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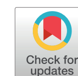
Considerations regarding the validation of chromatographic mass spectrometric methods for the quantification of endogenous substances in forensics



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ABSTRACT

The requirement for correct evaluation of forensic toxicological results in daily routine work and scientific studies is reliable analytical data based on validated methods. Validation of a method gives the analyst tools to estimate the efficacy and reliability of the analytical method. Without validation, data might be contested in court and lead to unjustified legal consequences for a defendant. Therefore, new analytical methods to be used in forensic toxicology require careful method development and validation of the final method. Until now, there are no publications on the validation of chromatographic mass spectrometric methods for the detection of endogenous substances although endogenous analytes can be important in Forensic Toxicology (alcohol consumption marker, congener alcohols, gamma hydroxy butyric acid, human insulin and C-peptide, creatinine, postmortal clinical parameters). For these analytes, conventional validation instructions cannot be followed completely. In this paper, important practical considerations in analytical method validation for endogenous substances will be discussed which may be used as guidance for scientists wishing to develop and validate analytical methods for analytes produced naturally in the human body. Especially the validation parameters calibration model, analytical limits, accuracy (bias and precision) and matrix effects and recovery have to be approached differently. Highest attention should be paid to selectivity experiments.

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1. Introduction

The requirement for correct evaluation of forensic toxicological results in daily routine work and scientific studies is reliable analytical data based on validated methods. Validation of a method gives the analyst tools to estimate the efficacy and reliability of the analytical method. Without validation, data might be contested in court and lead to unjustified legal consequences for a defendant. Therefore, new analytical methods to be used in Forensic Toxicology require careful method development and validation of the final method. Until now, there are no publications on the validation of chromatographic mass spectrometric methods for the detection of endogenous substances although endogenous analytes can be important in Forensic Toxicology. Endogenous substances relevant in Forensic Toxicology are alcohol consumption markers (i.e. ethylglucuronide and ethylsulfate in urine or hair, fatty acid ethyl esters (FAEE) in hair or phosphatidylethanol in blood [1–3]), congener alcohols, gamma hydroxy butyric acid (GHB) and its isomers, human insulin and C-peptide or creatinine. Furthermore, post mortal biochemical parameters which are mostly measured by immunochemical methods can also be measured by chromatographic methods (i.e. glucose, lactate, HbA1c acetone, beta hydroxybutyric acid or 1,5-anhydroglucitol [4] for the detection of a diabetic cause of death or parameters which indicate other causes of death [5]).

For these analytes, conventional validation instructions cannot be followed completely. Most of the validation experiments have to be changed and usual procedures cannot be conducted.

Special focus should be placed on selectivity experiments. Due to the fact that the analyte is mostly present in all samples and it is not possible to receive blank samples, regarding selectivity experiments, using chromatographic methods it is not always possible to completely exclude co-eluting substances with the same ion transitions in multiple ion monitoring. For EtG this is not a problem: although it has been described that small EtG concentrations could be also determined in urine of strict teetotalers, working with a LoD of 5 ng/mL in urine, completely negative samples on EtG and EtS can be found in teetotalers and

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used for normal validation [6]. Hair samples of strict teetotalers can also be negative on EtG using a Limit of Detection (LoD) of 4 pg/mg [7]. For GHB, endogenous serum and urine usually show concentrations <1 mg/L and there are also serum and urine available without any presence of analyte even when using low limits of detection. For FAEE, even in strict teetotalers or children hairs were tested positive, traces of FAEE with sum concentrations of the four esters <0.02 ng/mg were measured in all samples [8]. Endogenous ethanol or ethanol from hair cosmetics is assumed to be the reason. Additionally GHB is always present in hair samples endogenously in low ng/mg concentrations. Creatinine in urine is usually determined spectrophotometrically using the Jaffé reaction. A method comparison by Luginbuhl and Weinmann indicated that the spectrophotometric method was slightly overestimating the creatinine concentration when comparing to a LC–MS/MS method [9]. Chromatographic methods should be preferred, however for the validation of these methods there is no blank urine available. For some other analytes (congener alcohols) blank serum is commercially available. Furthermore, the instability of other endogenous analytes (human insulin and C-peptide) can be of advantage when validating analytic methods. A simple heating of the potential blank matrix can destroy the analytes, however, also destroys further – possibly disturbing – substances which can then be overlooked during validation process [10].

Furthermore, especially within the parameters calibration model, accuracy (bias and precision) and matrix effects and recovery, validation of new methods for the quantification of endogenous compounds in Forensic Toxicology is a challenge and requires special and befallen validation procedures. In this paper, considerations in analytical method validation for endogenous substances will be discussed which may be used as guidance for scientists wishing to develop and validate analytical methods for analytes produced naturally in the human body.

2. Validation procedure

2.1. Preparing a blank matrix or using a surrogate matrix

For analytes that are also endogenous compounds, the FDA (Food and Drug administration) states in its recommendations for method validation [11] that the best thing would be that the biological matrix used to prepare calibration standards etc. should be the same as the study samples and free of the endogenous analyte. To address the suitability of an analyte-free biological matrix, the matrix should be demonstrated to have (1) no measurable endogenous analyte using the method to be validated and (2) the same matrix effect and no interference when compared to the biological matrix. However, for some endogenous analytes in Forensic Toxicology, there is the possibility to receive analyte-free real biological matrix, for others it is impossible and surrogate matrices have to be used.

2.1.1. Removing of the analyte from authentic biological matrix

A possibility that was described previously to remove endogenous compounds from liquid matrices is the addition of activated carbon. Afterwards, the sample has to be mixed for several hours to days followed by centrifugation and filtering. Special care should be taken that all carbon particles are effectively removed before the matrix is spiked with analyte because the added analyte will readily bind to remaining traces of carbon resulting in a reduced concentration of analyte (see example testosterone in Ref. [12]). However, compounds which are bound to lipoproteins cannot be removed (e.g. progesterone) [13]. Furthermore, several problems come up with carbon treated blank matrix regarding validation: since more than only the analyte is removed from the matrix the addition of carbon results in a completely different matrix. The validation parameters matrix effects, limits of the method or recovery will not be the same as for authentic samples. Therefore, this procedure is not recommended by the authors.

Furthermore, affinity extraction can be used to clean matrix from the specific analyte. Sorbent- or magnetic bead bound antibodies like in case of insulin [10] or C-Peptide [14] can bind the analyte specifically and remove it from the matrix which then can be used as a blank matrix for validation. These approaches lead to the optimal situation of an analyte-free authentic matrix to be spiked with the analytes, however, are time consuming, expensive and in case of immunoaffinity purification there is a need of specific antibodies for the analyte. Therefore this approach did not find its way in forensic scientific validations.

Another approach which is possible for thermal instable analytes (i.e. insulins, [10]), is the slight heating which can lead to a complete destruction of the analyte, however, to the destruction of further matrix compounds which could interfere with the analysis, too. These could then be overlooked during validation process.

If the affinity extraction approach – the only one recommended here – is possible and wished to be used, well known validation procedures can be followed [15].

2.1.2. Quantification using surrogate matrix

If no analyte-free samples of the authentic matrix are available, calibration standards and quality controls were described to be prepared by spiking the analyte into a surrogate matrix.

Matrix most frequently used in Forensic Toxicology is blood or plasma/serum. Phosphate buffered saline (PBS) is frequently used as surrogate for plasma and serum in forensic publications. PBS can be buffered to a similar pH (7.4) and a similar ionic strength (150 nM) compared to plasma. To simulate protein content of blood, often bovine or human serum albumin is added at a concentration of 40–60 g/L [13]. However, due to the presence of lipids, sugars and proteins, plasma is very difficult to mimick completely. In addition to self-produced surrogate plasma, commercial suppliers (CST Technologie, USA; Irvine Scientific, USA) sell synthetic plasma. Furthermore, especially for polar analytes, water was used as a surrogate matrix [16]. For non-water-soluble analytes, organic solvents like ethanol can be used to replace authentic matrix [17].

Synthetic urine was prepared by Tanaka and Hayashi [18] by dissolving 14.1 g of NaCl, 2.8 g of KCl, 17.3 g of urea, 1.9 mL of ammonia water (25%), 0.60 g of CaCl, and 0.43 g of MgSO, with 0.02 mol/L hydrochloric acid (1 L) for the detection of silicon, magnesium and calcium in urine. For preparing calibration standard solutions of calcium or magnesium, synthetic urines which did not contain CaCl, or MgSO, were used. Cerebrospinal fluid has been prepared by Oka et al. [19] by spiking some salts, glucose and lactate into water.

Care should be taken using surrogate matrix since analyte solubility in a synthetic aqueous matrix may be low. Furthermore, extraction may be different in authentic and surrogate matrix. Wheeler et al. showed that testosterone is bound strongly to sex hormone-binding globuline (SHBG) and weaker bound to albumin in plasma. Depending on the extraction conditions, total testosterone or only fractions can be extracted [20]. Surrogate matrix would not copy protein fractions of the authentic matrix and extraction yields can be completely different for surrogate and authentic matrix. A similar effect can arise from differences in derivatization yield [21]. Therefore, extraction yields should be compared in real matrix and surrogate matrix before chosing the surrogate matrix as matrix for validation procedure. A potential way to compare the extraction yield of surrogate matrix and authentic matrix is to prepare a series of calibration points over the whole calibration range and determine the slopes of the calibration curves. Similar

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