Journal of Chromatography, 489 (1989) 3–9
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4597

MOLECULAR SPECTROSCOPY IN FORENSIC RESIDUE ANALYSIS: A GENERAL OVERVIEW OF RELIABILITY, APPLICABILITY AND COST EFFECTIVENESS

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SUMMARY

Classification of an analytical result for forensic purposes is briefly discussed, in addition to mandatory specificity and limits of appropriate analytical methods. Direct information on the molecular structure of the analyte is in general more reliable than indirect information. Direct information is obtained from molecular spectroscopic methods, in contrasts to chromatographic or immunochemical methods, which provide only indirect information. The cost effectiveness ratio as calculated per analyte is indicated for various analytical techniques.

INTRODUCTION

In Court the Judge generally only wants to know the truth and nothing but the truth. If this truth has to be based solely on the result of a chemical residue analysis, a situation that frequently happens, the analyst in court is faced with the problem of reducing all the information regarding the presence of the substance being measured (the analyte) finally into a single factor: positive or negative [1]. A positive result means the presence of the analyte in the sample is proved according to the applied analytical procedure and a negative result means a non-positive result. Also, a negative result does not prove that the analyte is absent from the sample.

For regulatory purposes within the European Communities (EC) quality criteria have recently been defined for chemical residue analyses, especially qualitative ones [2]. For positive results all criteria specified for the individual method of analysis have to be fulfilled. All criteria are focused on the prevention of false-positive results [3,4].

0378-4347/89/$03.50

SPECIFICITY AND RELIABILITY

The most important characteristic of a qualitative method of analysis is the reliability of its identification of the analyte. As the specificity of a method is the ability to distinguish between the analyte and all other substances, it is obvious that specificity is the most relevant quality parameter of a qualitative method, especially for reference, legislative and/or forensic purposes [5]. Specificity is the product of the selectivity of all the individual steps of the analytical procedure. If the analyte is a residue of a xenobiotic substance, such as some banned anabolic agents, present in the sample in trace amounts only, then the limit of applicability of a method is also very important as defined by its limit of detection and the sensitivity [2].

INFORMATION AND LIMITS

For reliable identification of a residue, detailed information on the molecular structure of the analyte is essential [6]. The number of analyte molecules present in the analytical system and the sensitivity of the final detection step, however, primarily determine the limit of applicability which, in general, is appreciably higher than the traditional, statistically defined limit of detection.

The total information on the molecular structure of the analyte is the sum of the information derived from each individual analytical step of the method. An-

TABLE I

RANKING OF VARIOUS KINDS OF MOLECULAR SPECTROSCOPY ACCORDING TO DECREASING DIRECT STRUCTURAL INFORMATION CONTENT OF THE FULL SPECTRUM

Spectroscopic technique	Abbreviation	Information on analyte structure from spectrum	Approximate applicability limit (mass fraction)[a]
Infrared spectroscopy	IR	'Fingerprint' of analyte Functional groups Intermolecular interactions	100 ppb
Nuclear magnetic resonance spectroscopy	NMR	Chemical and spatial position of (e.g.) hydrogen atoms in analyte	10 ppm
For hydrogen: proton magnetic resonance spectroscopy	^{1}H NMR	Intermolecular interactions	
Mass spectrometry	MS	Ion fragmentation pattern of analyte	1–100 ppt
High-resolution MS	HRMS	Element formula of analyte and fragment ions	
Low-resolution MS or mass-selective detection	MSD	Nominal mass of ions	
Ultraviolet and visible light spectrophotometry	UV-VIS	Multiple chemical bonds in analyte Conjugation of chemical bonds Intermolecular interactions	100 ppb

[a]Mass fractions: ppm = 10^{-6}; ppb = 10^{-9}; ppt = 10^{-12}.

TABLE II

SUMMARY OF STATEMENTS CONCERNING SPECIFICITY AND DIRECT VERSUS INDIRECT INFORMATION FOR THE IDENTIFICATION OF BANNED SUBSTANCES IN FORENSIC RESIDUE ANALYSIS

Specificity is the most important characteristic of qualitative methods to identify an analyte. Identification of an analyte as a chemical compound should be based on information about the molecular structure. Direct information is more reliable than indirect information.

Direct information	Indirect information
Obtained by: molecular spectroscopy	Obtained by: chromatography (immuno)affinity
↓	↓
Interaction between analyte and electromagnetic radiation	Interaction between analyte and chromatographic or (immuno)active agents
Authentic standard analyte is not needed	Authentic standard analyte is always needed
↓	↓
Absolute qualitative methods	Relative qualitative methods

alytical steps based on molecular spectroscopy all provide direct, more or less detailed information on the structure of the analyte. Frequently used selective analytical steps based on chromatography or immunoaffinity, however, provide only more or less general indirect information. For correct identification, relevant direct information on the molecular structure of the analyte is always more specific and hence more reliable than indirect information. Further, authentic standard analytes are always needed with chromatographic or immunochemical methods, in contrasts to molecular spectroscopic methods, where such standards are not strictly needed.

Common molecular spectroscopic techniques used for identification purposes, in order of decreasing general information content of the full spectrum, are: infrared (IR) spectroscopy [2,3,7], nuclear magnetic resonance (NMR) spectroscopy with proton magnetic resonance (^{1}H NMR) for hydrogen atoms, mass spectrometry (MS) in high-resolution, multi-ion (HRMS) [8] and low-resolution, mass-selective detection (MSD) [9–11] modes and finally ultraviolet (UV) [12] and visible (VIS) light spectrophotometry. The kind of direct structural information derived from the spectra of the analyte is summarized in Table I.

Approximate applicability limits of the various spectroscopic methods can also be indicated by assuming that the analyte is fully recovered during the analytical procedure and that the sensitivity of the spectral detection step is not influenced by co-extracted sample ('matrix') components [13]. Therefore, for residues of anabolic agents in a 1-g portion of sample, this limit for the various 'full spectrum' detectors as such or combined on-line with gas chromatography (GC), thin-layer

chromatography (TLC) and/or high-performance liquid chromatography (HPLC) is indicated, in order of decreasing analyte content (mass fraction), as follows: ^{1}H NMR, 10 ppm; GC–IR, IR, HPLC–UV, TLC–UV, 100 ppb; GC–MSD, 100 ppt; GC–MS, 10 ppt; and MS–MS, 1 ppt. This means that in general only mass spectrometry is applicable for the identification of analyte residues in the ppt range, a range to be expected in the edible parts of slaughtered animals treated with anabolic agents. In Table II the statements made so far are summarized.

COST AND BENEFIT

Another kind of 'applicability limit' to be kept in mind is the cost of an analysis. Routine GC–MS methods are very competitive with other methods such as TLC or (radio)immunoassays [(R)IA], especially if multi-residue trace analysis is needed [2]. With GC–MS up to 100 analytes can be detected and identified in a single analytical run, whereas with TLC in practice not more than ten analytes and with (R)IA only a single analyte can be detected. In The Netherlands, for continuous routine analyses this places the cost per analyte for GC–MS typically in the range Dfl. 3–4, for TLC in the range Dfl. 20–30 and for (R)IA in the range Dfl. 30–50, as calculated by commercial laboratories (1 Dfl corresponds to about US$ 0.5). More details on these costs are summarised in Table III.

For final regulatory identification of a single xenobiotic analyte on an ad hoc basis, however, completely different costs apply, such as Dfl. 900–1100 for HPLC–GC–MS and Dfl. 300–500 for an HPLC–RIA immunogram [14,15]. These are also the costs that one has to keep in mind for reference purposes.

After six years of comparative research with hyphenated techniques [2,6,9] such as HPLC–GC–MS and HPLC–(R)IA methods for the determination of anabolic residues in excreta [16] we reached the conclusion that GC–MS is to be preferred for reference purposes on the basis of its inherent high specificity and its cost/benefit ratio in multi-residue analysis.

TABLE III

INDICATION OF COSTS (IN Dfl.) IN THE NETHERLANDS FOR THE ANALYSIS OF RESIDUES OF ANABOLIC AGENTS IN URINE AND MEAT OF SLAUGHTER ANIMALS AND/OR DOPING AGENTS IN SPORTSMEN AND RACEHORSES AS CALCULATED BY COMMERCIAL LABORATORIES

Method	Analytes per sample	Cost		Samples per analytical run
		Per sample	Per analyte	
Continuous series of samples				
RIA	1	30– 50	30–50	5–30
TLC	10	200– 300	20–30	3–10
GC–MS	100	300– 400	3– 4	10
Ad hoc regulatory identification of a single analyte in a single sample				
HPLC–RIA immunogram	1	300– 500		1
HPLC–GC–MS	1	900–1100		1

ANALYTICAL STRATEGY

In our Institute we now combine the advantages of chromatography, immunochemistry and mass spectrometry in a general analytical approach to identify residues of anabolic agents such as nortestosterone (NT), methyltestosterone (MT), medroxyprogesterone (MP), trenbolone (TB) and/or diethylstilboestrol (DES) in various tissues of slaughtered animals at levels down to 100–200 ppt in a 1-g sample [17, 18]. This is achieved by a combination of enzymatic proteolysis of the tissue, solid-phase extractive defatting of the digest and isolation and concentration of the analytes by multi-immunoaffinity chromatography (MIAC) over anti-analyte antibodies immobilized on Sepharose. After derivatization of the eluted purified and concentrated analytes, final identification is performed by combined GC–MS [9] or GC–MSD [10].

FORENSIC CHEMOMETRY

The reliability of a qualitative method can be characterized by the probability of its creating a false-positive result, which means the identification of a compound other than the analyte as being this analyte. The regulatory analytical strategy applied in The Netherlands during 1982–1987 [19] for the control of DES residues in bovine urine consisted of a screening with combined column chromatography–RIA [16], confirmation by combined HPLC–GC–HRMS [8] and (if applicable) contra-expertise by combined HPLC–GC–multi-ion MSD [11]. The three completely independent analytical steps in this strategy were performed in different laboratories. On the basis of various Dutch comparative inter-laboratory studies, the probability of false-positive results of each step was estimated conservatively to be less than 1:100 for the screening, less than 1:1000 for the confirmation and less than 1:1000 for the contra-expertise. Therefore, the overall probability for false-positive results is estimated as much less than 1 out of 10^8 positive results. Whether this very small but non-zero chance is acceptable for regulatory or forensic purposes should ultimately be decided in the Courts. However, appropriate scientists have to provide the necessary experimental data on the reliability of the various methods of residue analysis.

CONCLUSIONS

Data on the reliability of methods of analysis should be available before any forensic 'residue trial' starts and should be based on strictly controlled cooperative validation programmes [5] according to good laboratory practices [20] and international guidelines making use of well defined samples [21]. Such programmes will be very expensive and time-consuming! However, it should be a challenge for all analysts involved to conduct such programmes worldwide in close cooperation in an intelligent way and as 'low budget' as possible. For this purpose the recent and forthcoming EC guidelines [2,6] provide an excellent and practical quality framework. Finally, the use of methods based on molecular spectroscopy has already been recommended for EC reference identification purposes

by a group of ten independent analytical experts from laboratories in five member states [6]. It is stated that: "Methods used for reference purposes should preferably be based on molecular spectroscopy (e.g., MS, NMR, IR, UV) providing direct information about the molecular structure of the residue. Special attention should be paid to methods based on mass spectrometry (MS). Inter-comparative trials should, if possible, always include MS, so that the applicability of this technique can be evaluated and assessed in comparison with other methods. In cases where MS has already been tested for certain substances by other organizations, the results of such tests should be taken into account. If validated MS methods are available, they should be the reference methods of choice".

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