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Review

The sacred cow: the questionable role of assay methods in characterising the quality of bulk pharmaceuticals

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Abstract

The specific and non-specific assay methods in the European and US Pharmacopoeias are critically evaluated. Emphasis is made on the discussion of the increasing role of impurity tests and decreasing, moreover questionable role of assay methods in characterising the quality of bulk drug materials. Various possibilities are also discussed for calculating the active ingredient content from the results of the assay and impurity tests. Only bulk drug materials are dealt with excluding from this study pharmaceutical formulations.

Keywords: Assay; Impurity test; Chromatography; HPLC; Spectrophotometry; Titrimetry

Contents

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1. Introductory remarks: assay methods in the pre-chromatography era

From the beginnings of official pharmaceutical analysis the aim of including assay methods in compendial monographs has been to characterise the quality of bulk drug materials by setting limits for their active ingredient content. Before the introduction of chromatographic methods into pharmaceutical analysis in the middle of the 20th century, almost exclusively classical methods such as titrimetry, gravimetry and later on UV spectrophotometry/colorimetry were available for this purpose. It was well known already in those years that, due to the poor specificity of these methods, the value of the percentage figures obtained in such a way for the active ingredient content were of limited value.

Nevertheless, due to the lack of specific chromatographic methods these assay methods were considered to be among the most important characteristics of the quality of a bulk drug substance. The purity was checked by means of physical constants, mainly by the melting point and the width of the melting range, limit tests for signal (mainly inorganic) impurities, clarity and colour of the solution of the material, etc.

2. The present state-of-the-art: assay methods in the chromatography era

2.1. Introduction

The invention and rapid spread of thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in the 1960s and 1970s [1], respectively, created an entirely new situation in this field. The reasons for this are as follows: (1) both methods enable the detection, separation, identification and quantitative determination of organic impurities which were up to that time not measurable [2]; (2) the selective chromatographic methods were found to be suitable for the reliable determination of also the main component.

The present state-of-the-art is reflected by the data in Table 1, based on the recent editions of the European [3] and US [4] Pharmacopoeias.

2.2. Non-specific methods

2.2.1. Titrimetric methods

As it is seen in Table 1, in the majority of cases classical, non-specific methods are still used, especially in the European Pharmacopoeia [3]. Of these, the non-specificity of titrimetric methods is evident: in the majority of cases organic impurities contain the same functional group on which the titration of the drug material is based. Signs of some modernisation are the spreading of non-aqueous titration methods expanding the field of application of titrimetric methods to (very) weak acids and bases as well as potentiometric (in the case of nitritometric titrations amperometric) end-point detection improving the precision of the methods. Advantages of these methods are saving time and labour, high precision and the fact that there is no need of using reference standards. However, due to their poor specificity the accuracy of titrimetric methods is also poor in the presence of related impurities.

2.2.2. Spectrophotometric/colorimetric methods

Another group of non-specific methods in pharmacopoeias are spectrophotometric methods based on natural UV–VIS absorption and to a lesser extent visual (VIS) spectrophotometric methods based on chemical reactions (colorimetric methods) [5]. The reason for their non-specificity is the same as in the case of titrimetric methods: most of the impurities of drugs contain the same or similar chromophoric systems as those of the drug material. The low time and labour consumption of the methods as well as good precision are advantages in this case also, especially if the method is based on natural absorption. There is no clear picture regarding the necessity of reference standards. In the majority of pharmacopoeial monographs of US Pharmacopoeia [4] the use of reference standards is prescribed, while in the European Pharmacopoeia the calculation of the content is mainly (but not exclusively) based on specific absorbance values given in the monographs. Although the principles of the validation of the determination of specific absorbance have been set up [6], and this is the less time consuming approach, this can be the source of further analytical error, if not high-level spectrophotometers are used for the assay.

It is worth mentioning that (although not too many) startlingly outdated colorimetric methods based on chemical reactions are still in use for the assay of bulk drug materials. For example, the blue tetrazolium assay was very popular in the 1950s and 1960s for the assay of corticosteroid drug formulations, moreover in their bioassay [7,8]. However, it would be difficult to find acceptable arguments for the use of this method for the assay of several bulk corticosteroids in the recent edition of US Pharmacopoeia (⟨351⟩ “Assay for Steroids” [4]). The specificity of this indirect method based on the reducing properties of the α-ketol side chain is not superior to the method based on the natural absorption of corticosteroids and at the same time the advantages of the latter method, i.e. low time and labour consumption as well as high precision are lost. The same applies to the isoniazide assay of several 4-ene-3-oxosteroids where the only “advantage” of this method is that the absorption maximum is shifted from about 240 nm to about 380 nm [4], the determination of cardiac glycosides by the classical picrate colour reaction [3], etc. The most absurd situation exists with the pair ethinylestradiol and its methyl ether (mestranol). The assay method for ethinylestradiol is an up-to-date HPLC method while for mestranol an absolutely outdated, non-stoichiometric colorimetric method using a methanol–sulphuric acid reagent is prescribed [4].

Table 1
Proportion of various analytical methods prescribed for the assay of bulk drug materials in Ph. Eur. 4 [3] and USP XXVII [4]

Method	Ph. Eur. 4 (%)	USP 27 (%)
HPLC	15.5%	44%
GC	2%	2.5%
Titration	69.5%	40.5%
Acid–base	57.5%	29.5%
Aqueous mixtures	21%	5.5%
Indicator	6.5%	4.5%
Potentiometric	14.5%	1%
Non-aqueous	36.5%	24%
Indicator	9.5%	14%
Potentiometric	27%	10%
Redox (iodometry, nitritometry, etc.)	6.5%	5.5%
Other (complexometry, argentometry, etc.)	5.5%	5.5%
UV–vis spectrophotometry	9.5%	8.5%
Native absorption	8.5%	6.5%
Colorimetry based on chemical reactions	1%	2.0%
Microbiological assay (antibiotics)	3%	2.5%
Other (IR, NMR, polarimetry, fluorimetry, atomic absorption spectroscopy, polarography, gravimetry etc.)	0.5%	2%

Included are in this survey organic drug materials and salts of organic acids and bases. Excluded are inorganic drugs, proteins, enzymes, and other macromolecules, radiopharmaceuticals, blood preparations, products of recombinant DNA technology as well as most of excipients.

2.2.3. Other methods

Although some other non-specific methods (polarimetry, polarography, fluorimetry, etc.) do not play an important role in the assay of bulk drugs, it is to be noted that even the precision of these methods is by no means sufficient for this purpose.

2.3. Specific chromatographic methods

2.3.1. High-performance liquid chromatography

HPLC methods appeared for the first time for the assay of bulk drug materials in 1980 [9]. As seen in Table 1, this has become the predominant method in USP XXVII [4] and—although to a lesser extent—it is one of the most widely used methods also in Ph. Eur. 4 [3]. The reason for this is certainly that in contrast to the above discussed non-specific methods the specificity of this method is excellent and at the same time sufficient precision is also attainable. Due to these advantageous features and the disadvantages of the methods discussed so far it can be stated that for the time being HPLC is the only generally applicable method for the Assay of drug materials which can afford accurate results. However, it has to be mentioned that the high specificity, precision and accuracy are attainable only if lengthy system suitability tests are carried out prior to the HPLC assay. For this reason the price to be paid for the high specificity, precision and accuracy is also high: the HPLC method is by about one order of magnitude more time consuming and labour extensive than the above discussed non-specific methods.

2.3.2. Gas chromatography (GC)

Due to the insufficient volatility and thermal stability of the majority of drug materials, gas chromatography can be used for their assay in a limited number of cases only, as reflected by the figures in Table 1. For the specificity, precision and accuracy as well as the time and labour consumption of this method the same considerations apply that are described in the preceding paragraph.

2.3.3. Thin-layer chromatography–UV spectrophotometry

Before the introduction and widespread adoption of HPLC, the high specificity of TLC was often exploited to quantitative analytical purposes using spot elution followed by spectrophotometric measurement. It is appalling that this outdated, very labour-intensive and less precise method is still prescribed in some cases in USP XXVII e.g. as “Single-steroid Assay ⟨511⟩” [4].

3. The value of assay methods in characterising the quality of bulk drug materials

3.1. The role of non-specific methods: terminological problem or more?

Typical statements regarding the active ingredient content of bulk drug materials taken from the US and European Pharmacopoeias, respectively, are shown here taking levonorgestrel as the example. The statement in USP XXVII after the formulae and names of the drug material is as follows: “Levonorgestrel contains not less than 98.0% and not more than 102.0% of $C_{21}H_{28}O_2$, calculated on the dried basis.” The wording is somewhat different, but the essence of the sentence is identical in Ph. Eur. 4 in the paragraph “Characters”: “Levonorgestrel contains not less than 98.0% and not more than the equivalent of 102.0% of 13β-ethyl-17β-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one, with reference to

the dried substance." It is evident that these figures are based on the data obtained from the paragraphs "Assay" (The European Pharmacopoeia contains a direct statement regarding this: "Where limits of content are prescribed, they are those determined by the method described under Assay"). Both pharmacopoeias use non-specific assay methods: USP XXVII [4] measures the UV absorbance at 241 nm (absorption maximum characteristic of the 4-ene-3-oxo chromophoric group) while the volumetric method used by Ph. Eur. 4 [3] is based on the titration of nitric acid liberated in the course of the reaction of the ethinyl group with silver nitrate:

$$-C \equiv CH + AgNO_3 = -C \equiv CAg + HNO_3.$$

The non-specific nature of both methods is evident. Of the six named impurities of levonorgestrel in Ph. Eur. 4 all contain ethinyl group(s) and two (as well as several more described in the literature [10–14]) contain the above mentioned or similar chromophoric groups. This means that the figures obtained by both methods do not refer to the active ingredient content but the sum of the active ingredient and (most of its) impurities. (Ph. Eur. 4 prescribes—on the basis of a TLC test—that no individual impurity may exceed 0.5% and at most two impurities are permitted to be between 0.2 and 0.5%; the number and total content of minor impurities is not limited. According to USP XXVII no individual impurity may exceed 0.5% and the sum of the impurities should be below 2.0%, based on a different TLC test.)

This means that in principle it is possible that the result of the assay is e.g. 98.1% meeting the above described requirements but the sum of the impurities is 1.9% (also meeting the requirements). In this case the real active ingredient content is not more than 96.2% and thus the sentences cited above are not valid. It should be mentioned that the term "active ingredient content" does not appear in the texts cited above. However, on the basis of this and the examples discussed above, the citations from the pharmacopoeias in the first paragraph of this section are meaningless and the requirements in these sentences do not refer to what they are intended to refer to. This seems to be an analytical (or moral?) rather than terminological question.

Innumerable examples could be taken from practically all monographs where the assay is based on non-specific methods, which represent according to the data in Table 1 the majority of cases.

3.2. The role of specific methods (HPLC)

On the basis of the facts summarised in Section 2.3.1 the problems described in the preceding section can be solved by using a specific method (in the overwhelming majority of cases HPLC) for the assay. This is certainly the reason for the fact that HPLC methods are step by step replacing the non-specific methods in the successive revisions of pharmacopoeias. This tendency is mainly characteristic of the US Pharmacopoeias where—as seen in Table 1—the proportion of HPLC method has raised to about 44% in the latest edition [4]. There are, however, at least two main problems with this approach. The first one (highly time- and labour-consuming nature of this method) was already shortly discussed in Section 2.3.1. The second question is whether the higher accuracy attainable by using HPLC for the assay is a real solution for the problematic nature of the value of the active ingredient content as a means for characterising the quality of a bulk drug material. With other words: is it really worth while to spend much extra time and energy to develop and perform HPLC for the assay?

It is not an easy task to give acceptable answer to this question. If the method is carefully elaborated and a sufficiently large number of critical impurities are available, the specificity of the method can be excellent. (The pharmacopoeias prescribe this to be checked routinely as part of the system suitability tests. It has to be mentioned, however, that not even the most specific pharmacopoeial HPLC assay methods are enantioselective.) This creates the basis for the elaboration of accurate assay methods. However, the analytical error highly depends also on the precision of the method. It would be difficult to give generally acceptable figures for the relative standard deviation of compendial HPLC methods. It is remarkable that in their system suitability tests in various monographs in European and US Pharmacopoeias the requirement as regards the relative standard deviation of replicate injections is that it should be less than 2% (in some cases 1 or 3%). A cautious estimation for the precision of compendial HPLC methods can be characterised by RSD of about 0.5–1%. The precision can be somewhat improved by using internal standards, but this possibility is relatively seldom used (in about 15% of cases). This means that the analytical error to be counted with is certainly above 0.5% and it is probably around 1%. This makes the value of assay results obtained even by the highly specific HPLC methods as a means for characterising the quality of bulk drug materials at least questionable.

3.3. The approach of the European Pharmacopoeia

The problematic points of using specific HPLC methods for the assay of bulk drug materials were probably taken into consideration by the European Pharmacopoeia Commission when they summarised their approach regarding this matter as follows: "Specificity of assays: For elaboration of monographs on chemical substances, the approach generally preferred by the Commission is to provide control of impurities via a well designed Test section rather than by inclusion of an assay that is specific for the active moiety. It is therefore the full set of requirements of monograph that is designed to ensure that the product is of suitable quality." [3]. Table 1 well reflects the differences between the approaches of the European and US Pharmacopoeias: while in USP XXVII the proportion of specific HPLC (+GC) methods is 46.5%, the same figure in Ph. Eur. 4 is only 17.5%. This means that the answer to the question formulated in the preceding section:

"... is it really worth while to spend much extra time and energy with choosing HPLC for the assay?" is at a much higher rate *no* in the case of the European than in the case of the US Pharmacopoeia.

Further to this (very agreeable) approach, The European Pharmacopoeia Commission attempts to give chemical-analytical interpretation of the results of the assay and the acceptance limits as means for the determination of the active ingredient content. For monographs where *non-specific* titrimetric or spectrophotometric methods are used, the following statement has been made: "When the substance to be examined contains only impurities that do not interfere with the assay, or when it contains only very low proportion of impurities interfering with the assay, the results can be used directly." [15]. The problem with this approach is that a situation described here practically never occurs: there are always impurities present which interfere with the assay and—as demonstrated in Section 3.1 taking levonorgestrel as an example—up to 2.0% of related impurities interfering with the non-specific assay methods is permitted. Another statement for the case of using *specific* assay methods is as follows: "In the case where a separation technique is employed both for the test of related substances and the assay, content limits are to be set taking into account the analytical error and the maximum permitted amount for impurities." [15]. This very agreeable sentence really makes a step forward to give chemical meaning to the figures of the limits but at the same time it raises further questions.

(a) Which are the related impurities to be taken into account? The US Pharmacopoeia contains information on related impurities in exceptional cases only. In contrast to this, a list of impurities known to be controlled by the (im)purity tests can be found at the end of about 60% of the monographs in the European Pharmacopoeia, represented in Table 1. These lists mainly include the impurities mentioned in the specification of the active substances of licensed products on the European market. The list may also contain some historically known impurities which are not detectable in the marketed products (other detectable impurities). The usefulness of these lists varies from monograph to monograph. In some cases up to 15 carefully selected potential impurities are listed taking into account even the different synthetic routes (such as e.g. in the case of piroxicam). In contrast to this, in addition to the 40% where such a list does not exist—in some cases the lists are of very limited value. For example, in the case of prednisolone only one impurity (hydrocortisone) is mentioned in spite of the fact that many impurities have been described in the literature just to mention two papers: a classical study based on off-line TLC-MS [16] and a recent paper based on an on-line HPLC–UV-MS study [13].

(b) Do the assay limits set in the monographs of Ph. Eur. 4 reflect the methodology of the assay and the level of related impurities? In the case of titrimetric methods the lower and upper limits of the assay are usually 98–99% and 100.5–102%, respectively (most typically 99.0–101.0%). When UV spectrophotometry is used as the assay method the most typical limits are 97.0–103% but others, such as 98.0–102.0% also occur. The limits set for HPLC assays are in many cases similarly strict e.g. 98.0–101.0% for alfadex, betadex, etoposide, fluoxetine hydrochloride, imipenem, methotrexate, 98.5–101.5% for ciclosporin, fenofibrate, 98.0–102.0% for acitretin, allopurinol, budenoside, doxorubicin hydrochloride, finasteride, ifosamide, indapamide, isomalt, maltitol, mannitol, mesterolone, propofol, sodium alendronate. These examples demonstrate that irrespective of the methodology used the limits more or less overlap. It has to be mentioned, however, that in many cases (mainly antibiotics and some peptides) the lower limit of the assay is much lower (down to 90–95%). The following examples [3] demonstrating this are in accordance with the principles of the European Pharmacopoeia [15] quoted in this section. The first example is cefadrine. In its monograph the requirement for the drug material is that it should be above 90% and the sum of cefadrine and cephaloxin should be 95.0–102.0%. The limits for aminodesacetoxycephalosporanic acid, cyclohexa-1,4-dienylglycine and any other individual impurities are 1.0% for each (TLC) and for cephalexin 5.0% (HPLC). For erythromycin the requirement is that the sum of erythromycin A, B and C should be 93.0–100.5%. The limit for each of B and C is 5.0%, any other individual impurity 3.0% and free lactobionic acid (titration) 1.0%. Finally, the assay limits for oxytocin are 93.0–102.0, the limit for individual impurities and their sum is 1.5% and 5.0%, respectively.

3.4. The relation between active ingredient content, compendial assays and the quality of bulk drug materials

As a consequence of the rapid development of methods for the identification and quantitative determination of impurities in bulk drug materials the focal point of characterising the quality of drug materials has shifted from assay methods to impurity tests. This is reflected by the quotation taken from Ph. Eur. 4 [3] in the first paragraph in Section 3.3. (The opinion that assay of bulk drug materials is a "dead issue" can be characterised by the fact that none of the 447 papers published in 2003 in the *Journal of Pharmaceutical and Biomedical Analysis* deals with the assay of bulk drug materials. This possibility is mentioned among other applications in 3 papers only.) This is why the treatment of the subject matter in this paper is based exclusively on pharmacopoeias. Taking into consideration the points discussed in detail in Chapters 2 and 3 of this paper, the following possibilities are available to present percentage figures for the active ingredient content of bulk drug materials:

$$\mathrm{Aic}_{\%} = A_{\mathrm{ns},\%} \quad (1)$$

where $\mathrm{Aic}_{\%}$ is the real active ingredient content and $A_{\mathrm{ns},\%}$ is the figure obtained by the compendial non-specific assay method. The invalidity of (1) is discussed in Section 3.1. From this discussion the following conclusion can be drawn:

$$\mathrm{Aic}_{\%} \neq A_{\mathrm{ns},\%}, \quad \text{i.e.} \quad \mathrm{Aic}_{\%} < A_{\mathrm{ns},\%}$$

The problem of the invalidity of (1) can be solved by subtracting the sum of organic impurities from $A_{\mathrm{ns},\%}$.

$$\mathrm{Aic}_{\%} = A_{\mathrm{ns},\%} - \sum_{\text{organic impurities},\%} \tag{2}$$

If specific chromatographic methods (mainly HPLC) are used for the assay to get $A_{\mathrm{s},\%}$ the invalidity does not exist:

$$\mathrm{Aic}_{\%} = A_{\mathrm{s},\%} \tag{3}$$

The problems and difficulties of this approach are discussed in Sections 3.2 and 3.3.

Eq. (4) is based on an entirely different approach:

$$\mathrm{Aic}_{\%} = 100 - \sum_{\text{impurities},\%} = 100 - \text{Volatile impurities},\% - \text{Residue on ignition},\% - \sum_{\text{organic impurities},\%} \tag{4}$$

The main advantages of using Eq. (4) are as follows:

(a) It would make it unnecessary to carry out assays. The time and energy thus spared could be spent to the more accurate determination of individual organic impurities e.g. by changing the presently existing semi-quantitative tests to their quantitative determination. Due to the very limited value of the data obtainable using the compendial assay methods, the lack of these would not be harmful to the safety of drug therapy. It is worth mentioning that the monographs of several drugs and related materials do not contain an assay test even in the presently existing edition of the European Pharmacopoeia [3] (e.g. butylhydroxyanisole, butylhydroxytoluene, camphor, clofibrate, eugenol, fructose, galactose, glucose, menthol, paraldehyde, sucrose, thymol, xylose). It is highly unlikely that in these cases the assay data are missing to anybody.

(b) The accuracy of $\mathrm{Aic}_{\%}$ obtained from (4) is higher than that obtained from (2) or (3) since the basis of the calculation is $100 \pm 0.0\%$, while in the case of (2) and (3) it is based on $A_{\mathrm{ns},\%} \pm$ analytical error and $A_{\mathrm{s},\%} \pm$ analytical error, respectively.

It is to be noted that this approach is not new at all: the $100 - \Sigma_{\mathrm{impurities},\%}$ concept is often used in those cases when a Reference Standard is not available or for the characterisation of Reference Standards themselves.The disadvantages of using Eq. (4) are also remarkable:

(a) Impurities can be present which are not taken into account by using (4). These are e.g. UV-inactive impurities not detectable by HPLC with long wavelength UV detection, some salts of organic and inorganic acids and bases, respectively e.g. ammonium acetate, alkylammonium salts, etc. In the majority of cases, however, these impurities are detected by other tests in the monographs or their quantity is negligible or at least the error caused by neglecting them is smaller than the value *analytical error* in $A_{\mathrm{ns},\%} \pm$ analytical error and $A_{\mathrm{s},\%} \pm$ analytical error.

(b) A more important disadvantage (?) is that the approach using Eq. (4) is inconsistent with the century old traditions of pharmaceutical analysis all over the world and with the spirit of the presently existing pharmacopoeias and is therefore highly unlikely to be acceptable by the drug registration agencies.

4. Conclusions

As pointed out in this paper, much time and effort is spent making assays of bulk drug materials with results of rather questionable value. There would be many other ways to use these resources to achieve maximum benefit as regards the analytical support to the safety of drug therapy.

Nevertheless, I have no proposals to end this paper that I expect to be accepted by the majority of the drug analytical community and especially by the drug registration agencies. In spite of the fact that the percentage figures obtained by the compendial assay methods for the active ingredient content are of very limited value in characterising the quality of a bulk drug material, which is mainly based on the identification and quantitative determination of impurities, assay methods (in the traditional sense of the word) are and will certainly remain parts of their compendial monographs. As for my personal opinion on this matter, I consider this concept as a sacred cow and summarise my opinion every year by telling my students: “If you will be drug analysts, you should carefully elaborate assay methods for bulk drug materials, carefully validate them, and carefully carry out your own and compendial assay tests routinely. However, you must not take your own results seriously as regards the real active ingredient content of drug materials and must not attribute great importance for the figures thus obtained as regards the quality of the drug material”.

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