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DEVELOPMENT OF APPROACHES TO VALIDATION OF UV-SPECTROPHOTOMETRIC METHODS OF QUANTITATIVE DETERMINATION IN FORENSIC AND TOXICOLOGICAL ANALYSIS: LINEARITY AND APPLICATION RANGE

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Summary: the approaches to choice of the method application range and to the procedure of experiment carrying out, which allow to estimate the linearity of UV-spectrophotometric methods of analytes quantitative determination in biological fluids used in forensic and toxicological analysis was offered.

Key words: validation, linearity, range, application range, UV-spectrophotometry, bioanalytical methods.

Introduction. This article is the continuation of authors' research [1, 2] in the field of development of the approaches to validation of methods of quantitative determination for purposes of forensic and toxicological analysis and devoted to the problem of range choosing and validation parameter «linearity» determination.

The purpose of this paper is to analyse the present approaches to determination of linearity and range according to the requirements of the international guidances [3 – 7], to form the approaches to the procedure of analytical range choosing and linearity determination, notably to the number of concentration levels within the range, to the number of replicate observations for each level, etc. when carrying out the validation of UV-spectrophotometric methods of quantitative determination for forensic and toxicological analysis.

Investigation methods. Such methods of scientific research as analysis, synthesis, systematic analysis, mathematical statistics, comparison and summarising were used.

Results and discussion. ***Linearity*** – is the ability of an analytical procedure (within a given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample (ICH) [3]. This parameter is present in all guidances, which give the directed recommendations in regard to validation of bioanalytical methods, – «Guidance for Industry: Bioanalytical method validation» (U.S. FDA, 2001) [4], «Standard Practices for Method Validation in Forensic Toxicology» (SWGTOX, 2012) [5], «Guidance for the Validation of Analytical Methodology and Calibration of Equipment used for Testing of Illicit Drugs in Seized Materials and Biological Specimens» (UNODC, 2009) [6] and «Guideline on validation of bioanalytical methods» (EMA, 2011) [7], but the different terms are used for its designation – see Table 1.

In three papers from four the word «linearity» is avoided to use and the terms ***«calibration curve»***, ***«calibration model»***, etc. are preferred that is connected, from our point of view, to the presence of bioanalytical methods, for which it is necessary to choose more complex calibration model, for example, when the concentrations range exceeds one order of magnitude or immunochemical methods of analysis are used. As a rule, linear models are more preferable, but, in case of need, using the nonlinear models is not only acceptable, but even recommended [5], therefore application of term ***«linearity/calibration model»*** is optimal in order to take into account all possible variants.

As regards ***analytical range of method application***, it is the interval between the upper and

Table 1. Requirements to the linearity determination according to the FDA, EMA, UNODC and SWGTOX papers

Paper	Term	The number of concentration levels	The number of replicates
FDA	Calibration/standard curve	6 – 8	–
EMA	Calibration curve	not less 6	not less 3
UNODC	Linearity and working range	5	not less 6
SWGTOX	Calibration model	not less 6	not less 5

lower concentration (amounts) of analyte in the sample (including these concentrations), for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity – according to ICH [3] definition. In the ICH guidance [3] this parameter is presented as individual, in the FDA, EMA, UNODC and SWGTOX papers [4 – 7] it is considered within the parameter «linearity», and the term is mentioned in the parameter name only in the UNODC paper [6]. From our point of view, for the purpose of ensuring the relative unity in the questions of terminology it is appropriate to mark out the parameter ***«range»*** as individual.

For validation of analysis methods for medicines the clear ranges of method application depending on the decided tasks – 80 – 120%, 70 – 130%, 50 – 130%, etc. – are foreseen by regulatory documents [8, 9], and nominal or rated concentration has been accepted as 100%; the number of concentration levels within the range of application – g = 9, and the number of replicate measurements for each concentration level are also standardized by the validation standardized procedures [9].

As regards validation of bioanalytical methods, none of the considered guidances [4 – 7] does not give distinct recommendations on the choice of range of method application – «the working range is predefined by the purpose of the method and may reflect only a part of the full linear range» (UNODC) [6], «before carrying out the validation of the analytical method it should be known what concentration range is expected» (EMA) [7], «concentrations of standards should be chosen on the basis of the concentration range expected in a particular study» (FDA) [4], «the calibrator samples shall span the range of concentrations expected» (SWGTOX) [5].

The requirements to the number of concentration levels g used for plotting the calibration curve are very various – see Table 1; it is specified additionally that «the number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship» [4], but the recommendations about increasing the number of concentration levels when extending the range of method application are absent in any of the considered papers [4 – 7]. It is especially accented that «the concentration levels shall be appropriately spaced across the calibration range» [5], but it is not pointed out in which way exactly. On the face of it, the conclusion inevitably comes to mind that concentrations should be spaced evenly (that is required by application of the least squares method), but the examples of validation reports given in Appendix [5] show that it is not the case.

The requirements to the number of replicates for each concentration level are also considerably differed (Table 1), and it is not clear, what is meant under the term «replicate» – replicate experiment or replicate measurement? The SWGTOX guidance [5] accents that each replicate is performed within the separate days/runs, but the set of questions arises:

- if this is the replicate measurement next day, is stability of the solution to be analysed under the storage conditions confirmed preliminary?
- if this is the replicate experiment, is the same matrix source used for its carrying out or another?
- if the same matrix source is used, under which conditions is it stored, is stability of its composition under the storage conditions confirmed, does not the nature of analyte interaction with the matrix after storage under the given conditions change, etc.?
- if this is the replicate experiment, in which way are the samples prepared – is the large volume of matrix spiked with analyte, and then the samples are taken for analysis, or firstly the necessary number of blank-samples is taken, and then they are spiked with analyte?

Besides, the SWGTOX guidance [5] allows to use fewer number of calibration samples (fewer number of concentration levels or fewer – up to 1 – number of replicates) for routine analysis, but with the set of limitations:

- the lowest and highest calibration levels should be used;
- no fewer than four concentration levels should be used;
- accuracy and precision for the given calibration samples should be confirmed.

Such approach contradicts the main validation principle – the method validation should be carried out under the same conditions as the method [10].

There are not clarity and unity in the texts of the considered papers [4 – 7] also in regard to the data that should be used for determination of calibration model. Thus, the UNODC guidance [6] recommends to plot the calibration curve using the mean values of responses for each concentration level; and it is necessary to exclude the outlying values of measurements on the basis of the Grub test or the Dixon Q-test. The FDA and EMA guidances [4, 7] require to plot the calibration curve for each analytical run, and they allow to exclude the values of measurements and concentration levels, if they do not satisfy to the requirements by the parameter «accuracy». In the event that the lowest and highest calibration levels are excluded, the whole run of measurements is rejected [7]. The SWGTOX guidance [5] says about the «combined» data. In this case it is not clear what does it mean – 6 ґ 5 =

30 response values for 6 concentration values or 6 mean response values for 6 concentration values? As it has been shown in [10], the first variant of data processing by means of the least squares method is meaningless as it masks the possible nonlinearity and creates the semblance of increasing the number of degrees of freedom.

Finally, all considered papers [4 – 7] suggest to plot the calibration curve for linearity determination using such biological matrix, for which the method is developed, but do not specify, from which sources the matrix is taken – from one or from different. The SWGTOX paper [5] foresees the possibility of replacement of biological matrix by more suitable or accessible, but only in the case of carrying out the corresponding experiments confirmed the adequacy of such replacement.

Thus, accumulated differences and contradictions when considering the international papers in the field of bioanalytical methods validation [4 – 7] require elaboration of the integrated approaches to the choice of analytical range of application and conditions of linearity verification, particularly, for UV-spectrophotometric methods of analytes quantitative determination in biological liquids for forensic and toxicological analysis.

As it has been stated before [1, 2], for validation of UV-spectrophotometric methods of quantitative determination for forensic and toxicological analysis we use the normalized coordinates (i. e. transition from the equation of $A_i = b_1 \cdot C_i + a_1$ type to the equation of $Y_i = b_2 \cdot X_i + a_2$ type), which advantages of application are widely reported [9] – the validation characteristics obtained in the normalized coordinates do not depend on the specificity of concrete analyte and can be regulated easier. In our case the expressions for the normalized coordinates have such appearance:

$$X_i = \frac{C_i}{C_{st}} \cdot 100\%, \quad C_{st} = C_{reference};$$

$$Y_i = \frac{A_i}{A_{st}} \cdot 100\%, \quad A_{st} = \frac{A_{reference} \cdot R}{100}.$$

I. e. for normalization of the obtained experimental data the reference solution with the concentration of analyte ($C_{reference}$) corresponded to its concentration in the end solution to be spectrophotometric measured under the condition of zero losses for the point of 100% in the normalized coordinates is used; the absorbance of such reference solution ($A_{reference}$) is corrected by the value of recovery R obtained at the preliminary stage of validation [2] and is used for normalization of absorbance values. Such approach is needed for decline of influence of the systematic error introduced by the components of blank-sample, which significance has been shown at the preliminary stage of validation [1].

When developing UV-spectrophotometric methods of analytes quantitative determination in biological liquids it is necessary to be guided simultaneously both by the analyte concentration in biological liquid that should be determined and by its concentration in the end solution to be spectrophotometric measured – it should provide the necessary working values of absorbance.

To base the approaches to the application range choosing for UV-spectrophotometric methods of quantitative determination in forensic and toxicological analysis it is necessary to present schematically the structure of such methods – see Figure 1.

Thus, the method in the general case consists of two parts – sample preparation and directly analytical operation; and the structure of sample preparation is such that knowing the analyte concentration in biological matrix it is possible to obtain the necessary analyte concentration in the end solution to be spectrophotometric measured in the way of increasing or decreasing the volumes V_1, V_2, V_3 and V_4. In the same way it is possible to solve the problems with the linearity loss on the ends of range of method application.

As 100% in the normalized coordinates we suggest to accept the mean toxic or lethal analyte concentration in biological liquid – depending on the purposes and tasks, for which the developed methods is intended. And it is necessary to have in mind that the range of toxic and lethal analyte concentrations in biological liquids can be wide enough, moreover, the lower concentrations are fixed more often, than respective mean [11]. On the other hand, UV-spectrophotometric methods can not provide the possibility of reliable analyte quantitative determination in the range of concentrations differed more, than in one order of magnitude [12]. Therefore it is appropriate to choose the range of application for UV-spectrophotometric method in the way that the point of 100% is nearer to its highest limit, and absorbance of the end solution to be spectrophotometric measured, which corresponds to this point, under ideal conditions (zero losses and absence of background absorbance) is equal to 0.7 – 0.9. Taking into account the reasoning in relation to the value of minimal absorbance stated before [13], the lower limit of the range of method application corresponds to the point of 25% in the normalized coordinates.

In turn the highest point of the method analytical range can be accepted equal to 125% (in the case

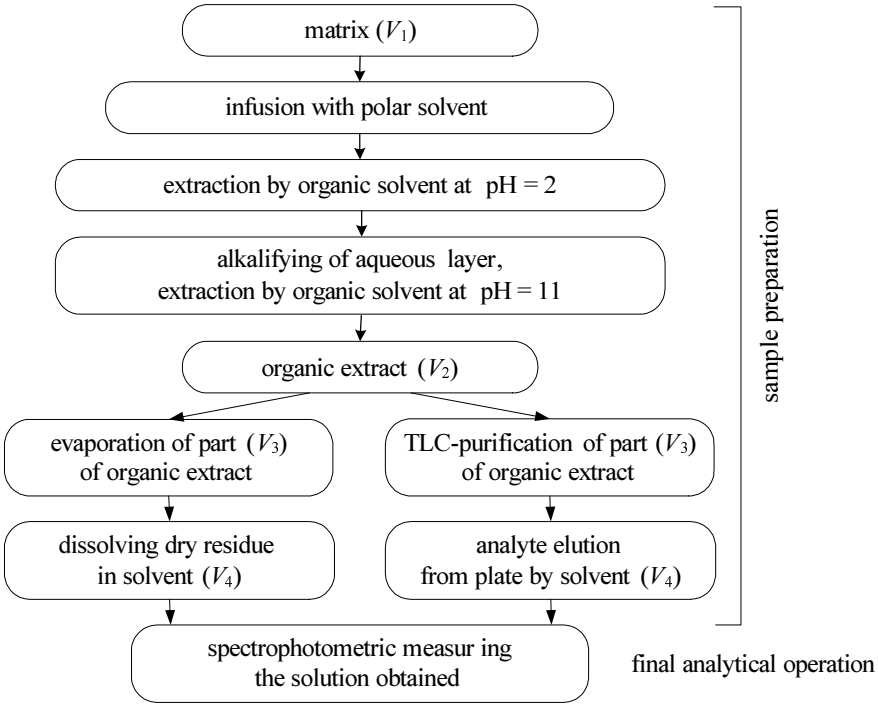

Fig. 1. **The main stages of UV-spectrophotometric methods of analytes quantitative determination in biological liquids in forensic and toxicological analysis**

of work with the samples to be analysed with higher concentrations it is possible to use diluting the samples for obtaining the absorbance values, which are within the range of method application). In the case if it is intended to carry out the analysis by the addition method, the range of method application should be extended in its high part to 150% or 175%.

The offered ranges of method application allow to dispose evenly within them the concentration levels in increments of 25% – in this way we obtain 5 – 7 points that corresponds to international recommendations [4 – 7]. Using the constant increment of 25% regardless of the chosen range of application allows dynamically to add or exclude the concentration levels – according to the obtained experimental data and stated purposes, i. e. in the case of decision making about changing the range it is not necessary to change the position of points within it and carry out the experiment over again, and that, in turn, substantially reduces the time and labour costs.

On the other hand, it is interest to show in theory sufficientness of the offered number of concentration levels for providing the necessary relative uncertainty of the method. For this purpose we proceed from the UNODC [6] requirements, according to which the correlation coefficient should be not less, than 0.99. Determining the range of method application we can calculate RSD_{range} [9]:

$$RSD_{range} = \sqrt{\frac{\sum_{i=1}^{g}(X_i - \overline{X})^2}{g-1}},$$

and using the formula [9]:

$$R_c = \sqrt{1 - \frac{RSD_0^2}{RSD_{range}^2}},$$

obtain the requirements to the value of RSD_0. In turn, applying the formula [10]:

$$\Delta_{cal} = t(95\%, g-2) \cdot RSD_0,$$

it is possible to calculate the calibration uncertainty $Д_{cal}$ [10]. In order to predict the total uncertainty of the method of analysis $Д_{As}$ (for the method of calibration curve) and estimate acceptability of its value, it is possible to use the approach offered in [10]:

$$\Delta_{As} = \sqrt{2} \cdot \Delta_{cal}.$$

And in the case of the method of standard or addition method $Д_{cal} = Д_{As}$ [9].

The results of such calculations for the offered variants of the range of method application are given in the Table 2.

Table 2. The results of $Д_{As}$ predicted for the offered variants of the range of method application

range	increment	g	RSD_{range}	RSD_0	$t(95\%, g-2)$	Δ_{cal}	Δ_{As}
25 – 125%	25%	5	39.53	5.58	2.3534	13.1%	18.6%
25 – 150%	25%	6	46.77	6.60	2.1318	14.1%	19.9%
25 – 175%	25%	7	54.01	7.62	2.0150	15.4%	21.7%

Under the concept «replicate» we suggest to understand the complete carrying out the replicate experiment, and in relation to the number of such replicate experiments for each concentration level – in terms of [10] it is possible to offer the next approach: to estimate acceptability of repeatability of the values of A_{sample} used for plotting the calibration curve – the relative uncertainty of repeatability of the values of absorbance obtained in replicate experiments, $D_{Anom,r}$ (against the nominal value of absorbance [13]) should not exceed the maximum allowed calibration uncertainty $Д_{cal}$, i. e. according to [9]:

$$\Delta_{A_{nom},r}(sample) \le \max\Delta_{cal} = \max\Delta_{As} / \sqrt{2}.$$

As [10]

$$\Delta_{A_{nom},r}(sample) = t(95\%, n-1) \cdot s_{nom,r}$$

$$(sample) / \sqrt{n},$$

we obtain the requirement to $s_{nom,r}$*:*

$$s_{nom,r}(sample) \le \max s_{nom,r} = 0.707 \cdot$$

$$\max\Delta_{As} \cdot \sqrt{n} / t(95\%, n-1).$$

I. e. after carrying out three replicate experiments for each concentration level it is necessary to calculate $s_{nom,r}$, to estimate its acceptability and make the decision about necessity or absence of necessity in carrying out additional batch/run of experiments.

The next question that needs discussion is the origin of the matrix used for linearity determination. As it has been already discussed before [2], the different degree of analyte extraction from the matrix, which, in turn, mainly depends on two reasons – the analyte amount in the matrix and the state of matrix – is, in the first place, the reason of unsatisfactory linearity of bioanalytical methods.

In order to estimate the separate influence of these two factors on linearity of the method to be validated we suggest to carry out the investigations for three replicate runs, each one consists of 5 – 7 samples of biological matrix obtained from the same source, i. e. for analysis of each run the individual source of biological matrix is used. We recommend to carry out the analysis of runs in different days (one day is one run) that corresponds to practical realities: 7 – 10 experiments is the maximum number of experiments, which can be carried out by analyst during the day in forensic and toxicological laboratory (taking into account the used procedures of sample preparation).

Such approach allows to avoid the necessity to store the samples of biological matrix, give the possibility to estimate the influence of analyte amount (in the case of determination of the linearity parameters for each run separately) and changing the matrix (in the case of determination of the linearity parameters by the mean values obtained for several runs) on the parameters of linear dependence.

Thus, for determination of parameter «linearity/calibration model» we suggest to use g = 5 – 7, but to obtain the equations $Y = b \cdot X + a$ both for each run and by the mean values of replicate experiments. By analogy with terminology of papers [4 – 7] it is possible to call these results the «parameters of within-run (within-day) and between-run (between-day) linearity».

Conclusions. Thus, the procedure of range choosing and linearity confirmation for UV-spectrophotometric methods of analytes quantitative determination in biological fluids used in forensic and toxicological analysis has been offered.

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РОЗРОБКА ПІДХОДІВ ДО ВАЛІДАЦІЇ УФ-СПЕКТРОФОТОМЕТРИЧНИХ МЕТОДИК КІЛЬКІСНОГО ВИЗНАЧЕННЯ В СУДОВО-ТОКСИКОЛОГІЧНОМУ АНАЛІЗІ: ЛІНІЙНІСТЬ ТА ДІАПАЗОН ЗАСТОСУВАННЯ

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Резюме: запропоновані підходи до вибору діапазону застосування методики та процедури проведення експерименту дозволяють оцінити лінійність УФ-спектрофотометричних методик кількісного визначення аналітів у біологічних рідинах для судово-токсикологічного аналізу.

Ключові слова: валідація, лінійність, діапазон застосування, УФ-спектрофотометрія, біоаналітичні методики.

РАЗРАБОТКА ПОДХОДОВ К ВАЛИДАЦИИ УФ-СПЕКТРОФОТОМЕТРИЧЕСКИХ МЕТОДИК КОЛИЧЕСТВЕННОГО ОПРЕДЕЛЕНИЯ В СУДЕБНО-ТОКСИКОЛОГИЧЕСКОМ АНАЛИЗЕ: ЛИНЕЙНОСТЬ И ДИАПАЗОН ПРИМЕНЕНИЯ

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Резюме: предложенные подходы к выбору диапазона применения методики и процедуре проведения эксперимента позволяют оценить линейность УФ-спектрофотометрических методик количественного определения аналитов в биологических жидкостях для судебно-токсикологического анализа.

Ключевые слова: валидация, линейность, диапазон применения, УФ-спектрофотометрия, биоаналитические методики.

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