

Recommendations from the Society for Toxicological and Forensic Chemistry (GTFCh) concerning the validation of methods for toxicological analysis in the context of determining brain death.

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1. Recommendations

In 2002, the Clinical Toxicology Committee of the Society for Toxicological and Forensic Chemistry (GTFCh) published recommendations for toxicological analysis in the context of determining brain death [1, 2]. In these, the necessity of method validation was established. As there are several, and in part very comprehensive validation recommendations for various areas of bioanalytics [3], the Clinical Toxicology Committee has agreed on a minimum consensus, which is recommended to laboratories when performing toxicological analyses in the context of determining brain death. In Table 1, the single validation steps regarded to be essential are summarised.

Table 1: Validation parameters and minimum requirements

Selectivity (Exclusion of Interference)

- Ten different blank samples (serum or plasma) must be analysed.
- Two zero samples (blank serum/plasma spiked with internal standard) must be analysed.

Linearity

Calibration (matrix-based)

- Lowest calibrator: A quarter of the lower limit of the therapeutic range
- Highest calibrator: At least as high as the upper limit of the therapeutic range
- Six replicate analyses at five different concentrations respectively
- Approximately equidistant spacing between the calibrators

Statistical Evaluation

- Verification of adequate linearity
- Checking of the y-intercept
- Use of a non-linear calibration model where necessary

Precision and Accuracy

Preparation of two spiked control samples

- Concentrations at the lower and upper limit of the therapeutic range

Two replicate analyses of each control sample on eight different days with full calibration performed daily employing freshly prepared calibrators

- Calculation of the concentration of the control samples via one-point calibration
- Selection of the optimal one-point calibrator
- If acceptance limits are not fulfilled for one-point calibration, calculation by full calibration

Acceptance limits

- 99% confidence interval within $\pm 50\%$ of the target value (includes precision and accuracy)

2. Justification

2.1. Selectivity (Exclusion of Interference)

The goal of analysing blank-matrix samples from various sources is the detection or exclusion of interference caused by endogenous (or exogenous) substances present in the matrix. In principle, the higher the number of blank-matrix samples analysed, the higher the probability is of finding rarer types of matrix interference in this validation phase, as undetected interference could later lead to considerable problems during routine application. At this stage, the procedure can still be modified, before the rest of the validation experiments are performed. The analysis of at least ten different matrix samples as set down in the minimum consensus constitutes a compromise between the effort required and the risk of possibly overlooking rarer kinds of interference. However, the analysis of further blank-matrix samples over and above this minimum requirement is advisable.

The goal of analysing zero samples is the detection or exclusion of interference arising from the internal standard. This is required particularly in mass spectrometry when deuterated analogues of the analytes are used as standards, as these cannot be fully separated from the analytes themselves by chromatography.

2.2. Linearity

In toxicological analyses in the context of determining brain death, only single samples are analysed, as they are seldom requested. Thus, performing a full calibration for each one of these single samples does not make sense or is not possible due to time or economic constraints. Ultimately, the target will be to develop methods that also lead to acceptable results with one-point calibration. This requires, that in the concentration range in question, a linear correlation exists between the analyte concentration in the sample and the signal response, and that the y-intercept is negligibly small.

The choice of the concentration of the lowest calibrator as being $0.25 \times$ the lower limit of the therapeutic range is necessary, in order to test the linearity down to concentrations below the limit of the measuring range ($0.5 \times$ the lower limit of the therapeutic range) as stated in the recommendations for toxicological analysis in the context of determining brain death [1, 2]. The concentration of the highest calibrator should be at least equal to the upper limit of the therapeutic range, to be able to reliably determine concentrations within the therapeutic range, and to allow estimation of the progression of non-sub-therapeutic concentrations in patients' samples if need be. Sometimes, choosing a concentration for the highest calibrator well above the upper limit of the therapeutic range can be advisable, for example when the method is additionally used for toxicological analysis in cases of poisoning.

The six replicate analyses of five different calibrators respectively – which ideally should be evenly spread across the aforementioned calibration range – are necessary for obtaining results which allow reliable judgement of the linearity due to their number (30 analyses) and character. As the sample matrix can considerably affect the calibration function, particularly in the absence of deuterated standards, it is necessary to perform the linearity experiments with matrix-based calibrators, whereby the matrix used should be as close as possible to the matrix of the samples later to be analysed. For statistical assessment of the linearity, there are a number of procedures described. For this, the program VALISTAT (<http://www.pts-gtfch.de/ben/b513.htm>) employs the linearity test according to Mandel, but requires that the variances across the concentration range are homogeneous. As a rule, however, this precondition is only fulfilled for concentration ranges within one order of magnitude. In the case where variances are inhomogeneous, other statistical procedures have to be employed. Basically, it has to be kept in mind, that where very precise methods of analysis are concerned, statistically significant deviation from a linear calibration model is not necessarily relevant in practice. If doubt exists concerning the latter, this can be checked via the data for precision and accuracy. If these are within the limits of acceptance after adopting a linear model, small deviations from the linear model can be neglected.

Of particular importance regarding the later use of a one-point calibration is the inspection of the y-intercept, as a one-point calibration is only reliable if the calibration line passes through the origin. However, here it is also true, that where very precise methods are concerned, a statistically significant

deviation of the y-intercept from zero is not necessarily relevant in practice. Here again, the fulfilment of the acceptance limits of the data for precision and accuracy are decisive.

2.3. Precision and Accuracy

The evaluation of precision and accuracy data at two concentrations corresponding to the lower and upper limit of the therapeutic range allows estimation of the capability of the method across the entire therapeutic range. By preparing a suitably large amount of control samples at each of the two concentrations, precision and accuracy can be determined by repeated measurement of the same sample material.

By the design of experiments incorporating duplicate measurements on 8 different days, a sufficient amount of data for statistical evaluation is acquired. In addition, with the aid of variance analysis (e.g. VALISTAT), this allows separate estimation of the repeatability and intra-laboratory precision (intermediate precision) from the same set of data.

Performance of the full calibration (with single measurements for each calibrator) is advisable on each of the 8 days for several reasons. Firstly, the estimation of accuracy should not be based on only one calibration. Secondly, the optimal one-point calibrator can be selected from the calibrators of full calibration. Thirdly, if the acceptance limits are not fulfilled with one-point calibration, re-calculation can be done using the data already available from the full calibration. In this way, it can be verified whether the problem has arisen solely due to the one-point calibration itself, or if the nature of the problem is fundamentally method-based.

As acceptance criterion, it was set down that the 99% confidence interval of the values measured (average $\pm 3 \times$ laboratory precision) must fit completely within an interval of $\pm 50\%$ of the corresponding target value. The feasibility of this criterion was demonstrated by several members of the Clinical Toxicology Committee in exemplary validations of their assays for the determination of midazolam, the analyte with the lowest measuring range. In practice, the acceptance criterion means that a concentration in the lowest therapeutic range can be differentiated from a concentration below the measuring range with 99% probability.

3. Bibliography

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