

Determination of Direct Oral Anticoagulants in Forensic Toxicology

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Oral anticoagulants have been used for more than 50 years as antithrombotic agents, particularly in the primary and secondary prophylaxis of thromboembolic disorders. Our study's purpose is to synthesize and analyze the toxicological methods used to determine the oral anticoagulants, described in the literature, so we can identify the optimal methods, taking into consideration the diversity of forensic medicine workload. In the electronic databases, for the period 1990-2019, there were identified 56 articles that included the searched keywords. Of these, 28 were included in our study. We excluded the articles that were not lining up with the objective of our study, the update-type articles and the case studies. After analyzing the literature, it was observed that liquid chromatography-mass spectrometry is the ideal method for detecting low-dosed drugs, such as oral anticoagulants.

Keywords: oral anticoagulants, toxicology, forensic medicine

Oral anticoagulants have been used for more than 50 years as antithrombotic agents, particularly in the primary and secondary prophylaxis of thromboembolic disorders. These drugs are highly effective and widely used. The response to these drugs is based on the inhibition of the synthesis of vitamin K-dependent clotting factors [1]. The great individual variability in response to oral anticoagulants is caused by many different factors, as: the rate of absorption and metabolic transformation, the diet and the genetically determined drug resistance [2, 3]. Therefore, prescribing these drugs should take into consideration a narrow, sensitive therapeutic index, and it implies a medical oversight, because of the increased risk of hemorrhage [4-9]. Indeed, therapeutic overdose can be a source of very serious hemorrhagic accidents, which can cause death, in case of delayed medical intervention [10, 11].

Monitoring the therapeutic effect of these drugs is usually done by measuring a blood coagulation parameter, and the most commonly used is the international normalized ratio (INR). The risk of hemorrhage increases significantly and linearly, as soon as this parameter is greater than 3 [12]. However, several situations may require direct identification and quantification of the anticoagulant agent, especially in forensic cases [13, 14]. Deaths may indeed be caused by excessive doses of oral anticoagulants, after voluntary or involuntary consumption [15]. Determination of the INR in post-mortem blood is not possible, therefore only the specific identification of the anticoagulants can confirm the intoxication [16].

Apart from the first line, represented by the automatic immunological analyzes, available for most common drugs, the procedures currently used for general unknown screening (GUS) in clinical and forensic toxicology involve chromatographic techniques, ideally coupled to specific detectors: gas chromatography-mass spectrometry (GC-MS) [12] or high-performance liquid chromatography (HPLC) coupled to W-diode-array detection (DAD) [17]. However, none of these methods taken alone is sufficient to identify all possible toxic compounds. For example, some polar compounds with little or no UV absorbency will not be detected by GC-MS or HPLC-DAD. With MS being more specific and more reliable than DAD, and GC being limited to volatile and thermally stable compounds, coupling MS with HPLC seems to be a way to widen the range of compounds that can undergo MS [18].

Electron impact ionization (EI) is the ionization mode generally considered the gold standard for MS detection specificity. Nevertheless, atmospheric pressure interfaces of the electrospray or APCI type that have superseded all other types of interface/ionization sources for LC-MS, cannot accommodate electron ionization [18]. Marquet presented in a literature review [19], several mass spectral libraries using tandem MS (MS-MS), which unfortunately due to processing requirements are not compatible with GUS. A solution to this limitation could be MS-MS with data-dependent or information-dependent acquisition (DDA or IDA) that are self-adaptive MS-MS production scan modes where the mass-to-charge ratios, the intensity of which is above a given threshold, are selected at each unit time [20, 21].

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Experimental part

Material and method

Our study's purpose is to synthesize and analyze the toxicological methods used to determine the oral anticoagulants, described in the literature, in order to identify the optimal methods, taking into consideration the diversity of forensic casuistry.

The articles were searched in the electronic databases platforms PUBMED, RESEARCHGATE and WEB OF SCIENCE, using keyword combinations in English, such as "legal medicine, forensic, toxicology, anticoagulant, oral, intoxication, death, method, chromatography, spectrometry". Studies containing data on toxicological methods for determining oral anticoagulants were selected.

Results and discussions

In the electronic databases, for the period 1990-2019, 56 articles were identified that corresponded to the above mentioned search keywords. Of these, 28 were included in our study. We excluded the articles that were not lining up with the objective of our study, the update-type articles and the case studies.

Choice of biosamples

The concentration of drugs is relatively high in the urine, so this is the sample of choice for comprehensive screening or for identifying unknown drugs, and more importantly, to identify the metabolites of these drugs [22-24]. Blood, plasma or serum is the sample of choice in the case of quantitative analysis, but, when the concentration of the drug is very high, this sample can also be used for screening. This can be advantageous, as frequently at the autopsy, it is the only sample that can be collected [25-37]. Toxicological analysis of hair shafts allows the detection of chronic drug use, but this sample is controversial due to external contamination [38, 39]. In the literature, several studies have been identified that used tissues taken from the body as a sample, such as liver or brain [40-43].

Sample preparation

In the toxicological determination of oral anticoagulants it is very important to find a suitable preparation method for each type of sample. This may involve cleavage of conjugates, extraction and derivatization preceded or followed by clean-up steps. For LC-MS it is known that the method of preparation of the sample is the least complicated. Nonetheless, a more or less selective extraction is also important for LC-MS [44], especially in order to avoid ion suppression effects [44-48]. Cleavage of conjugates can be performed by fast acid hydrolysis or by gentle, but time-consuming enzymatic hydrolysis [49]. Isolation can be performed by liquid-liquid extraction (LLE) at a pH at which the analyte is non-ionized or by solid-phase extraction (SPE) preceded or followed by clean-up steps. Depending on the type of the sample, for SPE, it is necessary to apply a pretreatment, for blood and tissues some steps of deproteinization and centrifugation are necessary, whereas for urine, a simple dilution or centrifugation is sufficient [50]. Most recent studies have introduced a new extraction method, called solid-phase microextraction (SPME), which is based on adsorption of the analyte on a stationary phase coating to fine rod of fused silica [51, 52]. Derivatization steps are necessary, if relatively polar compounds containing carboxylic, hydroxy, primary or secondary amino groups are to be determined, and if electronegative moieties have to be introduced into the molecule for sensitive negative ion chemical ionization (NICI) detection [53].

Chromatographic and electrokinetic procedures

The techniques for toxicological determination of oral anticoagulants analyzed in the literature are summarized in Table 1.

Table 1
TOXICOLOGICAL PROCEDURES FOR DIRECT ORAL
ANTICOAGULANTS DETERMINATION

References	Procedures	Sample	Work-up	Detection mode	Limit of detection
[53]	GC-MS	Urine	EX-ME	EI	25 ng/ml
[54]	LC	Serum	SPE	FL, 318/390	1 ng/ml
[55]	LC	Serum	LLE	FL, 318/390	1-2 mg/g
[56]	LC	Liver	LLE-SPE	FL, 310/390	2-10 ng/g
[57]	LC	Serum	LLE	UV, 285	20-75 ng/ml
[58]	LC	Serum	LLE	DAD	25-100 ng/ml
[31]	LC	Hair	SPE	DAD	-
[29]	TLC	Urine	SPE, LLE	DDCI, FeCl ₃	50-200 ng/ml
[22]	TLC	Serum	LLE	UV scanner	200 ng/ml

GC-MS – gas chromatography–mass spectrometry, LC – liquid chromatography;

TLC – thin-layer chromatography; EX-ME – extractive methylation;
SPE – solid-phase extraction; LLE – liquid-liquid extraction;
EI – electron impact ionization;
FL – fluorescence detector / detection; DAD – (photo) diode array detector / detection;
DDCI – 2,6-dichloroquinone-4-chloroimide

Linearity

Baranowska et al. [22] evaluated the calibration curves at six concentration levels, and for each concentration triplicate injections were applied. In the study of Fourel et al. [25], five replicates of spiked drug-free dog plasma samples for four different concentrations were performed for a total of twenty extractions and the peak area was then used in conjunction with the calibration curve to determine the concentration in contaminated plasma samples. Di Rago et al. [27] evaluated the linearity based on comparisons of regression analysis with no weighting, $1/x$ and $1/x^2$. In the study of Gonzales et al. [29], for calibration purposes, the most suitable solution was chosen by analyzing the relative error values of the calibration standard concentrations and the linearity of the calibration curves. Denoos et al. [31] used the absolute beta-expectation tolerance limits, with acceptance limits at 20% expressed in the concentration units.

Precision and accuracy

Baranowska et al. [22] evaluated this criterion using standard mixture solution of the drugs under optimal conditions six times in one day for intra-day variation and three times a day on three consecutive days for the inter-day variation, expressed as the relative standard deviation. In the study of Fourel et al. [25], the intra-day accuracy was evaluated by performing five replicates of four spiked samples and the inter-day precision was evaluated three different days by performing two replicates of a spiked sample chosen in the middle of the concentration range. Di Rago et al. [27] evaluated the precision and accuracy of performing calculations using a full seven-point calibration curve and compared with one-point calibrations. To evaluate the accuracy and precision of the method, Gonzales et al. [29] analyzed the spiked samples with low, medium and high drug concentrations.

Specificity

To assess the specificity of the method, Baranowska et al. [22] used drug-free samples, and the retention times of endogenous compounds in the urine were compared with those of direct oral anticoagulants. Fourel et al. [25] tested the specificity for the 14 compounds with 20 blank plasmas, and there were no interferences to be found. Di Rago et al. [27] used post-mortem blood samples sent to the laboratory for toxicological analysis and whole blood samples collected from drug-free volunteers, in order to evaluate the method's specificity. Gonzales et al. [29] used only 6 blank plasma samples from different healthy volunteers.

Applicability of the method

Baranowska et al. [22] analyzed samples taken from hospitalized patients who were treated with direct oral anticoagulants. The concentrations of the drug ranged between $3.14\text{--}6.81 \mu\text{g mL}^{-1}$. Di Rago et al. [27] analyzed 397 post-mortem blood samples and the cases that were above the highest calibrator were diluted appropriately to concentrations within the calibration range. Gonzales et al. [29] obtained plasma samples from 19 different patients under treatment with acenocoumarol between 1 and 20 hours after the oral intake of the drug. Besides studying quantitation and confirmation transitions, their ratio was measured and compared with the ratio obtained for a standard solution.

Conclusions

Systematic toxicological analysis covers the basic drugs, which are also the most important toxicants. However, it is necessary that drugs such as oral anticoagulants should be taken into consideration and be analyzed in forensic cases.

After analyzing the literature, it is observed that liquid chromatography–mass spectrometry is the ideal method to detect more polar, unstable or low-dosed drugs, such as oral anticoagulants. Moreover, high-performance liquid chromatography (HPLC) method with ultraviolet (UV) detection has been developed and validated for acenocoumarol, which is the antithrombotic of choice in Romania.

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