

Reprint from

RECENT ADVANCES
IN DOPING ANALYSIS
(11)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

Sport und Buch Strauß, Köln, 2003

P. VANEENOO, F.T DELBEKE:
Chromatographic and Mass Spectrometric Criteria in Doping and Related Areas
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping
analysis (11). Sport und Buch Strauß, Köln, (2003) 149-159

Chromatographic and mass spectrometric criteria in doping and related areas

Doping Control Laboratory, Ghent University, Salisburylaan 133, B-9820 Merelbeke,

1. Introduction

Gas chromatography-mass spectrometry (GC-MS) and liquid-chromatography (LC-MS) are hyphenated techniques that can provide unequivocal evidence of the presence of a prohibited substance in doping and residue analysis (1).

According to ILAC-G7, the identification of a prohibited substance must result from a direct comparison with a reference material analysed in parallel or series with the test sample using a mass spectrometric technique. Moreover, there must be written laboratory criteria of what constitutes “a match” (2). Indeed, due to instrumental and matrix parameters, small fluctuations in mass spectrometric and chromatographic data are observed. Hence, to identify a substance beyond any reasonable doubt with chromatography-mass spectrometry, a number of criteria need to be established.

The organisations shown in Table 1 have set up criteria for chromatography and mass spectrometry for related fields of analysis. In this paper, these criteria will be compared and the positive aspects as well as points open for improvement for each set of regulations will be discussed and illustrated with examples.

Table 1. Regulatory authorities and field of analysis

| Organisation | Field | ref |
|---|-----------------------|-----|
| European Council | Animal drug residue | (5) |
| Food and Drug Administration (USA) | Animal drug residue | (6) |
| Association of Official Racing Chemists | Equine doping control | (4) |
| International Olympic Committee | Human doping control | (7) |
| World Anti-Doping Agency | Human doping control | (8) |

Because of the unique specificity and selectivity associated with chromatography coupled to mass spectrometry, all of these organisations regard this hyphenated technique as the sole technique that is suitable on its own for use as a confirmatory method (2, 4, 5, 6, 7, 8). The European Council is the only organisation that allows the use of a combination of independent techniques to confirm the identity of a substance (5). In this case a minimum number of identification points (IP) associated with each technique needs to be obtained.

The rules of the AORC are minimum performance criteria (4), i.e. a laboratory can choose to use more stringent criteria. The rules of the European Union (EU) for residues in animals and animal products (5), the Food and Drug Administration (FDA) for veterinary residues (6) and the IOC (7) however, should be regarded as universal identification criteria. The criteria for mass spectrometry and chromatography in the technical document of WADA (8) are intended as “an example of acceptable criteria”. Hence, each laboratory can choose its own criteria, if it can justify their use. However, in the long term it seems advisable that all doping control laboratories should use the same criteria for reasons of harmonisation. Because the regulations of WADA are only intended as an example, it seems worthwhile for doping control laboratories to take other regulations under consideration as well when setting up their own criteria. Moreover, it seems impossible to create one set of rules that will be ideal for each specific case. The importance and practical experience of the scientist interpreting chromatographic and spectrometric data is one of the most important factors in the overall process and will probably remain so even if technology evolves. This paper therefore merely gives some examples of the different aspects an analyst needs to consider and help the analyst in making his/her decision.

2. Chromatography

The retention time of a substance can be regarded as characteristic. Hence, the retention times of the substance in a sample and of the reference compound should be comparable, if analysed under identical conditions. The retention time (RT) or relative retention time (RRT) of a substance can be helpful in differentiating between substances with similar mass spectra. The use of RRT instead of RT has been advocated to enhance reproducibility (9). Even in combination with mass spectrometry, retention time can be of great significance in discriminating between different substances, especially isomers which have very similar mass spectra. (e.g.

bis-TMS derivatised 19-norandrosterone and 19-noretiocholanolone, the two major urinary metabolites of nandrolone in humans).

Nowadays, capillary GC and HPLC are the most frequently used chromatographic techniques. As shown in Tables 2 and 3, several regulating authorities have set up criteria for these chromatographic techniques.

Table 2. Maximum tolerance for deviations in retention time (RT) and relative retention time (RRT) between a substance in a sample and the reference compound for gas chromatography (GC) according to regulatory body

| Regulatory Authority | Maximum allowed deviation |
|----------------------|--|
| AORC (4) | <ul style="list-style-type: none"> • 1 % for RRT • 1 % or 6 s for RT (whichever is the greatest) |
| EU (5) | <ul style="list-style-type: none"> • 0.5 % for RRT |
| FDA (6) | <ul style="list-style-type: none"> • 2 % for RT • 2 % for RRT |
| IOC (6) | <ul style="list-style-type: none"> • 1 % for RRT • 1 % for RT |
| WADA (8) | <ul style="list-style-type: none"> • 1 % or 12 s for RT (whichever is the smallest) |

Table 3. Maximum tolerance for deviations in retention time (RT) and relative retention time (RRT) between a substance in a sample and the reference compound for high-performance liquid chromatography (HPLC) according to regulatory body

| Regulatory Authority | Maximum allowed deviation |
|----------------------|---|
| AORC (4) | <ul style="list-style-type: none"> • 2 % for RRT • 2 % or 12 s for RT (whichever is the greatest) |
| EU (5) | <ul style="list-style-type: none"> • 2.5 % for RRT |
| FDA (6) | <ul style="list-style-type: none"> • 5 % for RT • 5 % for RRT |
| IOC (7) | <ul style="list-style-type: none"> • 1 % for RRT • 1 % for RT |
| WADA (8) | <ul style="list-style-type: none"> • 2 % or 24 s for RT (whichever is the smallest) |

Some authorities differentiate between GC and HPLC based on practical experience. Indeed, capillary gas chromatography has shown to provide a higher reproducibility in retention times than HPLC (10). Therefore stricter criteria can be applied.

It is clear, that the tolerance for differences in retention time expressed as an absolute difference by the AORC is only meaningful for rapidly eluting substances, i.e. with a retention time smaller than 10 minutes (4). In contrast to the AORC, WADA uses the combination of an absolute deviation and percentage tolerance to limit the difference in retention time for lately eluting substances, i.e. more than 20 minutes (8). Although from a theoretical and practical point of view neither of these situations (extremely fast/late eluting substances) is ideal for "classical substances" analysed in doping control, it is clear that the precautions made by both organisations are justified. Peptides, however, typically elute at retention times around 20 min (11, 12)

3. Criteria for mass spectrometry

Mass spectrometry is a detection technique based upon ionisation and fragmentation of molecules in an electric field. The fragmentation pattern of a substance (the relative abundance of the mass to charge ratios of the different fragments) can be regarded as a fingerprint of a substance.

Mass spectra consist of a series of m/z -values with different relative abundances, and, although a mass spectrum can be regarded as characteristic for that substance, different substances, especially isomers, can have similar mass spectra.

The diagnostic information in a mass spectrum is dependent on the detected m/z -values, observed mass differences, MS/MS transition, abundance ratios, isotopes, molecular ion, spectral pattern (15) and the mass spectrometric technique.

For each of these factors, criteria need to be developed that should be met to unequivocally identify a substance.

Loss of some functional groups (e.g. HOTMS, CH₃, H₂O,...) is less specific than the loss of other groups. Therefore most regulatory bodies have attempted to define which ions should be regarded as characteristic, by defining the term "diagnostic ion" (5, 6, 8) or by defining criteria for ions that can be used for matching (4). However, a clear definition of this term is not straightforward. Isotopic ions for example can be

diagnostic in biological samples, if derived from elements like Cl or Br, while C isotopes are far less diagnostic (17).

The loss of a HOTMS group e.g. is common for O-TMS derivatised substances. Hence it is most often regarded as having low specificity. However, loss of such a group also proves that the substance is derivatised and therefore provides evidence of the presence of a functional group (-OH or C=O in case of enolisation) in a molecule.

Because the relative abundances of the different ions in the mass spectrum of a substance can be influenced by instrumental parameters and matrix, the IOC has stated that the concentration of the reference substance should not differ by a factor 5 or higher than in the test sample (6, 13). Although no ratio is explicitly specified, this recommendation is followed by the AORC (4). Moreover, it should be noted that retention time behaviour is also concentration dependent in capillary GC (14). Therefore it seems necessary that the concentration ranges in the sample and reference are similar, to minimise the risks for false negative results.

As shown in Table 4, the minimum number of ions for unequivocal identification is 3 according to the AORC, IOC, WADA and FDA and 4 according to the EU in the full scan or partial scan mode with single MS (4, 6, 7, 8, 5). Although several organisations have defined full/partial scan, none of these organisations have identified a minimum scan range in their definition. The FDA states that all structurally specific ions should appear in the scan range (6). Although this definition depends on the interpretation of the term “structurally specific”, it is a recommendable extension of the definition of partial/full scan mass spectrometry. It is clear that such a definition is indeed extremely difficult. Several parameters need to be considered when setting up the scan range. Back ground ions at low mass in GC-MS (e.g. N₂ at m/z 28 and CO₂ at m/z 44) need to be avoided. However, this might also lead to low sensitivity for some amino compounds that exhibit a base mass peak at m/z 44 [C₂H₆N]⁺ and very low abundances at higher masses (e.g. amphetamine, nortriptyline) (14). Moreover, when quadrupole mass analyzers are used, the scan range affects chromatographic resolution, i.e. a wider scan range results in lower resolution because of the lower scan speed.

In contrast to full/partial scan, the definition of selected ion monitoring (SIM) is more straightforward. SIM is a technique that monitors a limited number of ions. Hence, the

number of data points for comparison is limited and the risk of misidentification could substantially increase. This was taken into account by some authorities (AORC, FDA) and hence smaller tolerances are applied for the ion abundances (6, 4) or the number of ions (4) by these organisations (Table 4).

Ions used for identification should have a signal to noise ratio (S/N) exceeding 3:1 (4, 5, 6, 8) and ions present in the reference spectrum with an intensity exceeding 10% should be included in the identification process (4, 5, 6), according to most regulatory authorities. However, the AORC is the only regulatory body that has determined that extraneous ions present in the test spectrum should not exceed 20% (4). If these ions are present at an abundance of at least 20% they should be treated as diagnostic and should be included for comparative purposes.

Background subtraction of spectra is often used. However, to unequivocally identify an unknown substance, it is necessary that background subtraction is performed consistently throughout a batch of samples (4, 5, 8), a procedure that is unexpectedly not prescribed by all regulatory bodies.

With respect to tolerance levels, differentiation between EI and CI techniques is only made in one document (5), while others specifically state that the rules are applicable for all common ionisation techniques, irrespective of the mass analyzer (4). However, differentiation between GC/MS and LC/MS is made by most authorities (Table 4).

There is general agreement that if the number of ions present in a mass spectrum is not sufficient for identification, additional techniques (ionisation, derivatisation, MSⁿ) can be used. However, each mass spectrum should at least contain two ions, i.e. one ion ratio in the case of single MS. WADA has clearly indicated the importance about the independence of the techniques used in such a case (8). The combination of two MSⁿ events generating different product ions can be useful in some cases (5, 17).

High resolution mass spectrometry (HRMS) can provide additional structural information based upon specific mass measurements that may reveal atomic composition. However to attain sufficient resolution this instrument needs to be operated in SIM. Hence for HRMS specific regulations have been provided by some bodies (5, 6), while for other organisations the same rules as for low resolution mass spectrometry apply.

For MS^2 spectra, the product ions are generated after fragmentation of a precursor ion. Therefore, the structural information of a product ion should be more specific than for ions obtained in single MS. Hence, the criteria for product ions are less tight or the required number of product ions for identification is lower (Table 4). However, the specificity of some fragmentations even in MS^2 can be relatively low. The ions at m/z 315 and m/z 225 in the product spectra of TMS-derivatised 19-norandrosterone and 19-noretiocholanolone, for example are generated by the loss of HOTMS, a common fragmentation pathway for O-TMS-derivatives. In MS^n ($n \geq 2$) spectra, the precursor ion is less specific than the product ions because it is by definition present in the product spectrum if the collision energy is sufficiently low. This has been recognised by several regulatory bodies by requiring a specific relative abundance range (10-80%) for the precursor ion (4) or attributing a lower number of identification points (IP) to a precursor ion (5), i.e. 1 IP to a precursor ion and 1.5 IP to a product ion. However, André *et al.* indicated that the identification point system of the EC automatically attributes 1 IP to the precursor ion in MS^n (16), whether the precursor ion is present in the product spectrum or not. Hence, the number of ions and the herewith connected number of ratios of ion abundances that need to be present in the MS^n -spectrum according to the EC regulations is smaller than for the other organisations. In contrast to the regulations for SIM, where most regulatory bodies apply more stringent criteria as compared to full scan MS, the AORC is the only organisation that applies stricter criteria (minimum 4 ions) for selected reaction monitoring (SRM) as compared to full scan MS^2 .

Although the minimum number of ions required for matching is generally well defined, some regulations can be interpreted in such a way that they allow for “ion shopping”, i.e. selection of the best matching ions out of all ions present in a mass spectrum. The AORC is the only organisation that has set up rules that completely rule out this possibility. Because WADA also states that when SIM is used, any of the ions present in the spectrum shall be within the tolerance limits for ion abundances, this organisation has ruled out such a practice in this case as well. Nevertheless, WADA’s regulations for full scan mass spectrometry are not as tight as for SIM and allow for ion shopping.

Library search algorithms can be applied under certain circumstances according to some organisations (8), while others explicitly prohibit its use (6).

Although all of the criteria described in this paper aim at preventing misidentification of a substance, chromatographic and mass spectrometric analysis of complex matrices for a wide range of substances as will still largely depend on the analysts' interpretation.

4. Conclusion

It is clear that differences in criteria exist between regulatory bodies. Some of these differences are due to specific requirements within the application field. Nevertheless it seems that all of the regulations try to achieve a common goal: to clearly identify what constitutes a positive finding.

Because the rules of WADA are non-binding criteria, it might be advisable to take the regulations of other regulatory bodies into consideration where appropriate.

Finally, a harmonisation among the different regulations, incorporating the strong points of each set of rules would be beneficial.

5. Acknowledgements

The financial support from the Flemish Ministry of Health is greatly acknowledged.

6. References

1. P. Marquet, G. Lachâtre (1999). Liquid-chromatography-mass spectrometry: potential in forensic and clinical toxicology. *J. Chromatogr. B.*, 93-118.
2. International Laboratory Accreditation Cooperation, Accreditation requirements and operating criteria for horse racing laboratories, ILAC-G7, 1996. <http://www.ilac.org/>
3. B.K. Logan, D.T. Stafford, I.R. Tebett, Moore, C.M. (1990). Rapid screening for 100 basic drugs and metabolites in urine using cation-exchange solid-phase extraction and high performance liquid chromatography with diode-array detection. *J. Anal. Toxicol.*, **14**, 154-159.
4. Association of Official Racing Chemists (2002). AORC guidelines for the minimum criteria for identification by chromatography and mass spectrometry. MS Criteria Working group – 19 June 2002 Version. Internal communication to AORC members.

5. Council Directive 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Communities*, L 221, 8-36.
6. US Department of Health and Human Services. Food and Drug Administration. Center for Veterinary Medicine. Guidance for Industry. Mass spectrometry confirmation of the identity of animal drug residues. <http://www.fda.gov/guidance/pulished.htm>
7. IOC, the International Olympic Committee (1998). Analytical criteria for reporting low concentrations of anabolic steroids. Internal communication to IOC accredited laboratories, Lausanne, Switzerland.
8. WADA, The World Anti-Doping Agency (2003). International standard for laboratories, version 3.0. <http://www.wada-ama.org/>
9. M. Bogusz (1999). Hyphenated liquid chromatographic techniques in forensic toxicology. *J. Chromatogr. B*, 733, 65-91.
10. M. Bogusz, M. Wu (1991). Standardised HPLC/DAD system based on HPLC retention index values of selected acidic and basic drugs measured in the 1-nitroalkane scale. *J. Anal. Toxicol.*, 15, 188-197.
11. A.J. Link, J. Eng, D.M. Schieltz, E. Carmack, G.J. Mize, D.R. Morris, B.M. Garvik, J.R. Yates (1999). Direct analysis of protein complexes using mass spectrometry. *Nat Biotechnol.* 17, 676-82.
12. C.S. Spahr, M.T. Davis, M.D. McGinley, J.H. Robinson, E.J. Bures, J. Beierle, J. Mort, P.L. Courchesne, K. Chen, R.C. Wahl, W. Yu, R. Luethy, S.D. Patterson (2001). Towards defining the urinary proteome using liquid chromatography-tandem mass spectrometry. I. Profiling an unfractionated tryptic digest *Proteomics*, 1, 93-107
13. R. Ventura, L. Damasceno, M. Farré, J. Cardoso, J. Segura (2000). Analytical methodology for the detection of β 2-agonists in urine by gas-chromatography-mass spectrometry for application in doping control. *Analytica Chimica Acta*, 418, 79-92.
14. A. Poletini (1999). Systematic toxicological analysis of drugs and poisons in biosamples by hyphenated chromatographic and spectroscopic techniques. *J. Chromatogr. B.*, 733, 47-63.

15. R. Bethem, J. Boison, J. Gale, D. Heller, S. Lehotay, J. Loo, S. Musser, P. Price, S. Stein (2003). Establishing the fitness of purpose of MS methods. *J. Am. Soc. Mass Spectrom.*, **14**, 528-541.
16. F. André, K. De Wasch, H. De Brabander, S. Impens, L. Stolker, L. van Ginkel, R. Stephany, R. Schilt, Courtheyn, Y. Bonnaire, P. Fürst, P. Gowik, G. Kennedy, T. Kuhn, J.-P. Moretain, M. Sauer. (2001). Trends in the identification of organic residues and contaminants: EC regulations under revision. *Trends in Analytical Chemistry*, **20**, 435-445.
17. P. Van Eenoo, F.T. Delbeke (2002). Detection of inhaled clenbuterol in horse urine by GC/MS-MS. *Biomed. Chromatograph.*, **16**, 475-481.

Table 4. Criteria for mass spectrometry according to regulatory body

| | Full-scan GC/MS | SIM GC/MS | MS/MS |
|------|---|---|--|
| FDA | n 3 tolerance 20% abs | n 3 tolerance 10% abs 4 15% abs >3 (isotopes, non-specific loss) 10% abs | n 2 tolerance 10% abs 3 20% abs |
| AORC | 3 10% abs or 30% rel | 4 tighter criteria should be applied | 3 (may include precursor) 20% abs or 40% rel |
| EU | 4 Relative abundance > 50% 20-50% 10-20% ≤ 10% EI-MS % rel 10 15 20 50 | no specific criteria | type n MS ² 1 M, 2 D 2 x MS ² 2 M, 1 D each MS ³ 1 M, 1 D/M, 2 D Relative abundance > 50% 20-50% 10-20% ≤ 10% MS ⁿ , CI-MS, LC-MS (% rel) 20 25 30 50 |
| IOC | 3 5% abs or 20% rel | no specific criteria | 3 (may include precursor) 10% abs or 25% rel |
| WADA | 3 Relative abundance > 50% 25-50% ≤ 25% EI-MS EI-MS 10% abs 20% rel 5% abs | 3 Relative abundance > 50% 25-50% ≤ 25% EI-MS EI-MS 10% abs 20% rel 5% abs | 3 Relative abundance > 50% 25-50% ≤ 25% MS ⁿ , CI-MS, LC-MS MS 15% abs 25% rel 10% abs |

M: precursor ion, D: product ion, rel: relative, abs: absolute