ACCURACY OF THE SEQUENCE OF STEPS IN THE ANALYTICAL PROCEDURE IN FORENSIC TOXICOLOGY. THE SIGNIFICANCE AND PRIORITY FOR THE INTERPRETATION OF THE FINAL RESULT OF SINGLE CASES OF POISONING

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ABSTRACT: The interpretation of the final result of the quantitative analysis determination within the forensic toxicological analysis, is characterised by a significant level of uncertainty because of the unique, single object of study having a high biological variability. This natural uncertainty is exacerbated still further by the inadequate preservation of the material for analysis and the lack of awareness as to which of the analytical steps significantly affects the precision of the final result. This results in the interpretational incomparability of the scientific studies and forensic quantitative analyses.

KEY WORDS: Uncertainty of results; Quality assurance; Accuracy of analytical steps.

Problems of Forensic Sciences, vol. XLII, 2000, 111–121
Received 9 September 1999; accepted 16 May 2000

INTRODUCTION

The progress in analytical techniques associated with the increase in sensitivity and precision and the limitations facing forensic toxicologists caused by the variability of the biological material analysed, point to the purposefulness of considering the differences which appear in this aspect in two activities within toxicology: scientific studies and forensic analyses in individual cases of fatal poisoning. It seems also beneficial to consider the causes of the differences in individual stages of analysis and the ability of an expert to effect its improvement.

SOURCES OF UNCERTAINTY OF QUANTITATIVE ANALYSIS RESULTS

In general, the sources of deteriorating precision and the uncertainty as to the result of quantitative analysis, can be divided into natural causes and those caused by a human factor. The former are brought in by biological variability both between individuals and within an individual which stem from the genetic variability of the general population. Some other can originate from a dynamic, unpredictable in time, variability in the

distribution of a xenobiotic in an individual living organism, and in its specific organs, which will depend on its current physiological and pathological state [3]. It is thus those sources of the uncertainty which are either outside the control of the expert or impose significant limitations to him.

The second group of the causes of increased uncertainty results from the shortcomings in the stringent application of common standards for the preserving of the material for forensic analysis. The inadequate preservation of the biological material constituting the evidence in trial proceedings affect the credibility of the evidence in the case and thus has both legal and economic implications. It disqualifies the object of the analysis as material evidence thus rendering the expenditure on analyses devoid of purpose.

On the other hand, the flawed preservation of the biopsy material after the post mortem, which includes the mixing the organs in one container, the mixing of them with the contents of the alimentary tract, or the lack of an unequivocal anatomical assignment of them, all constitute a source of errors which can be blamed on someone and which adversely affect the certainty of the result. This can be prevented by following the relevant procedures, implemented under Quality Assurance Systems.

The factor which can, to a certain extent, decrease the uncertainty of the result, is the gathering by the expert of the maximum available information about a person who died of poisoning [4], although this factor should not be overstated because of the high number of variable parameters on which the concentration determined in organs depend, this element should not be overestimated [3].

An essential insurmountable barrier' which results in the interpretational uncertainty pertaining to the results of the quantitative analysis of a xenobiotic in a living organism, is its uniqueness and non-repeatability. The uniqueness of fatal poisoning, given all the limitations stemming from biological variability, does not permit, for formal reasons, any statistical estimation of the level of uncertainty of the result obtained, let alone the ability to relate it to the dose taken. The dispersion of the concentration is determined by the parameter of variance, according to a well-known mathematical formula:

$$S^2 = \frac{1}{N-1}\sum_i \left(x_i - x_m\right)^2,\ N \geq 2\,.$$

The parameter contained therein, which describes the number of samples – N, evidently determines the possibility of drawing any statistical conclusions about the dispersion, for samples with N greater or equal to 2.

The death from poisoning of a person cannot be repeated even one more time. Hence, the factor (quotient) preceding the sum 1/(N–1) becomes, for obvious reasons, undetermined.

For this reason, a forensic toxicologist has only the possibility to compare particular concentrations determined for a current case with a set of other cases of fatal poisoning with the same substance. One should aware that the groups (populations) compared with

each other are incomparable because of the number of elements in the population, and their heterogeneity. This situation, as well the biological variability itself result in an cause enormously broad range of lethal concentrations, exceeding even the orders of magnitude of the parameters measured, compared with the acute poisonings and the range of therapeutic concentrations [6]. The cause is aggravated still further by the fact that the set of data includes cases from various laboratories using the quantitative analysis method and determination procedures which do not give a basis for comparability of the results. This issue had already been raised by the community of forensic toxicologists, the reaction to which was to refer to the Cochrane Collaboration group, established in 1993 within the international medical community [11]. This resulted in a special supplement to the TIAFT Bulletin [12] and a subsequent appeal published in the Bulletin [13].

Still superimposed on all these issue is one serious problem. Even the best and the most versatile toxicological expert's report, cannot give any assurance that certain unknown factors (interactions) capable of contributing to the lethal outcome, have not been omitted [2].

As a rule, the data on the predispositions resulting from the genotype of the deceased (poisoned) person are also scarce. These regard such manifestations as the increased resistance of the organisms (also involving training or habituation) or its over-sensitivity. And the full knowledge of the human genotype still seems to be a remote prospect.

The hard-won data will thus always be of an approximate nature worsened still by the fact that the number of fatal poisonings with a specific xenobiotic is very limited, compared with the general population.

It is also noteworthy to observe that in the present data on fatal poisonings, there is a tendency among authors to manifestly desist from providing an upper limit and they often only give the lower limit in an approximate form (a sub-range) or "peak" concentrations.

ACCURACY OF THE MEASUREMENT PROCEDURE FOR QUANTITY ANALYSIS IN FORENSIC TOXICOLOGY

Within the commonly known analytical procedure which is characteristic for forensic toxicology, several stages can be distinguished from the viewpoint of their accuracy [7]. They are presented in Table I.

TABLE I. INTERPRETATIONS UNCERTAINTY FOLLOWS STEPS OF FORENSIC TOXICOLOGICAL ANALYSES OF SINGLE CASES

No.	Stage	Reason of uncertainty	Estimate	Source
1.	Uniqueness of case	Biological variability	High, unmeasured	N

2.	Information about circumstances	Status of organism physiology	High, unmeasured	N/H			
3.	Information about deceased	Genetics properties of deceased	High, unmeasured	N/H			
4.	Preparation of organ samples	Distribution of poison in organ	Notable, difficult	N/H			
5.	Status of investigated samples	Putrification, hydration changes	Notable, difficult	H			
6.	Choice, preparation of material	Impropriety of choice	Notable, difficult	H			
7.	Xenobiotics isolation	Classic	SPE	Classic	SPE	H	
	Classic method	Solid phase extraction	No repetition No continuous Process pH control Large loses	Holding of Stability of separation conditions N ł 2	RSD = 20-100%	RSD = 5-20%	H
a.	Deproteinisa-tion $(NH_4)_2SO_4$		N = 1		RSD = 10-40%		H
b.	Liquid-liquid Extraction		N = 1		RSD = 10-30%		H
c.	Purification of extract (TLC)		N ł 2		RSD = 10-30%		H
8.	Qualitative analysis	No certainty of detecting all poisons, metabolites, derivatives, their influence, interaction	Real, difficult to define	N/H			
9.	Quantitative analysis (HPLC)	N ł 2	RSD = 1 – 15%	H			

N – for reason of natural cause.
H – for reason depending on human activity.

The initial stage of the availability of data about the course of the poisoning, the deceased person, and the procedure for preserving the material for analysis, introduces inaccuracy and interpretational uncertainty which is difficult to assess, even approximately. Perhaps a remarkable element of uncertainty is introduced by the preparation of post-mortem material for analyses. It does not always permit a precise description of: the object of the analysis, the anatomical structure, consideration of the morphological diversity of the same tissue, the exclusion of cross-contamination, the definition of the effects of lapsing time and storage conditions, or the level of dehydration compared with the time of death [5].

The accuracy of analytical procedures can be approximated much better.

The isolation of the xenobiotic itself from an organ bears the error resulting from the heterogeneity of the tissue and the distribution of the xenobiotic. The homogenisation operation can prevent this uncertainty, providing that it is carried out with the entire organ. This situation occurs only very rarely, and the fragments of organs presented are firstly not always unambiguously assigned anatomically, and secondly, it is not possible to discern the location of the fragments because of a lack of discernible structures. At the same time, morphological differences may occur among them (eg. degeneration of tissues).

The problem of the diversity of the objects subject to analysis appeared most manifestly when the toxicological studies started on a material such as hair, which is so difficult to interpret from the viewpoint of its diversity.

The stage of isolation of the xenobiotic from the tissue is characterised by low accuracy, particularly in the case of liquid-liquid extraction preceded by deproteinisation still commonly used to determine organic compounds. The low repeatability of the whole of the process is caused by the aforementioned differences among the fragments of organs, stemming from the differences in losses incurred during the isolation from the matrix, as well as from the differences in the distribution ratio, caused by even slight changes in the pH of the extraction medium which modifies the dissociation equilibrium constant of the xenobiotic. The difficulty with maintaining the pH level during throughout the process at the strictly defined, pre-determined level results from the different buffer capacity of homogenates, stemming from the unique composition of the tissue samples in individual cases of poisoning, as well as from the variable degree of their decomposition.

It is also difficult to control the phenomenon of the transformation of xenobiotics during the isolation, and producing derivatives other than metabolites, or to distinguish the compounds produced by a living organism from those resulting from the chemical processes during the isolation process.

In studies conducted in Cracow, many years ago, the losses during the isolation process using these methods for the matrix alone and next in the process of deproteinisation and extraction were estimated. The standard deviation values were there approx. 5% for the process carried out on the homogenate alone and with the artificial addition of the xenobiotic – Table I [1]. On the basis of the study by the author [7], the losses during purification of extracts were determined by TLC at approx. 36% with the standard deviation of approx. 13%. Thus the combined losses before reaching the quantitative analysis process were approx. 70% and the standard deviation – approx. 15%, for the extraction of the same homogenate. The pilot studies by the author indicate that in adverse conditions, in analysing different fragments of the same organ, the final value of the relative standard deviation in analyses done for actual cases of poisoning, can reach even approx. 100% [6].

Losses do not occur at the stage of the determination proper, and the standard deviation, particularly in automated, instrumental methods is small compared with the preceding stages and falls in the range from 1 to 10%, and sporadically even below 1% – Table I [7].

It should be noted that although using the internal standard in the course of the quantitative analysis itself, leads to the increased accuracy of the result, this role will not be that significant in the process of isolation.

The constantly developed and increasingly popular method of isolation – SPE, reduces the number of stages in the analysis. Although the standard deviation fluctuates within 10–15% limits, but in controlled and repeatable conditions of simultaneous isolation and purification of a specific xenobiotic it allows one to limit the overall

uncertainty of this critical stage of the quantitative analysis to a reasonable range. Other advantages of this method are that the repetition of the procedure of isolation in order to assess its accuracy and repeatability does not require great effort, and that the entire process can be automated. With the classic method of liquid-liquid extraction this is extremely troublesome and, to-date, not widely applied. Due to the low repeatability it causes the inaccuracy of the entire analysis, irrespective of the undetermined accuracy associated with a single sample in an individual case (N = 1).

The requirement of the statistics indicate that in order to retain the criterion of credibility, in the event of a higher dispersion of results, using a higher number of samples becomes a necessity to keep the same confidence level. For this reason, this is yet another argument for the universal introduction of the SPE method. To-date, when the highest uncertainty pertains to the deproteinisation and liquid-liquid extraction, a single course of this process with simultaneously successive repetitions and much more precise stages of instrumental analysis, has clearly been in contradiction with the principles of evaluating the accuracy and with the rationality of their application.

Other analytical methods applied in toxicology, omitting the deproteinisation and extraction processes, such as FPIA in this application, are also characterised by their limited precision of 10–15%, with regard to tests in the serum. In attempts to apply them to quantitative analyses in post-mortem samples of blood and the supernatants of tissue homogenates, the dispersion of the results vary within wide limits and exceed more than 30% [8]. As in the SPE extraction method, the advantage of these lies in the number of stages, each of which decreases the accuracy of the final result. By eliminating the drastic conditions of deproteinisation preceding the liquid-liquid extraction, the uncertainty of the result caused by the transformation of the xenobiotic has been decreased.

DISSCUSSION AND CONCLUSIONS

The unrepeatability of the course of poisoning, its dependence upon a large number of variable parameters, which are not always known, and the impossibility of repeated analyses in a single case of fatal poisoning, causes the evaluation of the quantitative analysis of the level of xenobiotic in biological material being a very rough estimate. Relating the result in a single case to an extremely variable random sample limited in its number compared with general population, produces a limited level of accuracy, although sufficient for interpretation purposes.

The value of the accuracy of the result assumes more importance in the region of extreme concentrations and overlapping ranges of particular categories: therapeutic, toxic and lethal. The natural origin of the causes listed superimposes itself on a great number of variable parameters, which are difficult to calculate, such as, for example, the difficult to predict possibility of interactions with other undetected xenobiotics, or unknown features of the organism e.g. its exceptional susceptibility or resistance. The

evaluation of the results, particularly in this last category will be of an approximate and, necessarily, alternative nature.

A great importance to the retention of the credibility of the results and the strict definition of the unequivocal origin of samples, is attached to properly securing the material for analysis. This element and its improvement is within the reach and capabilities of the persons participating in the proceedings. Its importance is great, due to the significant effect on the interpretation of the final result and the categorical value of it.

The final accuracy of the measurement in the course of analysis and the interpretation of the final outcome is a resultant of the precision of the individual stages of the process and cannot be better that the most inadequate of them. This becomes a critical stage in the whole process. With a comparable precision of the sequence of stages of the quantitative analysis, similar numbers of repetitions for each of them should be retained.

The accuracy of the final result, expressed by the number of significant decimal places of the numerical value of the result also depends on the resultant accuracy of the entire quantitative analysis process. For the reasons listed above, it is purposeful to make a clear distinction, based on the criteria given, between the two incomparable activities of the forensic toxicologist: scientific studies and the forensic analysis in individual cases of fatal poisoning.

TABLE II. ESSENTIAL DIFFERENCES IN PROBLEM OF ACCURACY WITH ANALYTICAL RESULTS BETWEEN FORENSIC CASES AND SCIENTIFIC EXPERIMENTS IN FORENSIC TOXICOLOGY

FORENSIC CASES	DIFFERENCES ELEMENT	SCIENTIFIC EXPERIMENT
1	N	1, 2, ..., n
None	Repeatability of case	Practically unrestricted
Impossible – accidental state	Reconstruct of circumstances	Precise repetition of experiment
Quantity of organs limited	Repeatability of investigations	Unrestricted in following series
Maximal	Biological variability of material	Limited to minimum
Maximal various	Relation of result to population	Moderately homogenous
Anatomically differentiated object	Investigated organ	Anatomically defined fragments
Beyond control	Number of variable parameters	Controlled, minimum
Strong limited	Statistical estimation of result	Possible

In scientific studies, a toxicologist can minimise the natural variability of the studied population (race, sex, age, diet, physiological condition, absence of pathology, strict adherence to experimental conditions) and match the number to the statistical requirements which guarantee the proof of the presented thesis with an assumed level of confidence. The accuracy of the final result will also depend on the magnitude of the

effect which is to be proved or rejected. When conducting forensic analysis, the toxicologist has no way of affecting the selection of any of the above factors.

There is no justifiable need to attain such high levels of accuracy as those offered by modern analytical techniques, in the forensic analysis of singular cases of poisoning.

Its objective is, thus, to establish a cause-and effect relationship between the xenobiotic detected and the fatal outcome. This relationship does not have a categorical value and is not very precise.

TABLE III. COMPARISON OF ABILITIES AND ACCURACY REQUIREMENTS FOR RESULT OF TOXICOLOGICAL ANALYSIS IN CASE OF A SINGLE LETHAL POISONING

Abilities (limiting factor)	Estimate of influence on limiting of accuracy	Influence of man	The need for expert opinions concerning the cause of death to be accurate
Biological variability	Considerably	No influence	Comparison with very wide concentration range exceeding orders of measured values with blurred boundaries and overlapping with other ranges : lethal and toxic
Preparation of section material	Considerably	Limited	
Isolation of xenobiotic	Considerably	Limited	
Quantity analysis	The lowest	Maximum	

Expert positive opinion variants:
1. Quantified concentrations of xenobiotic in tissue occur in lethal poisoning cases and are relatively high... It is very high probable that the death involved poisoning by this xenobiotic.
2. Quantified... are occuring in cases of lethal poisoning and are relatively low and on the border band of ranges of lethal and acute poisoning concentrations. The lethal effect could be due to interaction...
3. Quantified... have not been till now present in cases of lethal poisonings. They occured in acute poisoning concentration ranges. The death would occur:a) as a consequence of the unusual sensibility of the deceased; b) a consequence of interaction with other xenobiotics; c) from other causes, but the presence of a xenobiotic could contribute to the cause of death.

Forensic toxicology has for the long time now has given up trying to establish a precise and calculable relationship between a dose and its effect. A certain exception, applied in practice but with many limitations, is the forensic toxicology of alcohol. The last attempt at finding such relationship with respect to medicines was made in 1985 [10]. It seems that the position based on the philosophical stream of determinism has become a thing of the past, although not without resistance, and Man will have again to recognise his limitations in relations to Nature and her impassable limits.

There are many indications that the Heisenberg's uncertainty principle is applicable and relates not only to a microcosm, but is one of the fundamental rights of Nature. An exemplary relationship between the increase in the precision of quantitative analysis results and interpretational uncertaintyis presented in Figure 1.

Perhaps the point has been passed where the continued increase in technological advances will not culminate in any further improvement in the interpretational evaluation of the result. Instead it will raise still new doubts.

The international standards of the Quality Assurance System of the ISO 9000 series and others derived therefrom envisage the situation of differentiated requirements regarding processes to serve actual needs. This paper serves to support the notion that there are distinct differences between scientific studies and forensic analysis in individual cases of fatal poisoning.

The ISO 8402 standard [9] envisages in section 2.2 the concept of grade as a category assigned to the objects of the same functional purpose but with different quality requirements (section 2.3 of the standard).

The author of this paper does not argues in favour of abandoning the accuracy of the determinations and the quantitative analyses made during them, but advocates a complete stands for a full awareness of the accuracy and the need for it at every stage of the analytical process. The need for such an awareness should show in the maintenance of all the criteria which affect the accuracy of the results, and in the provision of the numerical values of these accuracy criteria for individual stages of the entire process.

The total accuracy of results in single poisoning cases does not have to be the highest, it can be even very low if it is sufficient, but it always must be known!

Similarly, in the court expert's opinion, the substantive value of the final conclusion about the cause of death should have reasonable foundations, based on the provided criteria for the accuracies of the stages and on the final precision of the result.

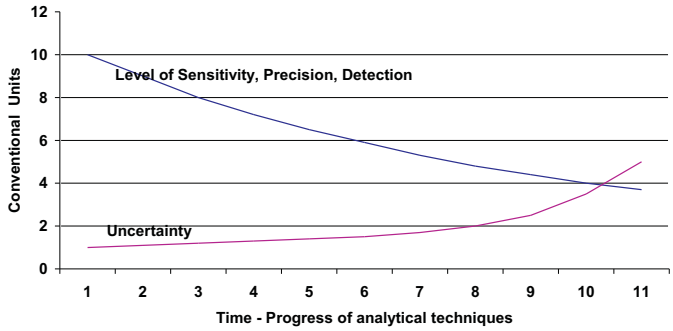

Fig. 1. An exemplary relationship between the increase in the precision of quantitative analysis results and interpretational uncertainty.

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