Quality Assurance of Forensic-Toxicological Analysis: Selected Issues

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Abstract

In the present paper, recent trends in the formulation and application of quality assurance (QA) parameters in forensic toxicology, as well as the selected validated applications published in the last five years, are reviewed. In the first part, QA aspects of the analysis of toxicologically relevant compounds in various materials are presented. In particular, QA aspects of analysis of various matrices, like seized street drugs, formalin fixed tissues, dried blood spots, oral fluid, hair, and nails are reviewed. The second part is devoted to identification as applied in general, untargeted and targeted screening; regulations and recommendations issued on international and national levels are presented in the third part, whereas existing QA programs applicable for forensic analysis are discussed in the fourth part.

Key words: Quality assurance, Forensic toxicological analysis, Quality control.

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Introduction

Quality assurance (QA) is a must in applied natural sciences, and plays a particularly important role in forensic disciplines. The result of forensic analysis may disrupt a person’s professional or family life, may ruin a good name of a sports champion or may send somebody to prison. For these reasons, a forensic expert witness while testifying in court is always exposed to the scrutiny of the court, the general public, the media, and his professional colleagues as well. This scrutiny is a matter of a permanent improvement of quality control and quality assurance...
procedures, leading to transforming the term “forensics” into “forensic science” [1-3].

The purpose of this review was to present new trends (from the past five years), which concern various aspects of QA as applied to forensic toxicology. The studies directly dealing with quality issues, as well the validated applications were reviewed. The review was divided into the following parts:

- QA aspects of the analysis of toxicologically relevant compounds in various materials.
- QA aspects of identification as applied in general, untargeted and targeted screening.
- Regulations and recommendations issued on international and national levels.
- Existing QA programs applicable to forensic analysis.

1. QA aspects of the analysis of toxicologically relevant compounds in various matrices:

1.1 Non-biological matrices:

In the United States, the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) published recommendations intended to assist forensic scientists in the development of analytical techniques, protocols, and policies applied in seized drug examination [4]. These recommendations are recognized to be minimum standards that may be expanded according to local requirements. The intention of SWGDRUG was to gain international acceptance of these recommendations as the foundation for good laboratory practice. These recommendations consist of the following chapters: Code of Professional Practice, Education and Training, Methods of Analysis (divided into Methods of Sampling Seized Drugs for Qualitative Analysis, Methods of Drug Identification, and Clandestine Laboratory Evidence), and Quality Assurance (divided into General Practices, Validation of Analytical Methods, and Uncertainty). It should be noted that the Quality Assurance chapter is the most comprehensive part of these recommendations. The recommendations should be adopted by any laboratory involved in the analysis of seized drugs. German authors [5] presented official guidelines for sampling traces of illicit drugs present on various surfaces and sampling loose chemicals, pharmaceuticals, or pharmaceutical preparations [6].

1.2 Biological matrices:

1.2.1 Isolation issues:

Fernandez et al. [7] developed and fully validated a procedure for the isolation of 19 opioids as well as four other drugs of abuse from urine samples. Automatically pretreated samples were injected on-line into the UPLC-MS-MS system, and Isotope dilution quantification was applied. Full validation parameters were published. Automation resulted in improved precision and accuracy, and shortened the preparation time by 80%. According to the authors, this procedure is of interest to accredited laboratories according to ISO 17025.

Molecularly imprinted polymers (MIPs) have been used for many years as material for selective extraction. Thibert et al. [8] synthesized several MIP supports for selective extraction of cocaine and benzoylecgonine from hair tissue. The limit of quantitation below 70 pg/mg of hair was reported for both compounds. Lendoiro et al. [9] applied MIPs for selective extraction of THC and THC-COOH from urine and oral fluid. The linearity range 1-500 ng/ml in oral fluid and 2.5-500 ng/ml in urine were reported.

Formalin-fixed tissues are sometimes subjected to forensic toxicological investigations. Two reviews summarizing the problems arising with the toxicological analysis of formalin-fixed or embalmed forensic samples were published. Takayasu [10] reviewed the influence of formalin fixation on the results of toxicological investigations in tissues. Several possible chemical reaction mechanisms of formalin with drugs were discussed. Nikolaou et al. [11] reviewed the studies on the stability of various drugs in formalin. The need for identification and evaluation of possible degradation products or formation of derivatives was stressed. Generally, the analysis performed on formalin-treated samples needs very careful validation, using calibration and QA samples prepared in parallel using the same or similar treatment.

1.2.2 QA aspects of dried blood spot analysis:

Dried blood spot (DBS) sampling technology was introduced by Bang in 1913 [12] and was used on a wide scale in newborn screening programs for around 50 years. The progress in forensic toxicology, particularly the introduction of LC-MS-MS on a routine scale, also facilitated the application of the DBS sampling method
Nevertheless, advances in analytical technology over the last few years have resulted in a growing acceptance of oral fluid as an important material in the workplace and in roadside drug testing. Many studies were devoted to quality assurance and quality control of results obtained with oral fluid testing. Clarke and Wilson prepared eighteen proficiency testing samples of oral fluid spiked with various drugs [21]. The samples were sent to 13 different laboratories and were analyzed by immunoassay and mass spectrometric methods. There was only one case of a false-positive result (for methadone), but many false negative results were obtained, probably because of the inadequate sensitivity.

In 2011, The European Workplace Drug Testing Society (EWDTS) published guidelines concerning the use of oral fluid for workplace drug testing [22]. It was recommended that, in addition to the general accreditation requirements as formulated in international standard ISO-EC 17025, the laboratories should also follow those guidelines as a template. The most important objectives of those guidelines were to provide a common framework for the European providers of oral fluid workplace drug testing services, promote and harmonize standards accepted at the European level, and to define common quality assurance and quality control criteria capable of being accredited by an external accreditation body. These guidelines cover the whole spectrum of activity, from oral fluid collection, laboratory procedures (sample processing, validity and adulterant testing, screening and confirmatory methods, reporting and storage), through quality assurance and quality control issues, to the interpretation of results. The guidelines also included laboratory organization, flaws in the chain of custody, recommended cut-offs for screening and confirmatory methods as well as the requirements and tasks of the medical review officer. These guidelines could be and should be implemented in forensic analysis of oral fluid samples.

Cooper et al. discussed these guidelines and their relevance in forensic toxicology [23]. According to Moore, the recommended cut-off values of the EWDTS are fully achievable for ELISA procedures and for GC-MS or LC-MS/MS as confirmatory methods [24]. In a European Commission co-funded research project, Pil et al. elaborated a proficiency testing (PT) program for DRUID (Driving under the influence of drugs, alcohol and psychoactive medicines) [25]. Twenty-two
compounds were included in this PT program. A common sample collection method, using a StatSure® Saliva Sampler, and a common GC-MS or LC-MS/MS analysis procedure were applied. Four rounds of proficiency testing were organized for eleven laboratories. This study showed a marked increase in the quality of results in comparison with previous PT studies. Lee and Huestis have reviewed recent knowledge on cannabinoids testing in oral fluid [26]. This review with 248 references covers practically all relevant papers published on this topic.

1.2.4 Hair and Nails:

1.2.4.1 Analytical and interpretation issues:

Toxicological analysis of hair is particularly useful in cases when the sampling is delayed for several days or even weeks after exposure to a particular drug or poison. This is particularly true in cases of drug-facilitated sexual assault, when the victim’s sample is only available a long time after the attack. In such cases, the drug in question is eliminated from blood or urine and only the analysis of hair provides a sufficiently broad detection window. Maublanc et al. published an LC-MS/MS procedure for the identification and quantitation of 35 psychotropic drugs, which were used as incapacitating agents in drug-facilitated sexual assault [27]. The procedure was fully validated and applied in forensic casework. The LC-MS-MS method was published for fast screening of 17 drugs of abuse in human hair. The procedure was fully validated and individual cut-off levels were established for each drug. The procedure has been applied in the routine monitoring of drug addicts who underwent rehabilitation therapy [28].

The differentiation between repeated and single exposure to drugs detected in hair samples is sometimes difficult, particularly in the case of drugs which cause excessive sweating. This is particularly true in the case of amphetamine derivatives. Decontamination procedures, like single or multiple wash steps, are not always effective in removing external contamination [29]. Kintz et al. presented a case of single, high-dosed exposure to 3,4-methylenedioxy-methamphetamine (MDMA), which caused excessive sweating in the subject causing subsequent contamination of the hair [30]. Toxicological analysis of the hair performed 7 days after the incident showed MDMA (but not its metabolite) throughout the entire length of the hair in fairly identical concentrations. Repeated analysis, done 6 weeks later, revealed MDMA in segments 1 to 3 cm, but not in the segment 0-1 cm. On the basis of these results, one expert testified that the subject consumed MDMA on several occasions. This opinion was challenged by the subject, who maintained that he took MDMA only once and that this occurred without his knowledge. The second opinion concluded that the results of the analysis indicate the external contamination of the whole hair due to excessive sweating, caused by the ingestion of a single high dose of the drug [30].

In another critical interpretation of two cases involving young children, possibly exposed to cannabis and methadone, Kintz stated that factors which may influence the results of the analysis include: changes in the mode of drug administration; increase in body weight due to natural growth with a subsequent lower concentration in hair after the same dosage; contamination of the child’s hair with the sweat of the mother; and in-utero exposure during pregnancy [31]. For these reasons, it was impossible to conclude whether the children were deliberately given drugs. Kintz and Nicholson [32] compared the reliability of two ethanol markers – ethyl glucuronide (EtG) and fatty acid ethyl esters (FAEE) – in 97 hair samples and the analytes were determined according to the Society of Hair Testing guidelines [33]. In over 30% contradictory results were obtained; 27 specimens were EtG negative and FAEE positive, and 4 specimens were EtG positive and FAEE negative.

Another aspect of the need for careful interpretation of hair analysis was raised by Hutter et al. who developed a fully validated LC-MS-MS procedure for hair analysis to determine the presence of 22 components of synthetic cannabinoids in a herbal mixture commonly offered as “Spice” [34]. The highest concentrations were found in the most proximal segments, even related to the time before the reported beginning of drug use. This evidenced the incorporation of cannabinoids in hair by sidestream-smoke during smoking. Therefore, any relation between the self-stated consumption and the observed concentration in the corresponding hair segments could not be established. New possibilities of drug testing in hair were revealed by Cuypers et al. who applied matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MSI) and Metal Assisted Secondary Ion Mass spectrometry (MetA-SIMS) to visualize
cannabis molecules in cross sections and longitudinal sections of hair samples contaminated with cocaine or taken from drug users [35].

Besides hair, nails offer another suitable source for drug analysis. Several drugs as well as alcohol consumption markers were detected in nails [36,37]. However, the mechanism of drug incorporation in nails is still unclear. Swiss authors undertook a systematic study on the incorporation of zolpidem in fingernails after the application of a single dose of 10 mg orally to nine volunteers [38]. Zolpidem was chosen due to its forensic relevance as an incapacitating drug, occurring often in drug-facilitated crime cases. Nail samples were collected once a week for a period of 3 to 5 months and analyzed with LC-MS-MS. Parallel with nail samples, head hair samples were analyzed. Three peak concentrations of zolpidem were observed in nails; the first was 24 hours after ingestion, presumably caused by sweat-mediated transport of the drug, the second peak was 2 to 3 weeks after ingestion, possibly caused by incorporation via the nail bed, and the third peak (the smallest one) 10 to 16 weeks after ingestion, presumably caused by incorporation via the nail matrix. The detection window of zolpidem after a single 10 mg dose was estimated as 3.5 months. The comparison with hair analysis showed that the concentration of zolpidem in hair was much higher, most probably due to the melanin presence in the hair. Shen et al. developed an LC-MS/MS procedure combined with frozen pulverization sample pretreatment for determination of five opiates in fingernails of subjects, whose urine was positive for morphine [38]. In 12 out of 18 specimens, 6-monoacetylmorphine, morphine, and codeine were found. The results were in agreement with the results of hair determination, however in hair the concentrations of opiates were higher.

1.2.5 Specimens used for the assessment of in-utero exposure to drugs:

Prenatal exposure to various drugs may affect the health of a fetus and neonate child. The assessment of such exposure through interviews and self-reporting is not reliable and should be supported by carefully validated analytical data, wherever possible. Chitamma et al. studied the in-utero marijuana exposure by GC–MS, ELISA, and LC–TOF–MS using an umbilical cord tissue sample [39]. A total of 16 tissue samples were analyzed. GC-MS was the most sensitive technique for the detection of prenatal exposure to cannabis (cut-off 0.05 ng/g), followed by ELISA (0.1 ng/g) and LC-TOF-MS (1 ng/g). The LC-TOF-MS procedure was not optimized for the determination of THC-COOH. Therefore, the THC-COOH-glucuronide was not analyzed.

Concheiro et al. studied 175 cases to compare the usefulness of various specimens, such as maternal hair, meconium, umbilical cord, and placenta for detecting in-utero exposure to cocaine, opiates, methadone, and amphetamines using LC-MS [40]. The highest frequency of positive results was obtained in maternal hair. The meconium, placenta, and umbilical cord samples also tested positive when the concentration of a given drug in hair was higher than the cut-off level defined by the Society of Hair Testing SoHT. Himes et al. analyzed hair specimens collected monthly from 29 opioid-dependent mothers and 4 of their infants enrolled in a methadone treatment program [41]. Methadone and its metabolites were analyzed by LC-MS-MS. Despite careful validation of the study, it was not possible to estimate neither the degree of methadone exposure during pregnancy based on maternal hair concentrations, nor the probability of adverse neonatal outcomes. Maternal and neonatal hairs were merely useful as good matrices for documenting methadone exposure during pregnancy.

Friguls et al. estimated the prevalence of drug use among pregnant women (n=107) living in Ibiza (Spain) by analyzing a segment of maternal hair in the last trimester of their pregnancy by GC-MS [42]. In addition, participants were also interviewed about their recent history of consumption of drugs. Hair analysis showed that 16% of hair samples were drug-positive, mostly for cannabis and cocaine. The analytical data were in striking contrast to the reported data about consumption of drugs, since only 1.9% of mothers declared using drugs of abuse during the pregnancy [42]. The group from the National Institute of Drug Abuse (NIDA) assessed the informative values of meconium analysis as an indicator of prenatal exposure to tobacco [43]. Self-reported prenatal exposure to tobacco smoke was correlated with the results of the analysis for smoking biomarkers in meconium and with neonatal growth outcome. Nicotine and its metabolites, cotinine and 3-hydroxycotinine, were determined by LC-MS-MS. It was found that reduced birth weight, height, and head circumference significantly correlated with the presence of meconium smoking biomarkers but not with individual or total marker concentrations [43].
2. QA aspects of identification as applied in general, untargeted and targeted screening:

The advent of LC-MS in analytical toxicology resulted in a substantial progress in the identification of known or unknown compound(s) in an evidence material. This technique is gradually replacing GC-MS in the field of drug screening. Various techniques have been used in the last decade: LC-MS, followed by LC-MS/MS, UP-LC-MS/MS, and LC-HR-LC-MS, applied as TOF or Orbitrap. Several reviews have been published on the application of these techniques for the identification of known and unknown compounds [44-47]. The quality aspects of possibly unambiguous identification of detected compounds were discussed by Bogusz [48].

In 2014, the Scientific Working Group for Forensic Toxicology (SWGTOX) issued “Standards for Mass Spectral Data Acceptance for Definitive Identification” [49]. These standards apply to mass spectrometric analyses of target analytes with molecular mass less than 800 Da, already separated with GC or LC, and ionized with EI, CI, ESI, or APCI. Several manufacturers of LC-MS-QTOF instruments (e.g., Agilent, AB Sciex, or Waters) are now offering libraries comprising over one thousand toxicologically relevant compounds, which can be identified on the basis of retention behavior, accurate molecular mass and corresponding fragments.

In the last few years, several studies on validated liquid chromatographic-mass spectrometric procedures for toxicological screening have been published, and most of those studies were based on LC-MS-TOF or QTOF. Pragt et al. developed an LC-MS-QTOF procedure for the screening of over 2,500 compounds from serum, blood, or urine after solvent extraction or acetonitrile precipitation [50]. Electrospray ionization in positive and negative mode was used, and three collision energy levels were applied. This procedure has been further adapted for toxicological screening in hair. Practical application in 80 forensic cases showed detection of 35 illicit and 154 therapeutic drugs. The method was fully validated and showed adequate robustness and sensitivity [51].

Guale et al. developed and validated an LC-TOF-MS screening procedure for the identification of 96 drugs and their metabolites belonging to ten various classes [52]. Blood or urine samples were subjected to automated SPE and injected into the LC-TOF MS system using positive ionization mode. The compounds were identified on the basis of accurate mass (mass error within 15 ppm) and retention time (difference less than 10s from the standard). The recovery varied broadly, even within the same group (e.g. for amphetamines 10 to 95%), and the matrix effect study showed high suppression in the case of opioids and cannabinoids. The procedure has been applied in 21 forensic cases and all positive results were confirmed by other methods.

Tsai et al. developed an UHPLC–QTOF-MS method for the screening and confirmation of 62 drugs of abuse and their metabolites in urine [53]. Urine samples were diluted 1:5 with water, centrifuged and injected into the LC-MS system. Chromatographic separation was performed on a Poroshell EC-C18 column in methanol-gradient. MS detection was done using an ESI source in positive and negative ionization modes. For screening purposes, the following parameters were used: Retention time, accurate mass, and isotopic pattern. For the confirmation, two product ions were used. The method showed satisfactory sensitivity for most compounds and low matrix influence. An automatic screening and confirmation procedure was developed using the software provided by the manufacturer (Agilent). The procedure showed higher sensitivity than immunoassays.

Benzodiazepines belong to the most frequently prescribed, consumed, and abused drugs. Nakamura [54] reviewed LC-MS and LC-MS/MS procedures for screening and identification of benzodiazepines and their metabolites, published from 2001 to 2010. The information about the biosamples assayed and their preparation, chromatographic conditions, ionization type, mass spectral detection mode, matrix effects and validation data for each procedure were summarized. Generally, the usefulness of LC-MS and LC-MS/MS for targeted benzodiazepine screening was stressed. The comparison of immunoassay with LC-MS/MS for the detection of benzodiazepines was performed as a National Proficiency Test in Italy with the participation of 178 laboratories. An overestimation of immunoassay results and high incidence of false positives was observed [55].

Guo et al. [56] developed an UPLC-quadrupole orbitrap high-resolution MS procedure for targeted screening and quantitation of eleven antidiabetic drugs in illegally adulterated herbal remedies. Identification and quantitation was achieved using full-scan mode, whereas product ion spectra were used for confirmation.
The method was fully validated using matrix-matched calibration curves. The procedure was applied for the analysis of 63 batches of herbal remedies and 34 batches of dietary supplements. In 7 cases positive results were observed.

Two papers were specifically devoted to postmortem forensic practice and were fully validated according to international standards: Di Rago et al. [57] developed an LC-MS/MS automated screening procedure for 132 acidic, neutral, and basic drugs from 0.1 ml blood. Sempio et al. [58] described an LC-MS/MS method for the detection and quantification of 88 psychoactive drugs and their metabolites in autopsy blood. The method was compared with GC-MS screening procedure and showed better sensitivity.

3. Regulations and recommendations on international and national levels:

All testing and calibration laboratories seeking accreditation ISO 17025, are obliged to provide measurement uncertainties for all types of laboratory measurements. This also concerns forensic toxicological laboratories dealing with the measurement of compounds of interest in forensic samples. Uncertainty of measurement occurs due to many factors: the uncertainty of the reference compound, the calibration curve, and the precision and accuracy. Some of these factors may be evaluated from the statistical distribution of the results of a series of measurements and can be characterized by experimental standard deviations. The other factors, which can also be characterized by standard deviations, are evaluated from assumed probability distributions based on experience or other information [59]. Kim et al. [60] studied the measurement uncertainty for the THC-COOH and its glucuronide determined in urine samples with LC-MS-MS after solvent extraction. The largest contribution to the overall uncertainty was from the recovery and calibration curve. The study identified the extraction procedure and construction of the calibration curve as the main factors affecting the quality of THC-COOH measurement. The reliability of a measurement was expressed as expanded uncertainty of the measurement result at 95% confidence level.

The scientific Working Group for Forensic Toxicology (SWGTOX) [61] was established in 2009 by the US Forensic Toxicology Council and was funded by the US Department of Justice (DOJ), and thereafter by SOFT and ABFT. The mission of SWGTOX is to develop and disseminate consensus standards for the practice of forensic toxicology. In 2013, the SWGTOX Committee for Research, Development, Testing, and Evaluation published recommendations [62] relevant to all forensic investigators, laboratory managers, policy makers, and governing bodies dealing with forensic toxicology. These recommendations presented the provisional list of focus areas for research, the list of priorities as well as the areas of practice in research. The need for oversight in research was stressed, which should cover the ethical use of human subjects and their biological specimens, privacy issues, and approval of animal experiments. The standards of research should be in agreement with legal requirements as formulated by multiple government and scientific agencies. SWGTOX also published minimal standards for developing a guide and code of professional conduct in forensic toxicology [63]. These standards cover ethical issues for individuals and laboratories.

In 2012, the US National Safety Council’s Drug and Alcohol Division undertook a survey of 96 out of 123 laboratories involved in the testing of drug-impaired driving cases. The results of this survey were published in the form of recommendations for toxicological investigations of drug-impaired driving and motor vehicle fatalities [64]. The council recommended blood and oral fluid as the preferred matrices for DUID investigations. Urine was the best suitable specimen to demonstrate prior drug use. Alcohol was recognized as the most prevalent drug. Other compounds were divided into two tiers on the basis of their prevalence in the casework. Recommended cut-off concentrations for screening and confirmation in blood and oral fluid were formulated. It was stressed that positive results of presumptive tests should always be confirmed and the scope of testing should be clearly defined in the laboratory test report.

Another important issue is to assure the quality of forensic experts. In US federal court procedures, admissibility criteria of expert testimony were defined by the US Supreme Court in the 1993 Daubert decision [65]. According to Rule 702, amended in 2011, “a witness who is qualified as an expert by knowledge, skill, experience, training, or education may testify in the form of an opinion or otherwise if: (a) The expert’s
scientific, technical, or other specialized knowledge will help the trier of fact to understand the evidence or to determine a fact in issue; (b) The testimony is based on sufficient facts or data; (c) The testimony is the product of reliable principles and methods; and (d) The expert has reliably applied the principles and methods to the facts of the case.” It is obvious that the requirements formulated in Rule 702 apply to forensic toxicology. Christensen et al. [66] recently discussed various aspects of technical, statistical, methodical, and human error in forensic science and testimony, mainly in the view of Rule 702.

4. Existing QA programs applicable to LC-MS forensic analysis:

Quality assurance (QA) is defined as “the practice that encompasses all procedures and activities toward ensuring that a specified quality of product is achieved and maintained”. Quality control (QC) is “a set of procedures designed to monitor the test methods and test results to ensure appropriate test system performance” [67]. Both these elements are included in the requirements for accreditation based on international ISO standards 17025 and 15189 [68]. In practice, QA is enforced through an internal and external QC program. External QC, known also as proficiency testing (PT) or external quality assessment (EQA), is a component of a laboratory’s total quality system that is intended to verify on a periodic basis that laboratory results are in conformity with the expectations for quality as set by the organizing body. Several international and national PT programs have been developed, such as the UK National External Quality Assessment Scheme (UKNEQAS), and the College of American Pathologists (CAP) Proficiency Testing/External Quality Assurance program. On the European level, “the European Proficiency Testing Information System (EPTIS) database lists around 1000 PT schemes, and many of them are applicable in forensic sciences.

Conclusion

Quality assurance is a moving target. Therefore, forensic scientists should observe all recent requirements formulated by accreditation agencies and implemented by local professional organizations. All forensic toxicological methods, procedures, and publications must follow up-to-date quality standards.

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