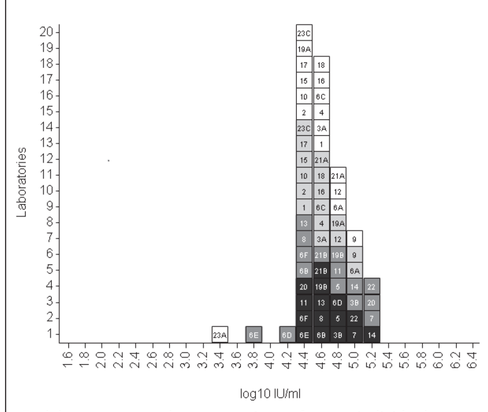
As expected, qualitative data were more variable than quantitative data.
In general, there was a good agreement between qualitative and quantitative assay data

Overall mean estimates (in $\log_{10}$ IU/mL) from quantitative and qualitative assays

CI: confidence interval; SD: standard deviation

	Estimate ($\log_{10}$IU/mL)	95% CI		Median	SD	Min	Max	Range
Quantitative (standard curve)	4.61	4.44	4.78	4.53	0.33	3.47	5.03	1.55
Quantitative (C_T)	4.62	4.51	4.73	4.57	0.21	4.38	5.07	0.69
Qualitative (end-point)	4.68	4.45	4.91	4.71	0.41	3.80	5.27	1.47
Qualitative (C_T and S/Co)	4.70	4.55	4.85	4.65	0.34	4.23	5.70	1.46
Overall	4.61	4.51	4.72	4.61	0.31	3.47	5.27	1.79

Histogram of mean estimates per assay and laboratory from quantitative and qualitative assays (expressed in $\log_{10}$ IU/mL)



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Conclusion

Based on these results, in February 2016, the Ph. Eur. Commission adopted the candidate material as the Hepatitis E virus RNA for NAT testing BRP batch 1, with an assigned unitage of 2.1×10^4 IU/vial (4.32 $\log_{10}$ IU/vial) [1, 4].

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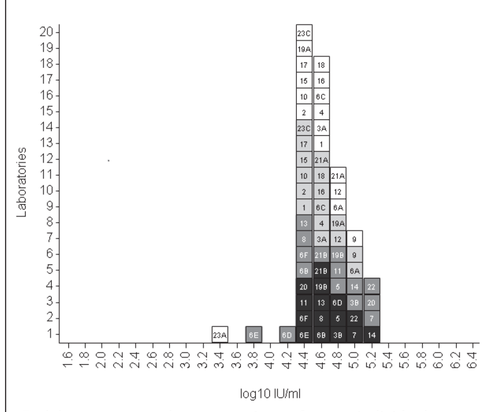

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Pharmacopoeial Reference Standards for Elemental Impurities

Matthias Weber[1,*], Andrea Lodi[1], Jean Charoud-Got[2], Olaf Rienitz[3], Anita Röthke[3], Silke Richter[4]

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2 European Commission, Joint Research Centre (JRC), Geel, Belgium
3 Physikalische-Technische Bundesanstalt (PTB), Braunschweig, Germany
4 Bundesanstalt für Materialforschung und -prüfung (BAM), Berlin, Germany

Abstract

Elemental impurities (EI) in medicinal products for human use are limited according to ICH guideline Q3D, which is in force since December 2017 in the European Union. As a consequence the relevant texts of the European Pharmacopoeia (Ph. Eur.) and the United States Pharmacopeia (USP) have been modified to reflect and complement ICH Q3D, providing details on the analytical methods to be used. In those chapters (Ph. Eur. 2.4.20., USP <233>), it is stated that for the quantification of elemental impurities, certified reference materials (CRM) from a national metrology institute (NMI) or reference materials that are traceable to the CRM of an NMI should be used.

The Ph. Eur. has so far implemented elemental impurity standards of this type for the four most important elemental impurities i.e. those corresponding to ICH Q3D Class 1: lead, cadmium, mercury and arsenic.

Details on the development of those four reference standards are provided, which was undertaken in partnership with an accredited CRM producer (JRC, European Commission), a national metrology institute (PTB, Germany), and a designated institute and accredited CRM producer (BAM, Germany). The reference standards were established and characterised according to rigorous metrological principles and are supplied with extended supporting information as required for the intended use.

After successful completion of the project, the four reference standards have been added to the Ph.Eur. catalogue and are in distribution. It is expected that another three elemental impurity standards will be implemented and made available to users within the next three years.

ICH Q3D Guideline for Elemental Impurities

ICH Q3D [1] is a harmonized guideline to ensure globally consistent control of elemental impurities in pharmaceutical products. The document describes a risk-based approach to ensure control for elements likely to be present in drug products and ingredients for human use by 3 routes of administration and divides them in three classes. In addition, safety-based limits for 24 elemental impurities are defined. The guideline was adopted by the participating regulatory authorities and published in December 2014.

Human toxicants that have limited or no use in the manufacture of pharmaceuticals.

Route-dependent human toxicants divided in sub-classes 2A and 2B based on their relative like-lihood of occurrence in the drug product.

Relatively low oral toxicity but could warrant considerationin the risk assessment for inhalation and parenteral routes.

Table V.1 (5.1): Elements To Be Considered in the Risk Assessment

Element	Class	If intentionally added (all routes)	If not intentionally added		
			Oral	Parenteral	Inhalation
Cd	1	yes	yes	yes	yes
Pb	1	yes	yes	yes	yes
As	1	yes	yes	yes	yes
Hg	1	yes	yes	yes	yes
Co	2A	yes	yes	yes	yes
V	2A	yes	yes	yes	yes
Ni	2A	yes	yes	yes	yes
Tl	2B	yes	no	no	no
Au	2B	yes	no	no	no
Pd	2B	yes	no	no	no
Ir	2B	yes	no	no	no
Os	2B	yes	no	no	no
Rh	2B	yes	no	no	no
Ru	2B	yes	no	no	no
Se	2B	yes	no	no	no
Ag	2B	yes	no	no	no
Pt	2B	yes	no	no	no
Li	3	yes	no	yes	yes
Sb	3	yes	no	yes	yes
Ba	3	yes	no	no	yes
Mo	3	yes	no	no	yes
Cu	3	yes	no	yes	yes
Sn	3	yes	no	no	yes
Cr	3	yes	no	no	yes

Implementation of ICH Q3D in the EU and Ph. Eur.

ICH guideline Q3D on elemental impurities was finally adopted by the EMA Committee for Human Medicinal Products in December 2014 [2]. The date of coming into effect for new marketing authorisation applications was June 2016 and forauthorised medicinal products December 2017.

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Implementation of ICH Q3D in the European Pharmacopoeia transformed the guideline into a legally binding document. The application date was January 2018 (Ph. Eur. 9.3.) and the following Ph. Eur. chapters and general monographs have been revised:

- 5.20. Elemental Impurities
- 2.4.20. Determination of elemental impurities
- 2619 Pharmaceutical Preparations
- 2034 Substances for Pharmaceutical use

Implementation of ICH Q3D by FDA, USP and JP [3,4]

ICH Q3D will be effective for existing products
– New NDA/ANDA effective June 01, 2016
– Existing products effective January 01, 2018

FDA

USP <232> Elemental impurities limits (August 2017)
USP <233> Elemental impurities procedures (January 2018)
(<231> Heavy Metals deleted January 2018)

JP draft for public comments, March to May 2018
New general test 2.66 Elemental impurities - Procedures
Publication of JP17 Supplement II in May 2019

JP Japanese Pharmacopoeia

(The harmonization of the elemental impurities procedure (G-07 Metal Impurities) is ongoing between EP, USP and JP within the PDG (Pharmacopoeial Discussion Group)).

Conclusion and Achievements

A highly successful collaboration has been established between leading European institutes to develop, characterise, produce and establish four elemental impurity reference standards that are traceable to the SI via the CRM of an NMI. These are the first pharmacopoeial reference standards suitable for the quantification of elemental impurities according to Ph. Eur. 2.4.20. or USP <233>. As of January 2018 these four standards are available in the EDQM CRS catalogue. Detailed information on the assigned value and the associated expanded uncertainty is included in the leaflets accompanying the reference standards.

Acknowledgement

The authors are very thankful for the great support and excellent work of the processing group of JRC-Geel, René Meinhardt, Maren Koenig, Dorit Becker, Antje Cossmer, Nicole Langhammer, Andreas Schulz, Volker Görlitz, Carola Pape, Ursula Schulz, Reinhard Jährling and Janine Noordmann.

References

[1] ICH Guideline Q3D (www.ich.org)
[2] EMA/CHMP/ICH/353369/2013
[3] Elemental Impurities in Drug Products Guidance for Industry FDA CDER; https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM509432.pdf
[4] JP draft for public comments, New general tests 2.66 Elemental Impurities – Procedures; https://www.pmda.go.jp/english/rs-sb-std/standards-development/jp/pub-comments/jp/0008.html

Requirements Elemental Impurities Reference Materials

Certified reference materials (CRM) from a national metrology institute (NMI), or reference materials that are traceable to the CRM of an NMI should be used.

Establishment of Pharmacopoeial Reference Standards

Reference materials or standards with a known content of the target element are required for the quantification of elemental impurities. The European Pharmacopoeia envisioned the establishment of suitable reference standards. However, there was a lack of specific know-how and technical equipment at EDQM. As a consequence a feasibility study was initiated to define the project scope and to identify competent and equipped partners:

European Commission
Directorate General Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Reference Materials Unit
Retieseweg 111, 2440 Geel, Belgium

BAM Bundesanstalt für Materialforschung und -prüfung

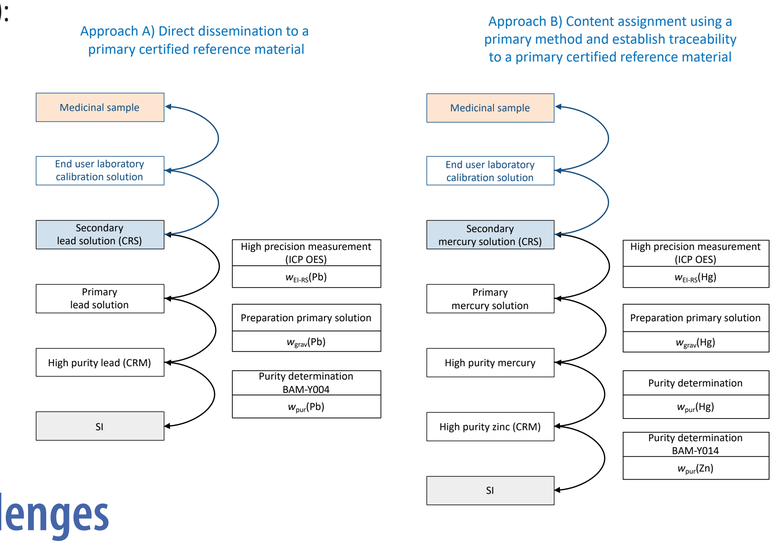
PTB Messen ▪ Forschen ▪ Wissen

First Project Phase for ICH Q3D Class 1 Elements

To mitigate the overall project risk at first the project focussed on the elements classified by ICH Q3D as class 1: lead, cadmium, mercury and arsenic.

Traceability to the SI

A key necessity was the traceability of the element content to the SI (International System of Units) to allow metrologically reliable and reproducible determinations. This included new and specific approaches to be developed by the partners in charge (BAM and PTB):

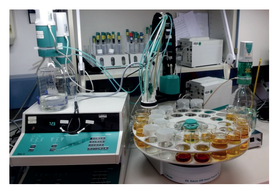


Project Challenges

At the beginning it was necessary to bridge the mind-set gap between metrologists and pharmaceutical analysts. Furthermore, concepts had to be tested and developed to achieve traceability to the SI without primary reference material of the same element. Technical solutions had to be implemented to ensure homogeneity of the concentration in the ampoules throughout the filling process. The possible leaching or absorption of element by contact materials had to be excluded by pre-testing. And finally, the synchronisation of the elemental impurity reference standards with the implementation of ICH Q3D in January 2018 demanded a tight timescale.

Outlook

On the basis of the established excellent cooperation another three elemental impurity standards will be implemented and made available to users within the next three years. Furthermore, long term stability studies are ongoing.



Element	Reference Standard	EDQM Catalogue code	w in mg/g	U(w) in mg/g	U_{rel}(w) in %	k	SI Traceability	High precision measurement	Purity determination
Pb	**Lead solution CRS 1**	Y00001996	0.9996	0.0050	0.50	2	Lead CRM BAM-Y004	ICP OES	(purity of BAM-Y004 well known)
Cd	**Cadmium solution CRS 1**	Y00001997	1.0012	0.0050	0.50	2	Zinc CRM BAM- Y014	EDTA Titration	EDTA Titration
As	**Arsenic solution CRS 1**	Y00002004	1.001	0.015	1.5	2	Copper CRM BAM- Y001	ICP OES	KI/$Na_2S_2O_3$ Titration
Hg	**Mercury solution CRS 1**	Y00002003	0.999	0.015	1.5	2	Zinc CRM BAM-Y014	ICP OES	EDTA Titration

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Importance of orthogonal methods for the characterisation of reference standards, illustrated by the example of α-tocopherol CRS

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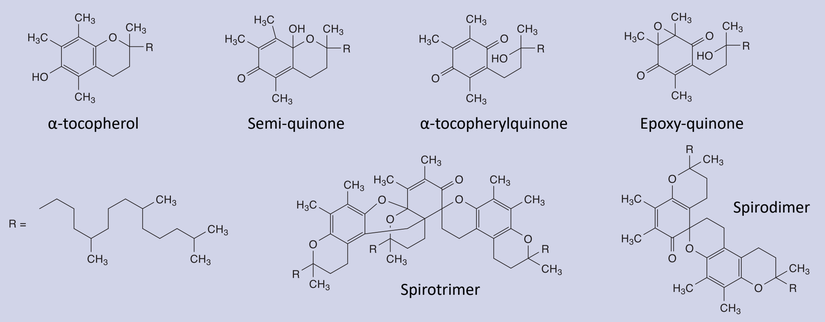
Introduction

The **assigned content** of a Ph. Eur. chemical reference substance (CRS) established for assay purposes is usually calculated from the results of the determination of impurities (organic, inorganic, water and solvents) by applying the principle of mass balance. When possible, the assigned content is verified by comparing with the result obtained by independent methods, in order to ensure the absence of a bias.

α-Tocopherol CRS is used *inter alia* as external standard in the gas chromatography (GC) assay of the Ph. Eur. monographs for *RRR*-α-tocopherol and all-*rac*-α-tocopherol. The establishment of α-tocopherol CRS is particularly challenging since the substance is a liquid and highly sensitive to oxidative degradation. It is of paramount importance that oxidation be avoided during handling and establishment of the CRS candidate material, and that methods appropriate for detection and quantification of potential impurities arising from oxidation be applied.

The **oxidation** mechanism of α-tocopherol is intricate and has not yet been fully elucidated. A major pathway includes the homolytic cleavage of the phenolic hydrogen bond that results in formation of a free radical, that is subsequently oxidised to semi-quinones and finally to α-tocopherylquinone. The latter is known to further oxidise into epoxy-quinones that in turn can dimerise and trimerise [1]. The chemical structure of α-tocopherol and some of its oxidation products is given in Figure 1.

Figure 1: Chemical structure of α-tocopherol and some of its oxidation products.



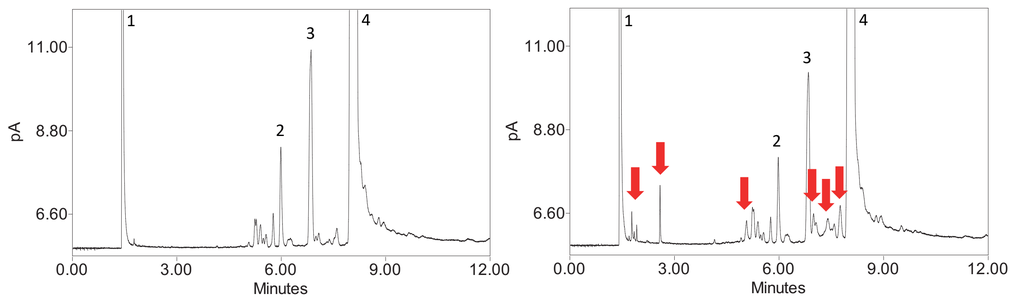
Mass balance / gas chromatography

The major impurities expected to be present in α-tocopherol are of organic nature, i.e. related substances, because usually the contribution of inorganic matter, water and solvents to the mass balance is negligible. In this study, **related substances** were quantified using the corresponding GC test described in the Ph. Eur. monographs for *RRR*-α-tocopherol and all-*rac*-α-tocopherol. Interestingly, the aforementioned oxidation products are not mentioned as specified impurities or other detectable impurities in the monographs.

The chromatograms obtained upon injection of test solutions of α-tocopherol provided by a manufacturer and intentionally oxidised* α-tocopherol are shown in Figure 2. Upon oxidation, the total amount of impurities, estimated by area normalisation, was found to increase by about 1 % and some additional impurity peaks were detected. However, employing squalane as internal standard the area of the peak due to α-tocopherol was found to decrease by about 5 %. The **disparity** between increase of impurities and decrease of α-tocopherol appears to point at oxidation products either having a lower response (unlikely) or not being detected by the GC method. α-Tocopherylquinone** was found to co-elute with α-tocopherol. GC/MS experiments could not elucidate the nature of the additional peaks nor pinpoint the location of other, known oxidation products in the chromatogram.

* Oxidation was performed in-house by exposure to air and heating. ** An authentic sample could be obtained.

Figure 2: GC analysis of α-tocopherol before (left) and after (right) oxidation. 1 = blank (sample solvent), 2 = related substance (impurity A), 3 = related substance (impurity B), 4 = α-tocopherol. Red arrows indicate additional peaks upon oxidation.



Liquid chromatography coupled to UV and MS

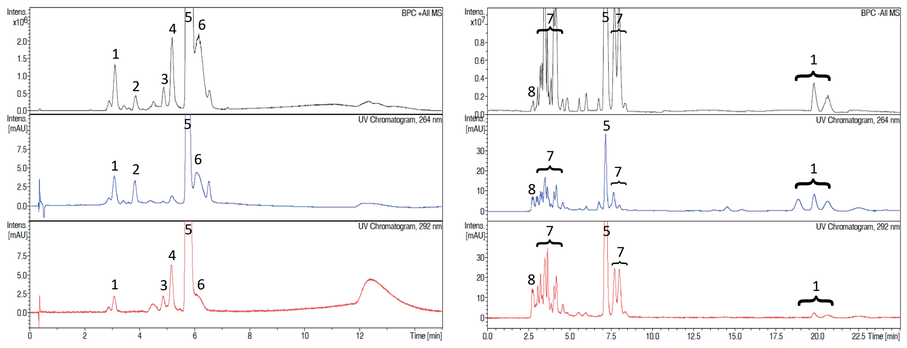
In order to further investigate the oxidation, two LC methods were applied:

- a **reversed-phase gradient LC method** used by a manufacturer to assay α-tocopherol in a finished product, employing an Acquity UPLC BEH C18 column and a mobile phase consisting of water and methanol.
- a **normal-phase isocratic LC method** reported to separate dimers and trimers of α-tocopherol [2], employing a Zorbax Rx Sil column and a mobile phase consisting of hexane and dioxane.

The UV absorbance was monitored using a diode-array detector. Furthermore, the LC instrument was coupled to a time-of-flight mass spectrometer (TOF MS). Typical chromatograms of oxidised α-tocopherol are given in Figure 3. Apart from α-tocopherylquinone, the peaks were only tentatively assigned based on their UV and mass spectrum.

The reversed-phase method could detect and separate several, monomeric oxidation products; dimers and trimers were however not readily detected. The normal-phase method on the other hand was able to detect and separate several dimers and trimers, as well as some monomeric oxidation products. The difference in UV response and absence of authentic samples of most oxidation products render quantification cumbersome.

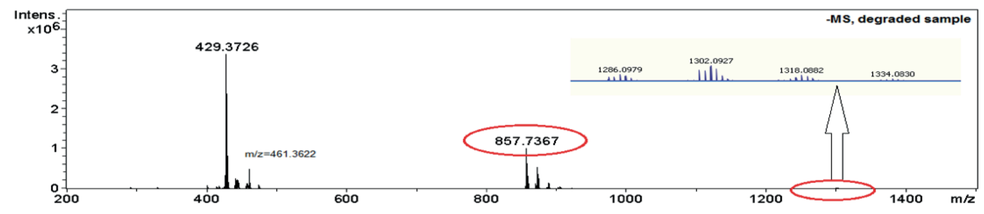
Figure 3: LC analysis of α-tocopherol after oxidation. Left: reversed-phase method; right: normal-phase method. 1 = epoxy-quinones, 2 = α-tocopherylquinone, 3 + 4 = related substances (impurities A and B), 5 = α-tocopherol, 6 = semi quinones, 7 = dimers, 8 = trimers. Peak assignment is tentative except for the underlined substances.



Direct infusion mass spectrometry

Direct infusion TOF-MS experiments were carried out using ESI and APCI in both positive and negative mode, focussing on the detectability of **dimers and trimers**. The best results were obtained using APCI in negative mode. Upon infusion of a solution of oxidised α-tocopherol, the m/z of dimers and trimers were detected, whereas these m/z were absent prior to oxidation. A typical mass spectrum of oxidised α-tocopherol is given in Figure 4.

Figure 4: Mass spectrum of α-tocopherol after oxidation.
The signals due to dimers and trimers are highlighted (red circles).



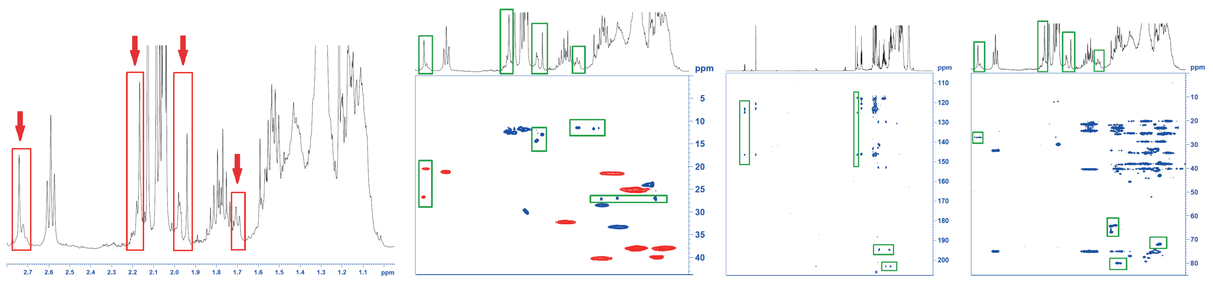
Nuclear magnetic resonance spectrometry

Nuclear magnetic resonance spectrometry (NMR) experiments were performed on a 400 MHz instrument using deuterated acetone as solvent.

Qualitative NMR

NMR spectra obtained for oxidised α-tocopherol are provided in Figure 5. Upon oxidation, additional characteristic signals were observed in the 1D proton spectrum, most prominently in the chemical shift region between 1.5 and 3.0 ppm, and in the 2D proton-carbon spectra. The nature of the signals remains however to be elucidated; it could be excluded that they are due to α-tocopherylquinone.

Figure 5: Excerpts of NMR spectra of α-tocopherol after oxidation.
From left to right: 1D proton spectrum; 2D proton carbon spectrum 1 (HSQC); 2D proton carbon spectrum 2 (HMBC); 2D proton carbon spectrum 3 (HMBC). Additional signals that appear upon oxidation are highlighted (red arrows and green boxes).



Quantitative NMR

Quantitative NMR (qNMR) was performed using an internal standard (1,2,4,5-tetrachloro-3-nitrobenzene). Only the proton signal at about 2.6 ppm, corresponding to the -CH2- group of the dihydrobenzopyrane moiety closest to the unsaturated ring, was found to be sufficiently separated to allow for quantification. However, due to slight overlap with other, minor signals the qNMR results were only considered to be an estimation. Since oxidation takes place close to the chosen protons, the signal is expected to be selective for α-tocopherol vis-à-vis of its oxidation products. Comparing the content estimated by qNMR to the one estimated by applying mass balance taking account of the impurities detected by GC, the former content was found to be lower (by about 5 to 6 %) for intentionally oxidised α-tocopherol, whereas it was within about 0.5 % prior to oxidation.

Conclusion

Based on the results obtained, it appears that the content of organic impurities in α-tocopherol CRS candidate materials may be underestimated applying the GC test for related substances of the corresponding Ph. Eur. monograph, if impurities arising from oxidative degradation are present. Several independent methods including LC coupled to UV and MS, direct infusion MS and NMR were each found to provide complementary, qualitative information regarding the presence of oxidation products. In addition, quantitative NMR seems to be a valuable tool for selective determination of the content of α-tocopherol. Correlation of the information obtained from each method and quantification of oxidation products is complicated by the complex mechanism of α-tocopherol oxidation, the plethora of potential oxidation products present, for most of which authentic samples are not available, and differences in UV response. Upon characterisation of α-tocopherol CRS candidate materials and monitoring of the fitness for purpose of the CRS, application of multiple, orthogonal methods in addition to the aforementioned GC test for related substances seems to be key in order to detect a potential bias on the assigned content related to potential oxidation by-products.

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[2] Doudin K. and Al-Malaika S. Vitamin E-stabilised UHMWPE for orthopaedic implants: quantitative determination of vitamin E and characterisation of its transformation products, Polymer degradation and stability 125: 59-75 (2016)

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Combining qNMR with LC-UV for better accuracy

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Introduction

qNMR: solution-state 1HqNMR with a certified reference material (CRM) as an internal standard is a primary ratio method with universal molar response. An uncertainty level as low as 0.1 % can be obtained when certain criteria are respected (e.g. pulse delay time, baseline separation and correction, integration region, signal-to-noise ratio, etc. [1]). The peak area of the analyte selected for quantification may include the contribution of related structure impurities (RSIs) which is a drawback in qNMR. Signals due to water, residual solvents and inorganics (not detectable) are in general separated from the analyte signal, resulting in a *% m/m* "as is" content which requires a correction for included RSIs ,if any.

LC-UV: one of the advantages of LC-UV is its high selectivity; many Ph. Eur. monographs describe methods with full separation between the analyte and its RSIs. One of the options to quantify such impurities is by using a dilution of a test solution (e.g. 0.1 % V/V or 2.0 % *V/V*) as an external standard. The result of % peak area RSIs is used as a chromatographic purity (CP) in calculating the mass balance (MB) result in *% m/m* "as is" content of the analyte in the sample:
MB = [100 − % *m/m water* − *% m/m* residual solvents − *% m/m* inorganics] × (100 − *area* % RSIs)/100 (1)

It is proposed to use LC-UV results on RSIs to correct qNMR results in order to improve accuracy.

Experimental conditions

NMR:
Instrument: Ascend 400 MHz Avance III HD Nanobay equipped with a 5 mm BBI probe (Bruker)
Software: Topspin 3.4 (Bruker)
Internal standards: TraceCERT® CRMs for quantitative NMR (Merck)

LC-UV:
Instrument: Alliance e2695 Separation Module with a 2487 Dual λ Absorbance Detector set at 210 nm (Waters)
Software: Empower 3 (Waters)
Stationary phase: Inertsil ODS-2 column of 250 x 4.6 mm, particle size 5 µm
Mobile phase, flow rate, injection volume and temperature settings: see Ph. Eur. monograph No 0582 [2]

The concept

NMR analyses of a folic acid (FA) sample had revealed the unexpected presence of about 0.2 % m/m of a small impurity which could be characterised as pterin-6-aldehyde [3]. Figure 1a shows the chemical shifts of the two singlets caused by this impurity in the aromatic region of the NMR spectrum of FA which was zoomed in on the baseline.

What if the singlet of proton P7 was overlapped by the FA signal as simulated in figure 1c? Then a qNMR result for FA No 7 would be overestimated and, subsequently, could be corrected with the aid of the amount of pterin-6-aldehyde as determined by LC-UV. Bearing in mind that qNMR displays universal molar response, one should subtract 0.2 × 441/191 = 0.5 % m/m (% *m/m* content impurity × $M_{FA}/M_{impurity}$) to obtain an accurate result. Small molecules give rise to higher integrals than large molecules at similar concentrations.

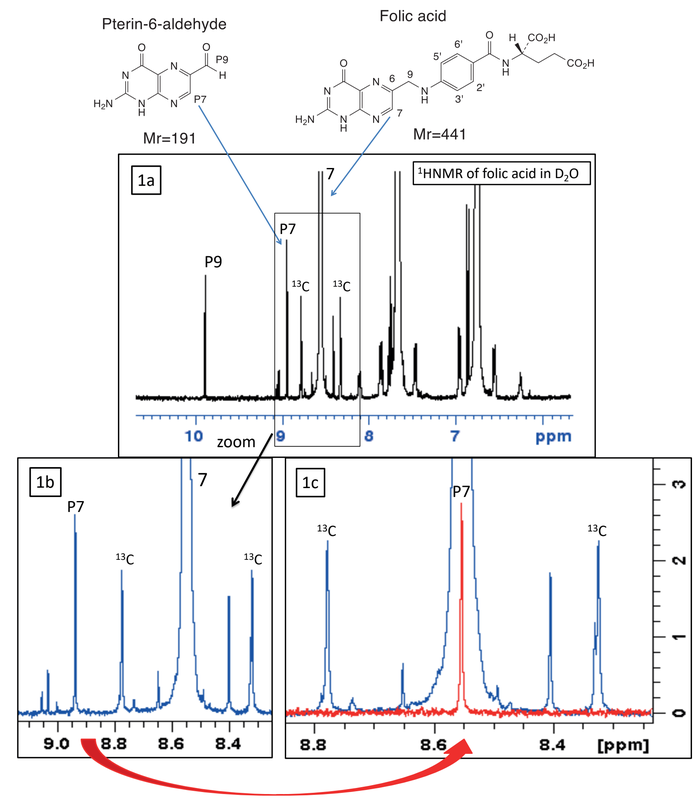

Figure 1: NMR spectra of the simulated overlap of impurity proton P7 by proton No 7 of folic acid.

Conclusion

qNMR results of analytes that are biased can be corrected by subtracting the contents of overlapped RSIs as determined by LC-UV. The information given in Ph. Eur. monographs on such analytes proved to be very helpful.
LC-UV and qNMR are complementary techniques; the combination brings about perfection.

References

[1] http://www.validnmr.com/w/index.php?title=1H_High-Precision_Quantification

[2] Council of Europe (2016) Clindamycin hydrochloride. In: European Pharmacopoeia, 9th edition. Strasbourg, France, pp 2110-2111. https://www.edqm.eu/en/european-pharmacopoeia-ph-eur-9th-edition

[3] Andrea Lodi et al, Characterisation of folic acid by mass balance versus quantitative NMR, Accreditation and Quality Assurance, Volume 23, pp 211–218 (2018)

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Calculations in qNMR

Content of X in % *m/m* = $I_X/I_{IS} \times N_{IS}/N_X \times M_X/M_{IS} \times a_{IS}/a_X \times P_{IS}$, (2)
where X stands for the analyte, I is the integral value, N is the number of nuclei, M is the molar mass, a is the amount weighed and *P* is the purity of the internal standard (*IS*).
Correction for RSIs found by LC-UV and overlapped in qNMR:
corrected content of X = qNMR content of X − (*% m/m RSI* × M_X/M_{RSI}) (3)

An example: clindamycin HCl

The Ph. Eur. monograph on Clindamycin HCl [2] describes an LC-UV method for the evaluation of its RSIs by the 2.0 % V/V dilution of a test solution. Three structures of potential RSIs are given in this monograph: impurities A, B and C as depicted in figure 2. The anomeric proton of clindamycin (see ↙ in figure 2) was selected for quantification by NMR. The anomers of the impurities were expected to be (partly) overlapped by the main signal. Therefore the qNMR result had to be corrected by subtracting the amounts of impurities A, B and C as determined by LC-UV, which resulted in a slightly negative bias of 0.3 % with respect to the mass balance. Figure 2 shows the satisfactory separation of clindamycin and its RSIs by LC-UV and, in contrast, their overlap in the integration region of the NMR spectrum.

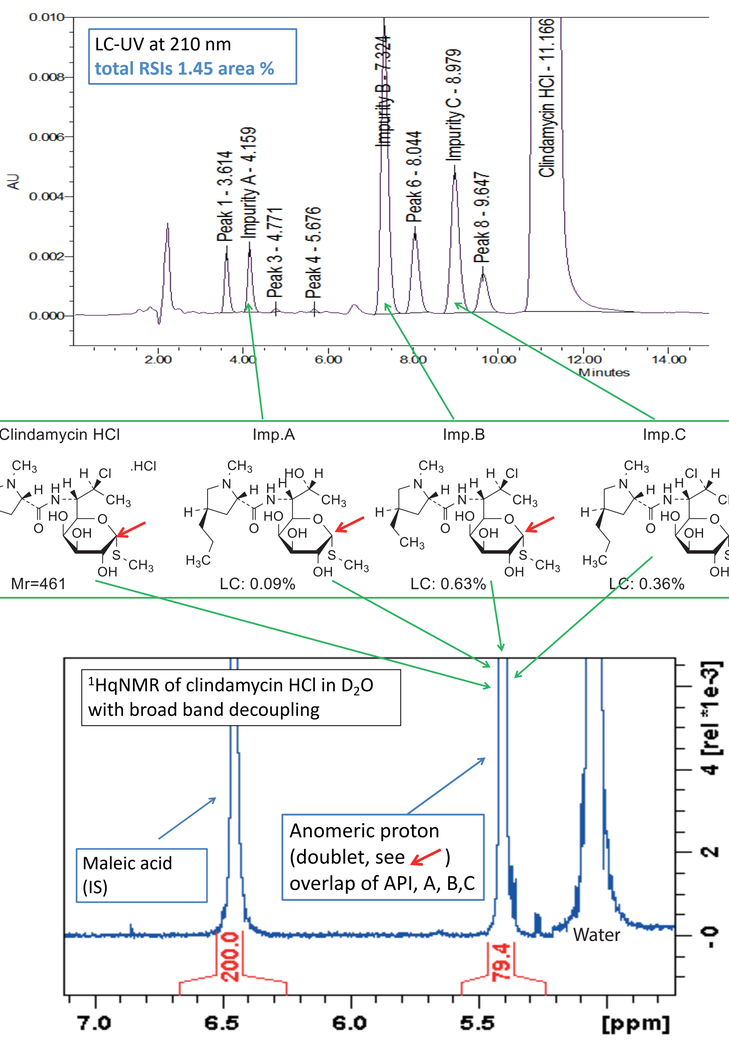

Figure 2: LC-UV chromatogram (top), structures of clindamycin and RSIs (middle) and qNMR spectrum (bottom).

Content of clindamycin HCl in % *m/m*:
MB (1) found: [100 − % *m/m* water − % m/m residual solvents − *% m/m* inorganics] × (100 − *area* % RSIs)/100 = [100 – 3.89 – 0 – 0.03] x (100 – 1.45)/100 = 94.7 %
qNMR (2) found: 95.4 %; correction for overlapped RSIs, A + B + C (3): 95.4 – 1.0 = 94.4 % (ratio $M_X/M_{RSI} \approx 1$)

Acknowledgement

The authors are grateful to all staff of the EDQM Laboratory, in particular to Ms Stephanie Moneret and Dr Matthias Weber of the NMR team for the scientific advice provided.

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Survey of Peptide Quantification Methods and Comparison of their Reproducibility: A Case Study Using Oxytocin

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[1]USP, [2]NRC, [3] Swedish Medical Products Agency, [4]NIST, [5]TGA, [6]PMRJ, [7]NIBSC, [8]Centre for Vaccine Evaluation, Health Canada, [9]BCN Peptides, [10]Aspen Oss BV
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Abstract

The assigned value of USP's peptide reference standards (RS) is typically determined using a mass balance approach in which the peptide of interest is quantified by HPLC assay against an external standard.

To explore the suitability of alternative analytical methods to assign values to peptide RS, the UPS Biologics Department conducted a collaborative study. It aimed to evaluate the accuracy and precision of quantitative nuclear magnetic resonance (qNMR) spectroscopy, and amino acid analysis (AAA), as compared to HPLC assay for the quantification of the nonapeptide oxytocin.

The three methods provided comparable quantification results for the high purity RS. It must be noted that if peptide-related impurities had been present in significant percentage, the assigned value would have required a correction, since, unlike HPLC, qNMR and AAA cannot discriminate between the target peptide and peptide-related impurities. The study also indicated that the traditional HPLC method showed the lowest inter-lab variability. Higher variability of qNMR seemed to be related to the type of internal standard used by the participating lab. The selection of a suitable internal standard could help improve precision. In the case of AAA, variability seemed to be linked to the type of quantitation method and type of pre- and post-derivatization.

Results suggest that qNMR should be further explored as potential candidate for primary method for peptide RS value assignment. It measures peptides directly against an internal standard, it is a simpler method to operate under common laboratory conditions and it has shorter analytical time.

Methodology

PARTICIPATING LABORATORIES

- Aspen Oss BV, Netherlands
- Australian Therapeutic Goods Administration (TGA), Australia
- BCN Peptides S.A, Spain
- Health Canada, Centre for Vaccine Evaluation, Canada
- Ipsen Manufacturing Ireland Ltd, Dublin, Ireland
- Pharmaceutical and Medical Device Regulatory Science Society of Japan (PMRJ), Japanese Pharmacopoeia Reference Standards Laboratory (JPRS Lab), Japan
- Swedish Medical Products Agency (MPA), Sweden
- National Institute for Biological Standards and Control (NIBSC), United Kingdom
- National Institute of Standards and Technology (NIST), United States
- National Research Council of Canada (NRC), Canada
- University of Nebraska, United States
- US Pharmacopeial Convention (USP), Reference Standard Laboratory, United States
- US Pharmacopeial Convention (USP), Reference Standard Laboratory, India
- US Pharmacopeial Convention (USP), Global Biologics Laboratory, United States

Testing material: Lyophilized USP Oxytocin Reference Standard candidate

1D ^{1}H NMR Method

- Oxytocin 3.6 mmol/L
- Internal standards: 2, 3, 4 mmol/L caffeine, 2 mmol/L maleic acid or 0.05 wt % TSP-d4
- Acquisition: 30° pulse, 30-sec relaxation delay, 64 scans (Lab 3: 90° pulse, 50-sec relaxation delay, 32 scans)
- NMR spectrometers' magnetic fields: 300-700 MHz

AAA Method

- Oxytocin and samples in 6-12 mol/L HCl
- 110-115°C for 12-24h
- Quantification by LC-MS/MS with (isotope) labeled and un-labeled Asp, Glu, Gly, Ile, Leu, Phe, and Pro standards or by LC/derivatization with commercially available amino acids standard used for quantification (nmol/mL)

HPLC Method

- Sample and Standards: 0.02 mg/mL
- Column: C_{18} (25-cm×4.6-mm 5-µm), T: 25° or 40°C
- Gradient mobile phase (1.5 mL/min):
  (A) Sodium dihydrogen phosphate 0.1 mol/L
  (B) Acetonitrile and water (50:50, v/v)
- UV detection at 220nm
- Injection vol.: 100 µL

Results

This study was aimed to evaluate the inter-laboratory precision (reproducibility) of HPLC, qNMR and AAA methods for peptide quantitation. Also, to evaluate the suitability of these three methods for the quantitation of lyophilized peptide RS, oxytocin in this study.

AAA and qNMR appeared to have higher intra-lab and inter-lab variability, compared to HPLC (Table 1, Figure 1). All seven labs performing HPLC used the same method (USP compendial method). Five out of six labs performing qNMR tests used similar analytical conditions and the same internal standard (caffeine).

Although it has been reported that caffeine may be solvent and/or concentration dependent, the results obtained in this study indicate that it is a useful internal standard for quantification of oxytocin in D_2O solution. For this purpose, caffeine was compared to maleic acid. Both resulted in comparable oxytocin quantitation, but maleic acid provided better within-lab precision. Moreover, malic acid displays one well resolved singlet peak in an often signal free part of the spectrum (Figure 2)

AAA results indicate that accuracy and precision are highly dependent on the quantitation method. The LC-MS method using ^{13}C isotope-labeled amino acids as an internal standard is far superior to the derivatization method, but it is hard to be adopted by pharmaceutical industry labs. Pre- or Routine post-column derivatization methods have variability, reducing its suitability for peptide quantitation.

AAA is widely used for peptide and protein quantitation. However, a conclusion about using AAA for peptide quantitation can not be derived from this study due to the low number of participating labs (4) performing the test.

Table 1. Statistical analysis (mg of oxytocin per vial)

Analytical method	Mean (mg oxytocin)	95% CI (mg oxytocin)	Source	Variance	SD	%RSD
qNMR (n= 6 labs)						
	1.798	1.707 - 1.888	Reproducibility (lab to lab)	0.0072	0.0851	4.73%
			Repeatability (sample to sample)	0.0011	0.0332	1.85%
AAA (n= 4 labs)						
	1.733	1.580 - 1.884	Reproducibility (lab to lab)	0.0089	0.0942	5.44%
			Repeatability (sample to sample)	0.0009	0.0308	1.78%
HPLC (n= 7 labs)						
	1.807	1.769 - 1.845	Reproducibility (lab to lab)	0.00168	0.0410	2.3%
			Repeatability (sample to sample)	0.00015	0.0123	0.7%

Figure 1. Plot of individual data for the peptide reference materials.

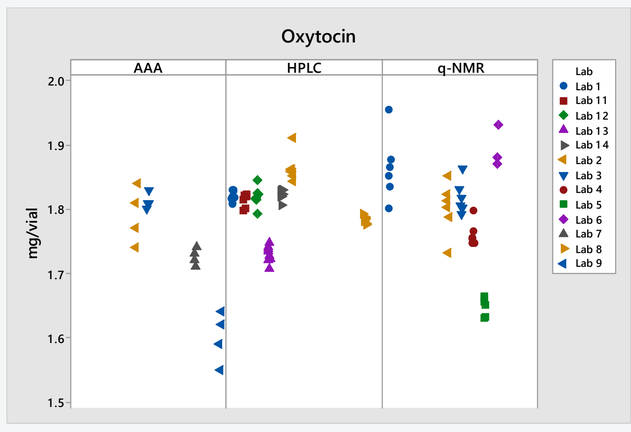
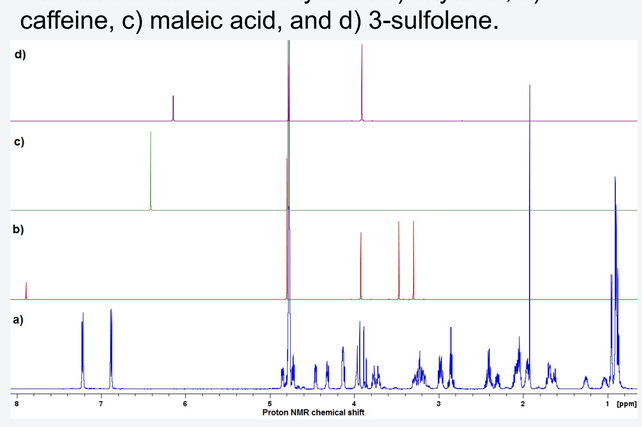


Figure 2. Comparison of 300 MHz ^{1}H NMR Spectra of internal standards and oxytocin. a) oxytocin, b) caffeine, c) maleic acid, and d) 3-sulfolene.



Information

The complete information about this study is available in the *Journal of Pharmaceutical and Biomedical Analysis* 166 (2019), 105–112. Please scan the following QR code to download a copy or visit **http://bit.ly/2SUGnxH**

Conclusions

- This study demonstrates the use and suitability of three distinct analytical methods for peptide quantification: HPLC, qNMR, and AAA. The HPLC assay (using the same peptide bulk material as the standard) resulted in the lowest inter-lab variability.
- The advantages of qNMR: (1) measuring peptides directly, (2) it is easy to perform under common laboratory conditions. Its accuracy and precision can be enhanced by selecting appropriate signals from the peptide or selecting an internal standard that has non-exchangeable and non-overlapping signals in D_2O. The scope of qNMR analysis can be also applied easily to peptide reference standards packaged by lyophilization.
- Unlike HPLC, especially LC-MS, qNMR is generally not suitable for quantification of product-related impurities. However it can detect and quantitate non-product-related impurities (e.g. solvents). In this study, the values assigned by NMR and AAA were not corrected for peptide related impurities, which in theory could contribute a bias comparing with the results by HPLC.
- The current study provides a promise for compendial adoption of the qNMR methodology, although it needs the development of validation strategy and further exploration as a primary method for peptide assay.

Microcrystalline Wax: Identification of a Falsified Bulk Material by GC-EI-MS and GC-CI-MS

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Abstract

Microcrystalline wax (MCW) is a refined mixture of solid saturated aliphatic hydrocarbons obtained by solvent fractionation of the still bottom fraction of petroleum by suitable dewaxing or deoiling. It is predominately comprised of branched-chain hydrocarbons with minor amounts of straight-chain and cyclic hydrocarbons. It is used as coating and stiffening agent in cosmetic and pharmaceutical formulations, and after further refining as a masticatory substance in chewing gum base, protective coating, and defoaming agent in foods defined in the Food Chemicals Codex as “Petroleum Wax”. In an effort to modernize the USP–NF monograph for MCW as an excipient USP has been investigating the use of IR as an identification test for this excipient used in conjunction with a USP RS. To evaluate USP RS bulk candidate materials USP developed two GC-MS methods, using electron and chemical ionization. These methods were used in addition to the current MCW USP–NF monograph tests to characterize a candidate bulk material for USP RS development. The bulk material passed the current monograph tests, while the GC-MS methods identified the bulk material as being consistent with paraffin wax instead of MCW because of higher than expected amount of straight-chain alkanes compared to branched alkanes in this material. Results of this study have improved our understanding of the chemical composition of MCW, provided new GC-MS tools for characterizing bulk materials, and pointed to the need to strengthen the USP–NF monograph to ensure it can distinguish MCW from other wax materials in the marketplace.

Methods

1 EI — 1 mg/mL of MCW bulk material in tetrahydrofuran

2 CI — 5 mg/mL of MCW bulk material in tetrahydrofuran

GAS CHROMATOGRAPHY PARAMETERS

**GC conditions adapted from a GC-FID method developed by USP-China R&D laboratory*

- Column: G27, VF-5ht UltiMetal with retention gap, 30m x 0.32mm x 0.10µm
- Oven temperature program: from 80°C to 420°C (CI: from 80°C to 350°C)
- Injector temperature: 400°C
- Injection volume and mode: 2 µL splitless (CI: 15 µL split 2:1)
- Carrier gas: Helium

GC-EI-MS
(Gas Chromatography- Mass Spectrometry-Electron Ionization)

- Transfer line temperature: 370°C
- Ion source temperature: 230°C
- Quadrupole temperature: 150°C
- Electron energy: 70 eV
- Range (m/z): 15 - 715

GC-CI-MS
(Gas Chromatography-Mass Spectrometry-Chemical Ionization)

- Transfer line temperature: 350°C
- Ion source temperature: 250°C
- Quadrupole temperature: 150°C
- Electron energy: 92 eV
- Range (m/z): 42 - 850
- Reagent gas: Methane

Results

Three series of alkanes were observed in the microcrystalline wax bulk material:

- ♦ Straight chain alkane
- ♦ Branched chain alkane
- ♦ Branched/cyclo alkane

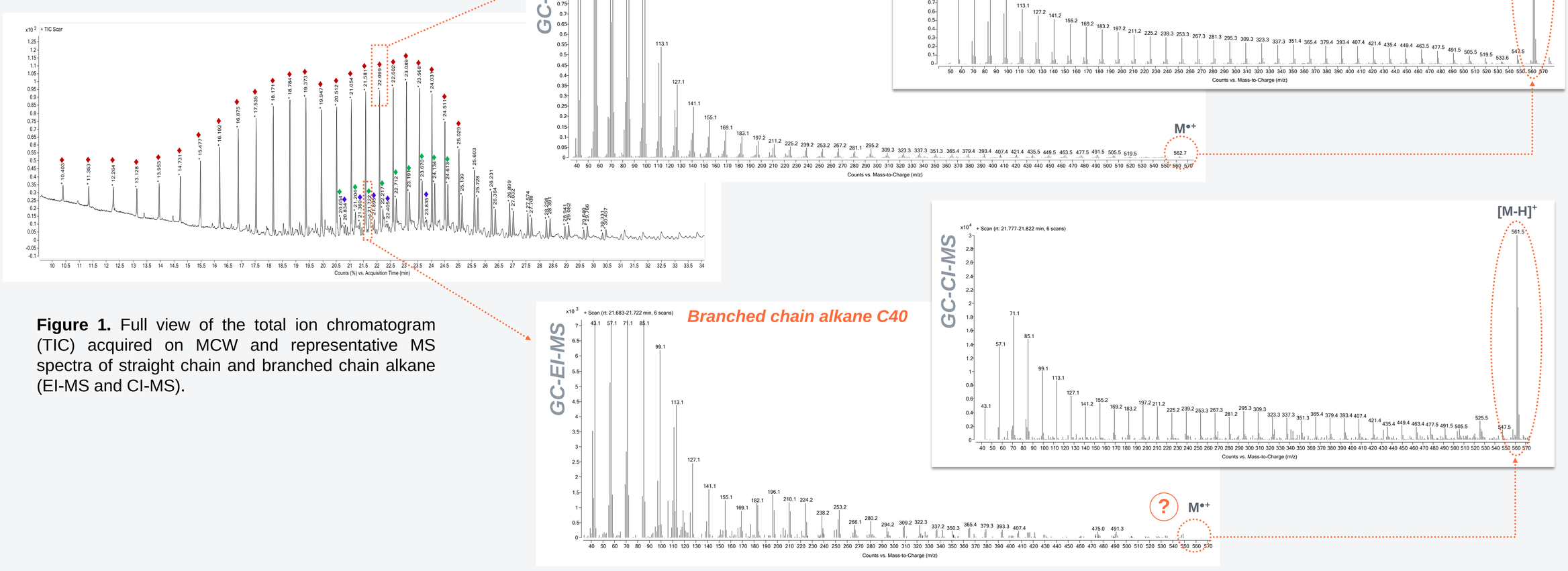

Figure 1. Full view of the total ion chromatogram (TIC) acquired on MCW and representative MS spectra of straight chain and branched chain alkane (EI-MS and CI-MS).

GC-EI-MS KEY POINTS

Branching chains cause a decrease in $M^{+\bullet}$

- Typical series of C_nH_{2n+1+} fragment ion for straight chain alkanes and to a lesser extent the series of $C_nH_{2n-1}^{+}$

GC-CI-MS KEY POINTS

- [M-H]+ showed up in high abundance in the MS spectrum contributing to assign the molecular weight of alkanes.

STANDARD CO-INJECTION

- Retention time and MS fragmentation profile of straight chain alkane in MCW bulk material match to its respective n-alkane straight-chain standard (C7-C40) co-injected.

PROPOSED DEFINITION

- MCW is composed from a complex mixture of: $C_{30\leq n\leq 120}H_{2n+2}$ with an average carbon chain length of C_{50}
- Predominantly branched-chain hydrocarbons.
- Molecular weight: from about 400 to 1700 with an average of 700.

Conclusions & Next Steps

All these observations and strategies supported the assignment of the alkane content in the bulk material under investigation, and confirmed the presence of an unexpected high amount of straight-chain alkanes instead of branched alkanes. The bulk material candidate was characterized as paraffin wax instead of MCW. The strategy of using CI, a “soft ionization” technique contributed to the confirmation of the molecular weight for the identified alkanes and provided evidence for the assignment of their classification as linear versus branched. In coordination with the data acquired with GC-EI-MS, the Cl results showed that there were only a minimal amount of branched alkanes. This work contributed to the selection of an appropriate bulk material and in providing an authentic public reference standard.

- The bulk material passed the specified testing in the microcrystalline wax monograph, however this testing showed that the monograph specifications for Paraffin and MCW need to be strengtened.
- The GC-MS results were reported in a stakeholder meeting between USP and a trade organization. The member companies of this organization demonstrated great interest in the results and further collaboration with USP to understand better the composition of Microcrystalline Wax. Four companies are currently working with USP on a Round Robin study for composition determination of Microcrystalline Wax excipient products.

ACKNOWLEDGMENTS

We specially thank Kelly Sakiara, Wanderson Guimarães, Bruno Horta and Bruno Rondon for supporting this research at USP-Brazil site.

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2. Barker, A. The chromatographic analysis of refined and synthetic waxes. Chapter 3: *Chromatography in the petroleum industry*, v.56, 1995.

To Determine Handling Conditions through Hygroscopicity Study: USP Clarithromycin RS

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Abstract

Hygroscopicity is a physicochemical property describes the ability of a material to take up moisture from surrounding atmosphere.

USP Clarithromycin Reference Standard (RS) lot R07810 bulk was packaged in a glove box at NMT 10% relative humidity (RH) as Dynamic Vapor Sorption (DVS) results showed the material is hygroscopic. The water content in the packaged vials was found to about 0.6% lower than that of the bulk material, which was used during the collaborative testing. The water content appears to be dependent on the ambient lab %RH at which the test was conducted. This change in water content would affect the assigned value as the RS is used on the as is basis. Hence, to evaluate appropriate handling conditions for the use of USP Clarithromycin RS, static vapor sorption experiments at specific %RH (10%, 20%, 30%, 40%, 50% and 60%) levels were performed. The weight changes were monitored at each %RH level till the sample reaches to equilibrium (approximately for 1 hour). Finally, water content was performed by Karl Fischer (KF) at each level for the equilibrated samples.

Static vapor sorption profiles of % weight change versus time at 10%, 20%, 30%, 40%, 50% and 60% RH levels showed sample was equilibrated at each level in <10 min. Hence, 15 min vial equilibration time was selected to handle this RS.

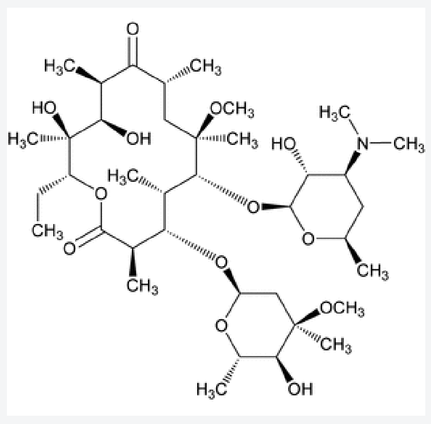
The water content values obtained by KF were plotted against the %RH. At each %RH level the water %w/w values were determined from the linear regression equation. This is to determine the appropriate handling humidity range needed and to ensure the assigned value is accurate. Based on the results, 20% to 40% RH was determined to be the preferred handling range. The water content of 0.90% w/w obtained at 30% RH level was used in the assigned value calculation. The allowed water content range was set at NMT ±0.2% based on the use of USP Clarithromycin RS as an assay standard. This requires that the water content should be within the range of 0.7% to 1.1%, which will be within ±0.2% from the 0.90% w/w water value used in the assigned value calculation. The %w/w water content results determined from linear regression at 20%, 30% and 40% RH were found to be within the acceptable range of 0.7% to 1.1%.

Based on the overall study, appropriate handling conditions “After opening, allow to equilibrate to room temperature for at least 15 minutes within a range of 20% to 40% RH. Do not desiccate, handle between 20% and 40% RH. Discard unused portion after opening” were recommended to the customers to ensure that the assigned label value is appropriate when the USP clarithromycin RS is used on as is basis.”

Introduction

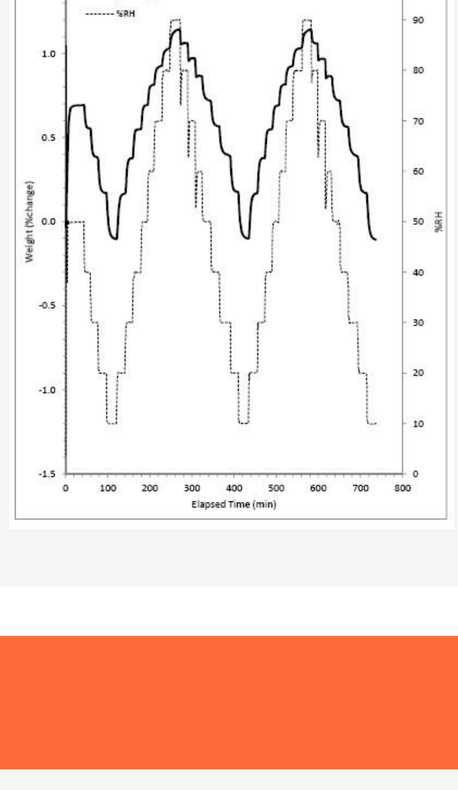
Hygroscopicity is a physicochemical property describes the ability of a material to take up moisture from surrounding atmosphere. These studies usually performed to determine handling requirements and the need for drug substance potency calculations in quantitative methods.

Fig 1. Clarithromycin



Clarithromycin is a macrolide antibiotic, Fig 1. USP Clarithromycin RS is used as an assay standard, lot R07810 bulk was packaged under glove box, NMT 10% RH based on the DVS results, Fig. 2. The water content performed for the packaged vials was found to be less than that of the bulk material, which was used for the collaborative testing. The water content was found to be dependent on the lab %RH which would affect the assigned value. Therefore, hygroscopicity at specific %RH (10%, 20%, 30%, 40%, 50% and 60% RH) levels and weight changes of sample from packaged vials were monitored at each %RH level till the sample reaches equilibrium (approximately for 1 hour). Finally, water content was performed by KF at each level for the equilibrated samples.

Fig 2. Dynamic Vapor Sorption Isotherm



Methodology

A glove box was equipped with a coulometric Karl Fischer (KF) titrator and a microbalance, a dry-air inlet, and a humidifier.

The glove box was equilibrated to a target humidity level (10%, 20%, 30%, 40%, 50% or 60% RH) by introducing a weak, constant flow of dry air into the main chamber and using a humidistat (±3% RH accuracy) to power the humidifier on/off when humidity readings were below or above a specified range.

A sealed vial containing clarithromycin that was present in the equilibrated humidity chamber was opened and the contents were immediately transferred into a tared cup on the microbalance. Weight and %RH values were recorded immediately and at subsequent regular time intervals. Samples were then analyzed by KF after equilibration occurred (after approximately one hour of weight monitoring).

Conclusions

Static vapor sorption profiles of % weight change versus time at 10%, 20%, 30%, 40%, 50% and 60% RH levels showed the sample gained weight for <10 minutes at each %RH level and after that the weight change was negligible, Fig. 3.

Based on this study 15 min vial equilibration time was selected to handle this RS.

The water content values obtained by KF were plotted against the %RH, Table 1 & Fig. 4. The plot showed water content results were dependent on the %RH.

Based on the results, 20% to 40% RH was determined to be the preferred handling range.

The water content values obtained by KF at 20 to 40% RH were plotted against the %RH, Fig. 5. The water %w/w values were determined from the linear regression equation, Table 2.

The assigned value was calculated with the water content of 0.90% w/w determined at 30% RH level from linear regression equation. The allowed water content range was set at NMT ±0.2% based on the use of USP Clarithromycin RS as an assay standard. This requires that the water content should be within the range of 0.7% to 1.1%, which will be within ±0.2% from the 0.90% w/w water value used in the assigned value calculation.

The %w/w water content results determined from linear regression at 20%, 30% and 40% RH were found to be within the acceptable range of 0.7% to 1.1%, Table 3.

Based on the overall study, appropriate handling conditions “After opening, allow to equilibrate to room temperature for at least 15 minutes within a range of 20% to 40% RH. Do not desiccate, handle between 20% and 40% RH. Discard unused portion after opening” were recommended to the customers to ensure that the assigned label value is appropriate when the USP clarithromycin RS is used on as is basis.”

Results

Fig 3. Weight change vs Time at various static RH levels (10%, 20%, 30%, 40%, 50%, and 60% RH)

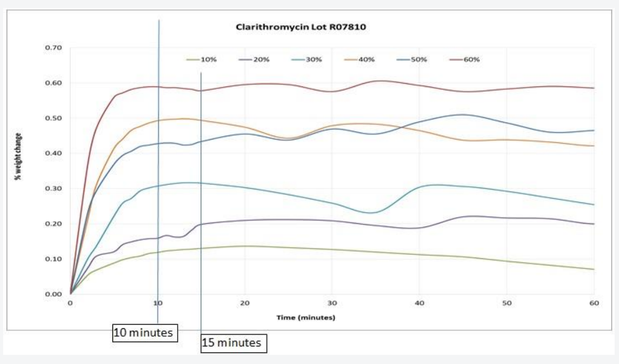


Fig 4. KF data vs Static RH level (10%, 20%, 30%, 40%, 50%, and 60% RH) from all determinations.

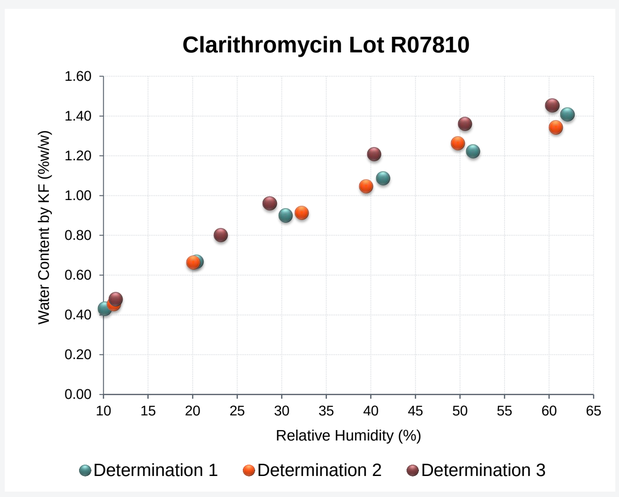
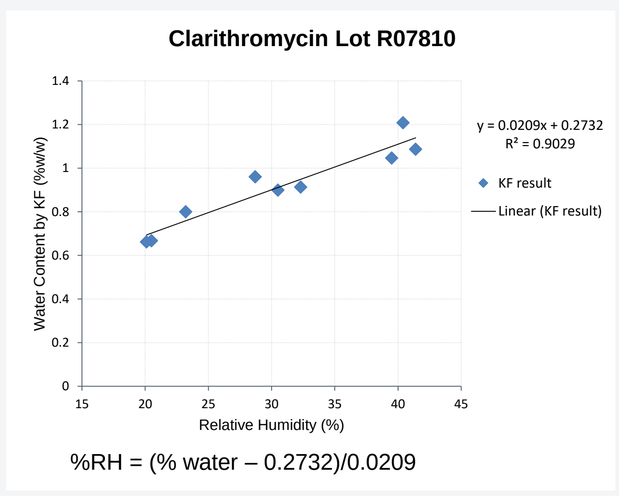


Fig 5. %RH vs KF results



%RH = (% water – 0.2732)/0.0209

Table 1. KF Results for Equilibrated Samples from 10% to 60% RH

%RH	Water content from KF (% w/w)
10.2	0.43
11.2	0.45
11.4	0.48
20.5	0.67
20.1	0.66
23.2	0.80
30.5	0.90
32.3	0.91
28.7	0.96
41.4	1.09
39.5	1.05
40.4	1.21
51.5	1.22
49.8	1.26
50.6	1.36
62.1	1.41
60.8	1.34
60.4	1.45

Table 2. KF Results for Equilibrated Samples from 10% to 60% RH

%RH	Water content from KF (% w/w)	Water values determined from linear regression (%w/w)
10.2	0.43	0.70
11.2	0.45	0.69
11.4	0.48	0.76
20.5	0.67	0.91
20.1	0.66	0.95
23.2	0.80	0.87
30.5	0.90	1.14
32.3	0.91	1.10
28.7	0.96	1.12
41.4	1.09	1.14
39.5	1.05	0.70
40.4	1.21	0.69

Table 3. Water content determined from linear regression equation

%RH	Water %w/w determined from linear regression	Calculated assigned value (µg/mg) from water %w/w (includes Organic Impurities, Residual Solvents, ROI) (Label assigned value is 984 µg/mg)
20	0.69	986
30	0.90	984
40	1.11	982

Revisit to the molecular structure of USP Kanamycin Sulfate RS

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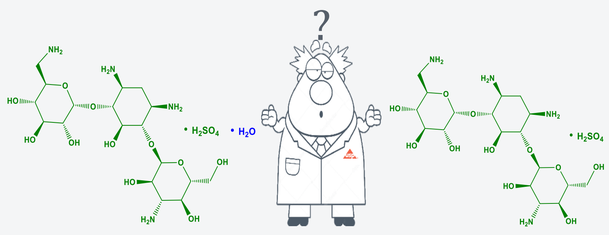
Abstract

Antibiotic reference standards are widely used in pharmaceutical industries, to ensure the quality of drug substances and drug products. The chemical Identity is an integral part of the USP RS Certificate; which provides the molecular structural details of the reference material. Kanamycin Sulfate is known to exist as monohydrate. In this study, water content was determined at elevated temperature (170°C) to verify if the water content was at least equivalent to theoretical monohydrate content (3.0% w/w) in the USP Kanamycin Sulfate RS. The Loss on drying and elemental analysis results, supported by the data obtained from thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC), indicate that the USP Kanamycin Sulfate RS contains a water molecule which is tightly bounded in the crystal lattice. Powder X-ray diffraction experiments produced a significantly different XRPD pattern for dried material (6hr @ 170°C) vs. undried material, and showed that the crystal lattice was disturbed due to the loss of water molecule. To study the role of the bounded water molecule in the crystal lattice in more detail, solid-state nuclear magnetic resonance (NMR) was used to examine the differences between the dried and undried material. Solid-state NMR spectra of both the materials showed minor, but readily detectable, differences in the 13C spectra due to the loss of water molecule. Interpretation of all these analytical data suggests that the USP Kanamycin Sulfate RS contains a water molecule that is tightly bound in a network of hydrogen-bonding with the sulfate oxygens. The loss-on-drying conditions in the monograph [3 hours at 60°C under vacuum (5 mm Hg)] have been shown to be insufficient for complete removal of moisture content from the material. The presence of crystal water in USP kanamycin sulfate RS molecule has no impact on the compendial uses and the value was assigned taking into account LOD.



Introduction

Kanamycin is a broad-spectrum antibiotic produced by *Streptomyces kanamycetius*, the sulfate salt of Kanamycin is used to treat gonorrhea, salmonella, tuberculosis, and many other diseases. Historically, the USP Kanamycin Sulfate RS molecular structure has been referenced as the anhydrate form. In our recent literature search it was revealed that Kanamycin Sulfate is a monohydrate referred to as Kanamycin Sulfate, although water molecule (1:1 mole ratio) is known to be present in the material[1].The water molecule is tightly bound in a network of hydrogen bonding with the sulfate oxygens[2]. It is very difficult to remove the crystalline water, for this sample needs to be dried to constant weight at 170^{O}C under vacuum[3]. Hence ,we have undertaken the task of determining the structure and confirming the presence of water molecule in the Kanamycin Sulfate RS using modern solid state techniques.

Methods

DATA VERIFICATION

CAS Survey and Compendia Cross Reference

	EP	BP	IP	USP
Monograph Title	Kanamycin Monosulfate	Kanamycin Sulfate	Kanamycin Monosulfate	Kanamycin Sulfate
Formula	$C_{18}H_{38}N_4O_{15}S \cdot H_2O$	$C_{18}H_{38}N_4O_{15}S \cdot H_2O$	$C_{18}H_{36}N_4O_{11} \cdot H_2SO_4 \cdot H_2O$	$C_{18}H_{36}N_4O_{11} \cdot H_2SO_4$
Molecular Weight	601	601	600.6	582.58
CAS No.	25389-94-0*	25389-94-0*	58207-20-8**	25389-94-0
Form	Monohydrate	Monohydrate	Monohydrate	Anhydrous
Loss on Drying	**Dry at 60°C for 3 hours under reduced pressure (about 5 mm mercury)**			
Acceptance	NMT 1.5%	NMT 1.5%	NMT 15mg/g (1.5%)	NMT 4.0%
Assay Specification	750 IU/mg (dried substance)	750 IU/mg (dried substance)	750 IU/mg (dried substance)	NLT 750 µg/mg (calculated on dried basis by HPLC)

* CAS No belongs to anhydrous.
** At present Scifinder deleted this CAS number and replaced with 5965-95-7.

USP RS Lot R01190 analytical data at the time of release

- Elemental analysis/Counter-ion → Do not match with theoretical ($C_{18}H_{36}N_4O_{11} \cdot H_2SO_4$)
- Loss on Drying → 0.2 – 0.7% (w/w) (Dry at 60^{O}C for 3 h under vacuum)
- Water content (KF) → Not successful (<921> method Ia & Ic)

TESTING STRATEGY

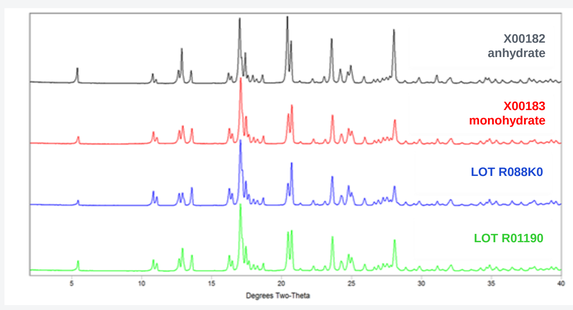
- External samples in different forms:
  - **Sample X000182 – Anhydrous**
  - **Sample X000183 – Monohydrate**
- Tested USP Kanamycin Sulfate Lot R01190, Lot R088K0 and Samples:
  - Elemental analysis (CHN), LOD and an orthogonal KF coulometric oven method <921> Ic used for characterization.
  - The advanced solid state techniques such as SSNMR[solid state NMR, DSC-TGA and XRPD used for confirmation.

Testing Conditions

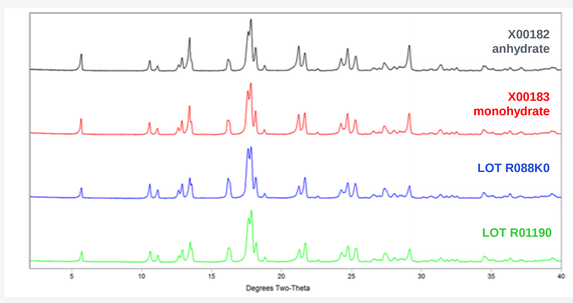
Test	Conditions
CHN	As is , Dried @ 150°C, Dried @ 170°C
Loss on Drying	Dry @ 150°C for 6h Dry @ 170°C for 6h Dry @ 60°C for 3h under vacuum
TGA	As is
SSNMR	As is, Dried @ 150°C, Dried @ 170°C
PXRD	As is, Dried @ 150°C, Dried @ 170°C

Results

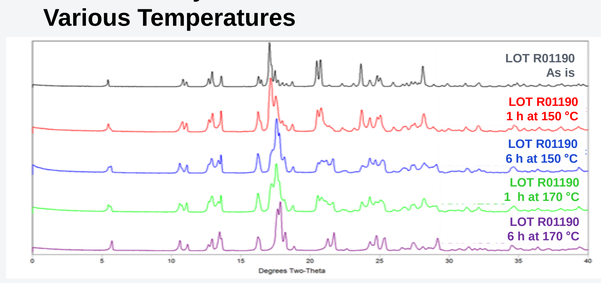
Powder X-Ray Diffractions (As is)



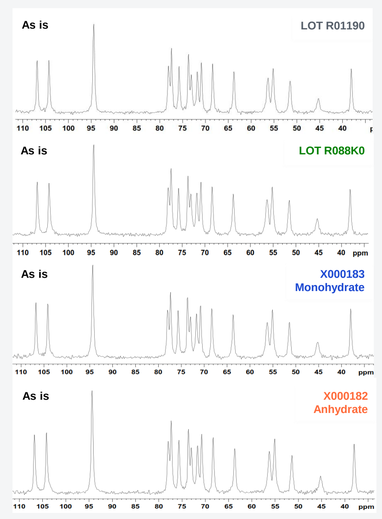
Powder X-Ray Diffractions (Dried @ 170^{O}C)



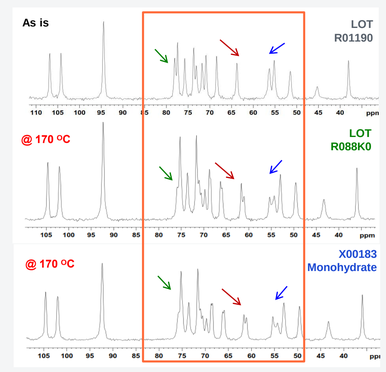
Powder X-Ray Diffractions Lot R01190 at Various Temperatures



Solid State ^{13}C NMR (As is)



Solid State ^{13}C NMR (Dried @ 170^{O}C)



Loss on Drying at Various Temperatures

Sample Name	LOD @ 60°C 3h vac.	TGA @ 170°C	LOD @ 150°C 6h	LOD @ 170°C 6h
USP Lot R01190	0.32	3.74	1.76	3.43
USP Lot R088K0	0.72	4.15	2.50	3.85
X000182 (anhydrate)	0.51	3.62	2.06	3.67
X000183 (monohydrate)	0.69	4.03	2.31	3.50

KF Test

KF Method Ic (Oven temp 170°C)
2.81
3.48
2.84
3.20

Theoretical water content value for monohydrate ($C_{18}H_{38}N_4O_{15}S \cdot H_2O$) = ~ 3%

CHN Analysis (Dried @ 170^{O}C)

	Theoretical % Anhydrous	USP Lot R01190	USP Lot R088K0
C	37.11	37.15	36.82
H	6.57	6.68	6.60
N	9.62	9.65	9.39
S	5.50	5.52	5.45

CHN Analysis (Anhydrous)

USP Lot R01190	USP Lot R088K0	X000182 (anhydrate)	X000183 (monohydrate)
36.56	36.63	36.59	36.56
6.58	6.55	6.61	6.56
9.25	9.37	9.24	9.27
5.39	5.41	5.44	5.41

Conclusions

- Based on the analytical test results presence of water molecule in the USP RS has been confirmed.
- Chemical Identity (**Molecular Formula**, **Molecular Weight, Structure** and **CAS No.**) of the Kanamycin Sulfate in the USP RS Certificate (lot R01190) has been revised.
- We have submitted our recommendations to Scientific Liaison to updated the Chemical Identity in the Kanamycin Sulfate Monograph and align with the other compendia (**EP/BP/IP**).

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1. Crystal Structure, conformation, and absolute configuration of kanamycin A; Yoram A. Puisu, et al; Carbohydrate Research 341 (2006) 2871-2875.
2. The Chemistry of Kanamycin; Annals New York Academy of Sciences; M. J. Cron, et al. Research Division, Bristol Laboratories Inc., Syracuse, NY.
3. Kanamycin and Processes for the Preparation Thereof; U.S. Patent 2,931,798; Patented Apr. 5, 1960. Hamao Umezawa, Kenji Maeda, and Masahiro Ueda, Tokyo Japan.

Loperamide Related Compound F

A Case Study for the Application of Organic Solid State Techniques in USP Reference Standards Characterization

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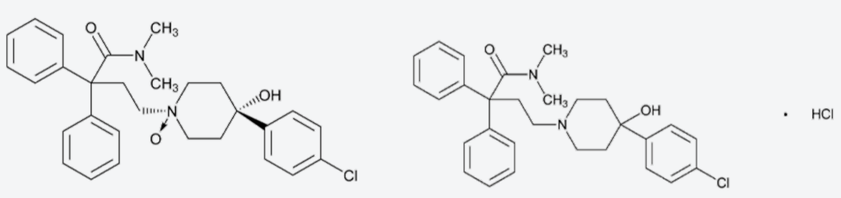
Abstract

Organic solid-state techniques (Dynamic vapor sorption, X-ray powder diffraction, Differential Scanning Calorimetry, Infrared spectroscopy, Thermogravimetric analysis, and Hot-stage microscopy) are extensively used in the pharmaceutical industry for the characterization of drugs and drug products. As part of USP's continuing global mission to deliver "highly characterized" and high-quality reference materials, the organization has increased its use of solid state techniques in the development process. The characterization of a replacement candidate for Loperamide Related Compound F using solid state techniques will be provided as a case study. The analysis will illustrate how these techniques may be applied to provide critical data on the solid-state attributes of the candidate lot and the current lot of *USP Loperamide Related Compound F RS.*

Introduction

Loperamide related compound F [**I**] (also known as Loperamide oxide), is a prodrug of Loperamide [**II**], which is a specific, long-acting anti-diarrheal drug.[1] Loperamide related compound F is used to support the *Assay* and *Organic Impurities* test methods in the **Loperamide Hydrochloride Tablets** monograph. It is used quantitatively as an impurity standard, and qualitatively as an elution marker and resolution probe standard. Loperamide related compound F is known to exist in different polymorphic forms (hydrates) and morphology (crystalline and amorphous). It is essential to know the form of the reference standard being used as different polymorphs can confer different physicochemical properties, which in turn could impact attributes such as solubility and particle size. Those differences could affect a customer's use of the standard. This study examines the characterization of the current and replacement lots of Loperamide related compound F using organic solid-state techniques.



[I]

[II]

Conclusions

Based on the solid-state data, the following conclusions may be drawn:

- The candidate and current lots of Loperamide related compound F are different polymorphs.
- The current lot is amorphous in morphology and exists as the anhydrate.
- The candidate is crystalline in morphology and exists as the 9/4 hydrate. In the crystal structure, there are endless chains in the *b*-direction (plane). The water molecules are hydrogen-bonded in the lattice, and is shown to have partial occupancy. As a result, the stoichiometry is not a whole number.

REFERENCES

(1) O.M. Peeters, N.M. Blaton, C.J. De Ranter, *Acta Crystallographica*, *Section C: Crystal Structure Communications*, **1996**, *52*, 2100.

Results

The differences in the spectra (highlighted by red arrows) are due to the different water content for the candidate (about 6%) and the current lot (about 1%) [see Table I]. These differences are significant enough not to be simply due to poor peak resolution. Another reason for the difference in the spectra is due to the morphology of the two materials. XRPD analysis of the candidate and current lot indicates that the candidate is crystalline, while the current lot is amorphous.

Figure 1. ATR overlay spectra for the candidate and current lot. Differences in the spectra are shown by the red arrows.

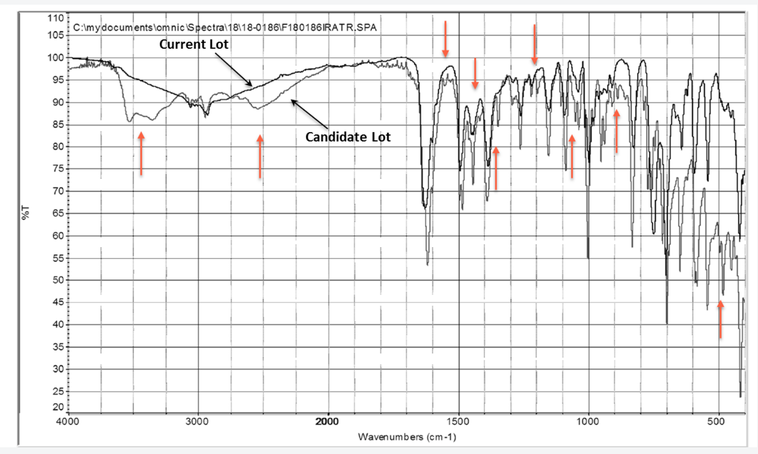


Table I. KF values for the candidate and current lot

Sample Name, Sample ID	Appearance	KF Water content	Water uptake by DVS (5% RH – 95% RH)	Form change after DVS
Loperamide RC F, Sample Lot R089C0	White powder	6.35% 6.18% 6.04%	4.6%	No
Average		6.19%		
Loperamide RC F, Standard Lot F017R0	White powder	1.32% 1.41% 1.36%	8.4%	No
Average		1.36%		

Dynamic vapor sorption analysis was performed for undried samples of the candidate (R089C0) (Figure 2 – upper panel). The sample showed a 4.6% water uptake from 5% RH to 95% RH at 25° indicating that the sample was hygroscopic. Based on the pre- and post-DVS and XRPD data, no form change was observed after the DVS evaluation (Figure 2 – lower panel). The data also suggest that the candidate may be a stable hydrate.

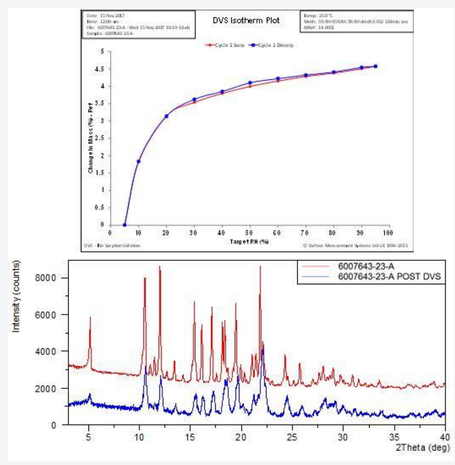


Figure 2
DVS and XRPD for the candidate

Dynamic vapor sorption analysis was performed for undried samples of the current lot F017R0 (Figure 3 – upper panel). The sample showed an 8.4% water uptake from 5% RH to 95% RH at 25° indicating that the sample was hygroscopic. Based on the pre- and post-DVS and XRPD data, no form change was observed after the DVS evaluation (Figure 3 – lower panel).

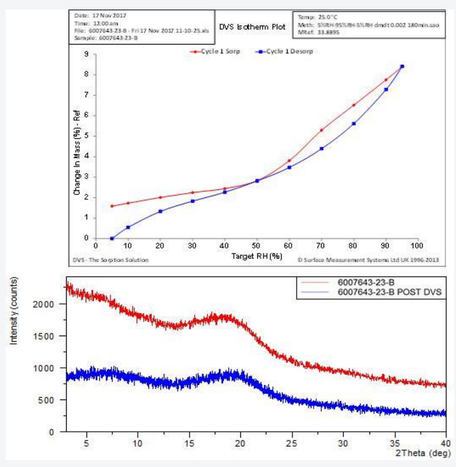


Figure 3
DVS and XRPD for the current Lot.

The mean D_{50} for the candidate was found to be 8.9 µm, with a narrower particle size distribution ranging from 0.23 to 144 µm. The particle size distribution profile is shown in Figure 4 – top panel.

The mean D_{50} for Lot F017R0 was found to be 312 µm, with a wider particle size distribution ranging from 90 to 689 µm (Figure 4 – bottom panel).

These differences in the particle size distribution between the candidate and the current lot could have an impact on the solubility characteristics of the candidate in certain solvents.

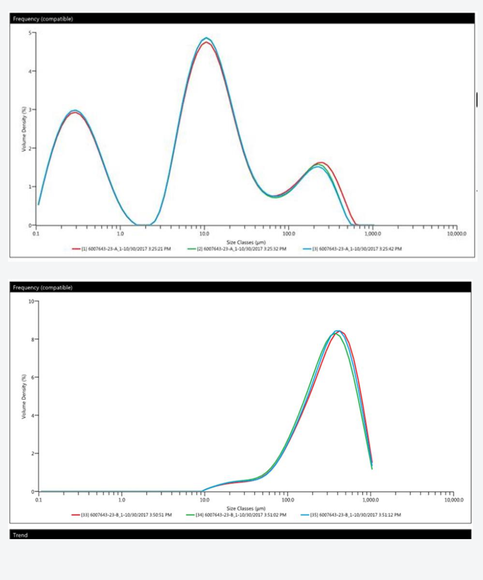


Figure 4
Particle size distribution for the candidate and current lot.