9

LC-MS in doping control

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Introduction

Definition of doping

Doping analysis comprises a diversity of substance classes with different pharmaceutical and chemical properties. Therefore, the discussion of the suitability of liquid chromatography-mass spectrometry (LC-MS) in doping analysis needs to distinguish various categories.

According to its formal definition, a doping violation in sports can be caused by various events, e.g.:

- the detection of a prohibited substance or metabolites or markers of that substance (as defined by the recent document [1] of the World Anti-Doping Agency [WADA]) in the athlete's specimen
- the use of prohibited substances or methods
- possession or trafficking prohibited substances
- refusing without compelling justification to submit a sample.

This definition is clearly legally motivated and does not support the discussion of technical issues.

The number and classes of prohibited substances is very complex in human sports, where selected stimulants, narcotics, hormones, β_2-agonists, anti-oestrogenic agents and diuretics are covered. The situation becomes even more confusing if the term 'doping' is extended to animal (e.g. equestrian) sports, where any application of pharmaceutical drugs is totally prohibited and even substance classes like muscle relaxants or mild stimulants are included.

Moreover, doping analysis is closely related to adjacent fields with similar analytical prospects, like veterinary residue control (predominantly dealing with identification of growth promoters in various matrices), forensic sciences (the majority of doping-relevant substances are scheduled as controlled substances in most countries), environmental analysis (e.g. steroids in waste water) or clinical chemistry (e.g. due to the increasing relevance of steroid hormone replacement therapy).

This chapter describes the key fields of application of LC-MS in routine doping control (i.e. screening analysis, confirmation and quantification of positive results) extra to particular research activities. The latter are focused on the intended technical improvements (e.g. extension of detection time windows, reduction of turnaround times and costs) of conventional analytical procedures and, in particular, on the detection of prohibited substances that cannot be adequately identified so far (e.g. growth hormone).

The arrangement follows mainly historical and technical considerations, and does not necessarily represent the frequency or relevance of the application of LC-MS.

LC-MS in doping control – historical and technical aspects

Some peculiar legal and technical principles in doping control influence analytical strategies in athletes drug testing:

- The substance-based doping definition prioritises target analyses compared with general unknown screening procedures. Sensitive and specific selected ion monitoring (SIM) or

selected reaction monitoring (SRM) experiments are much more frequent than scanning experiments.
- Urine, which is the preferred specimen for doping control due to the ease of sample collection and relatively high concentrations of xenobiotics, requires a careful consideration of substance metabolism, including conjugation. Minor biochemical pathways leading to long-term metabolites are often more important than active parent compounds. The relevance of quantitative analyses is reduced to a few 'threshold substances'. This group comprises compounds that are accepted below certain threshold concentrations, because low amounts may be due to a permitted administration (e.g. inhalation of salbutamol) or an endogenous origin of the substance (e.g. natural levels of testosterone). In general, qualitative substance identification is a sufficient proof of a doping offence.
- The differentiation between substance prohibition 'in competition' and 'out of competition' requires modified analytical procedures with respect to numbers of included substances and threshold concentrations.
- A major analytical challenge consists in the verification of the prohibited administration of endogenous substances like testosterone, human growth hormone (hGH) or erythropoietin (EPO). In such cases, minor quantitative (e.g. amount of steroids compared with endogenous precursors or biochemical byproducts) or qualitative (e.g. glycosylation of proteins) deviations need to be identified.
- MS plays an outstanding role in doping analysis and was originally considered as a mandatory analytical technique for confirmation of substance identity. Exceptions were later acknowledged in the field of peptide hormones.

Approaches to the application of an LC-MS coupling in the framework of doping control were already reported in 1981 [2], when a combination of LC-MS equipped with a moving belt was used for MS confirmation of corticosteroids. The relevance of LC-MS application in doping control was ruled by practical demands, resulting in an early implementation of the technique in anti-doping research and routine [3] analyses. The issue of peptide hormones was already tackled in the mid-1990s, because gas chromatography (GC)-MS analysis could not solve the problem of identification of macromolecular compounds. The potential of LC-MS to differentiate intact growth hormone obtained from different manufacturers, quantify the insulin-like growth factor (IGF-1) and characterise human chorionic gonadotrophin (hCG) after tryptic digestion had already been reported in 1994 [4, 5]. However, these 'proofs of principles' demonstrate the general usefulness of LC-MS for the identification of peptide hormone doping, but are not used routinely to date, mainly due to sensitivity limitations.

The subsequent developments were mainly characterised by practical improvements. Substances (e.g. mesocarb [6]) and substance groups (e.g. diuretics [7]) causing severe analytical problems (stability) or inconvenience (time-consuming derivatisation reactions) were covered by efficient LC-MS assays, while other screenings remained unchanged, due to the availability of well-established and validated GC-MS procedures.

In contrast, there is an obvious preference to use LC-MS in cases of upcoming new substances (like the 'designer steroid' tetrahydrogestrinone [THG] and the stimulant modafinil) or substance groups (e.g. corticosteroids, included in the list of prohibited substances in 2003).

There is no preferred default LC-MS instrumentation in doping control analyses. Almost any technical variant of ionisation – electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) or photo-ionisation (APPI) – has been applied in combination with quadrupole (Q), ion trap (IT) or time-of-flight (TOF) mass analysers, whether as single or tandem mass spectrometers.

Certain reports of related technical developments of LC-MS seem to be just coincidentally associated with doping, e.g.:

- The introduction of isotope ratio MS linked to LC enables the identification of the origin of substances. In particular, a differentiation of endogenous production from synthetic material becomes possible in principle. However,

applications presented so far [8] are rather insensitive (requiring 400 ng substance on-column) and therefore of no practical value in routine cases.
- The introduction of Fourier-transform ion cyclotron resonance (FTICR) MS to identify corticosteroids [9] is probably technically motivated. The high expense of this technique does not permit routine applications. Nevertheless, it is clear that high-resolution (HR)-MS is essential for identification of multiply charged intact peptide hormones (hGH) [10]. Affordable routine instruments could greatly improve the detection and characterisation of proteins.

Additionally, progress in chromatographic separation (e.g. column switching [11], use of graphitised carbon [12] or chiral [13] columns) needs to be achieved, particularly in the field of peptide hormones. The improvement of ionisation efficiency appears to be a crucial aspect of steroid analysis. Derivatisation (dansylation [14]) of steroids and attempts to improve APPI ionisation (e.g. using anisol as dopant gas) are both specifically focused on a sensitivity enhancement [15].

Small molecules

The majority of doping-relevant substances belong to the category of small molecules. The most common definition of substance groups is based on pharmaceutical activity, distinguishing stimulants, narcotics, cannabinoids, anabolic agents, β-agonists, anti-oestrogens, masking agents and glucocorticoids. However, this schedule is not suitable for analytical consideration as structurally and analytically unrelated species may be arbitrarily grouped together (e.g. clenbuterol and testosterone as anabolic agents) because of their equal intended activity. However, one substance may be scheduled in different groups. The β_2-agonist salbutamol, for instance, may be considered as a permitted anti-asthmatic, as a stimulant or as an anabolic agent, depending on the occasion of the doping control (in/out of competition) and on its urinary concentration. The group of masking agents is rather complex too. It summarises any substance that may interfere with doping analysis by:

- diluting the urine and accelerating excretion (diuretics)
- suppression of reabsorption of xenobiotics (uricosurics, e.g. probenicid)
- manipulation of endogenous steroid profiles (administration of epitestosterone, used as an endogenous reference of urinary steroid concentrations, is able to conceal elevated levels of testosterone).

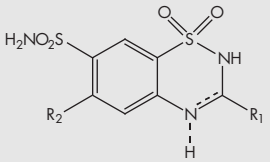
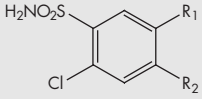
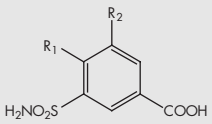
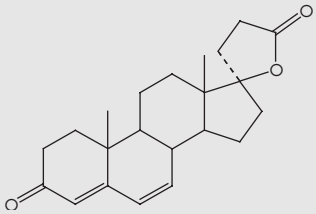
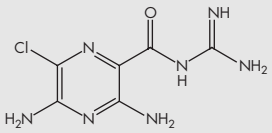
Diuretics

The main motivation to prohibit the use of diuretics in sports is the intended reduction of body mass in weight-classified sports. The second reason is a masking effect. Due to forced diuresis, the clearance of prohibited substances (e.g. anabolic steroids) may be accelerated and the urinary concentration may drop below the detection limit or threshold. Different types of diuretics (thiazide-like, loop and potassium-sparing diuretics) may be distinguished on the basis of their pharmacological properties (Table 9.1). However, these classes are not differentiated with regard to their doping relevance. The first two groups are supposed to be most efficient in doping because of their high potency. They are characterised by acidic groups (acid amides, typically sulphonamides) and are therefore suitable for negative ionisation. In contrast to these compounds, the group of potassium-sparing diuretics is characterised by steroid structures (aldosterone antagonists, e.g. canrenone) or cycloamidine structures (triamterene), and is more appropriate to protonation and subsequent detection in positive-ionisation mode.

Other compounds with diuretic (side) effects, like osmodiuretics (mannitol) or xanthine derivatives (caffeine), are no longer mentioned in the list of doping substances, because prohibited use could not be certainly discriminated from their permitted applications as food ingredients.

The identification of diuretics in doping control was originally carried out by GC-MS and LC-UV diode array detection (LC-DAD) [7, 16]. The reduced polarity of the methyl derivatives

Table 9.1 Typical classes and examples of prohibited diuretics

Class	Typical modifications	Examples	Chemical structure
Thiazide	R_1 = H, alkyl, subst. phenyl R_2 = Cl, CF_3	benzthiazide hydrochlorothiazide	
Thiazide analogues	R_1 = subst. amide R_2 = H, OH	mefruside xipamide	
Loop diuretics (furosemide type)	R_1 = Cl, phenoxy R_2 = amine	furosemide bumetanide	
Potassium-sparing diuretics, aldosterone antagonists		canrenone spironolactone	
Cycloamidine derivatives		triamterene amiloride	

permitted a GC separation and subsequent identification of the majority of diuretics in SIM mode. This approach includes analytical limitations, in addition to the requirement for a time-consuming derivatisation step. Certain substances do not form stable, reproducible and uniform methyl derivatives. Chromatographic artefacts (Figure 9.1) need to be factored into the screening [17] and there remained at least one diuretic (benzthiazide) undetectable in GC-MS. Therefore, LC-DAD was additionally applied as a complementary analytical technique. The obvious benefits of LC-ESI-MS are revocation of derivatisation, improvement of comprehensiveness, reduction of turn-around times and increase of sensitivity [3, 18]. As stated earlier, there are strongly acidic as well as basic compounds among the class of diuretics, requiring either negative- or positive-ionisation modes, respectively. Basic diuretics may well be combined with other screening procedures (e.g. anabolic steroids) to avoid a re-injection of samples in different ionisation modes. Alternatively, the option of a scan-to-scan polarity switching was utilised to detect both groups of diuretics simultaneously [19, 20].

The relatively high urinary concentrations combined with the sensitivity of the technique reduces chromatographic separation to a minimum and diminishes turn-around times to a

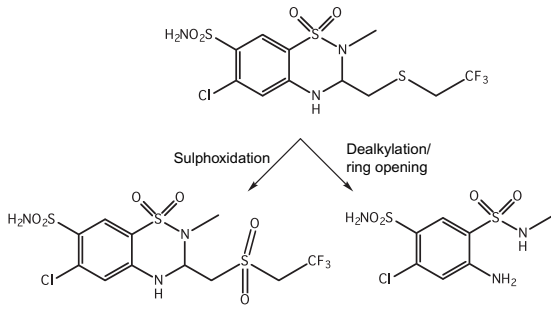


Figure 9.1 Diuretics with thiazide structure are converted by hydrolysis or sulphoxidation during sample transportation, storage or preparation, which may cause analytical problems in GC-MS. Respective artefacts need to be factored into LC-MS screening.

few minutes. A combination with automated solid-phase extraction (SPE) may be applied to establish high-throughput LC-MS screening [21]. The assay is sufficiently selective to differentiate concentrations above a minimum required performance level (MRPL [17]) of 250 µg/L from a blank matrix (Figure 9.2). The potential influence of ion suppression is not critical, because there are no threshold values and the quantification of diuretics is not required in screening analyses.

β_2-Agonists

The classification of these sympathomimetic agents in doping control has been frequently modified. At present, there is a differentiation between substances that are permitted by inhalation to treat asthma (requiring a therapeutic use exemption), while others are prohibited due to their potential stimulating or anabolic effect. Clenbuterol, which is supposed to be the most potent anabolic β-agonist, constituted a particular analytical challenge as typical urinary concentrations are of the order of magnitude of 1 µg/L. Due to its two chlorine atoms, it is well suited for HR-MS and its trimethylsilyl (TMS) derivative was preferably identified by either GC-HR-MS or GC-MS-MS. Typical LC-MS screening procedures are slightly less sensitive than GC-MS procedures, but more comprehensive, which appears to be more important in the field of horse testing [22] because of the general prohibition of all β_2-agonists, regardless of their intended action, concentration or administration route. Typically, β_2-agonists (Table 9.2) are identified in positive ESI mode using the protonated molecule as precursor ion. Characteristic fragmentation reactions are losses of the terminal isobutene group, resulting in $[M - 56]^+$ fragments, whether or not combined with losses of water [23].

Approaches to differentiate between inhalational and prohibited systemic (e.g. oral) application of salbutamol were based on quantitative examinations (values greater than 1 mg/L were, according to the WADA regulations, considered as an adverse finding) or investigations of salbutamol enantiomers. A discrimination function was derived from the higher amounts of the *S*(+) relative to *R*(–) isomer after oral administration. This approach requires the combination of chiral LC separation with MS detection, whether on-line or off-line [13].

β-Blockers

β-Adrenergic blocking agents (β-blockers) are prohibited due to their reduction of heart rate, blood pressure and hand tremor. Doping controls are consequently restricted to competition controls in particular sports where steadiness is important (archery, shooting, etc.).

β-Blockers are characterised by a very similar chemical structure. With a few exceptions (e.g. sotalol or carvedilol), they represent derivatives of oxypropanolamine terminated by *t*-butyl or isopropyl groups and aromatic substituents (Table 9.3).

GC-MS was the conventional screening technique for β-blockers in doping control. Derivatisation with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), if necessary combined with *N*-methyl-bis-trifluoroacetamide (MBTFA), was applied after hydrolysis of the conjugates and isolation from urine [24]. The application of LC-MS represents a useful alternative to avoid this time-consuming derivatisation step, and the formation of unstable derivatives and artefacts in some cases (e.g. acebutolol). All β-blockers contain a secondary amino group accounting for

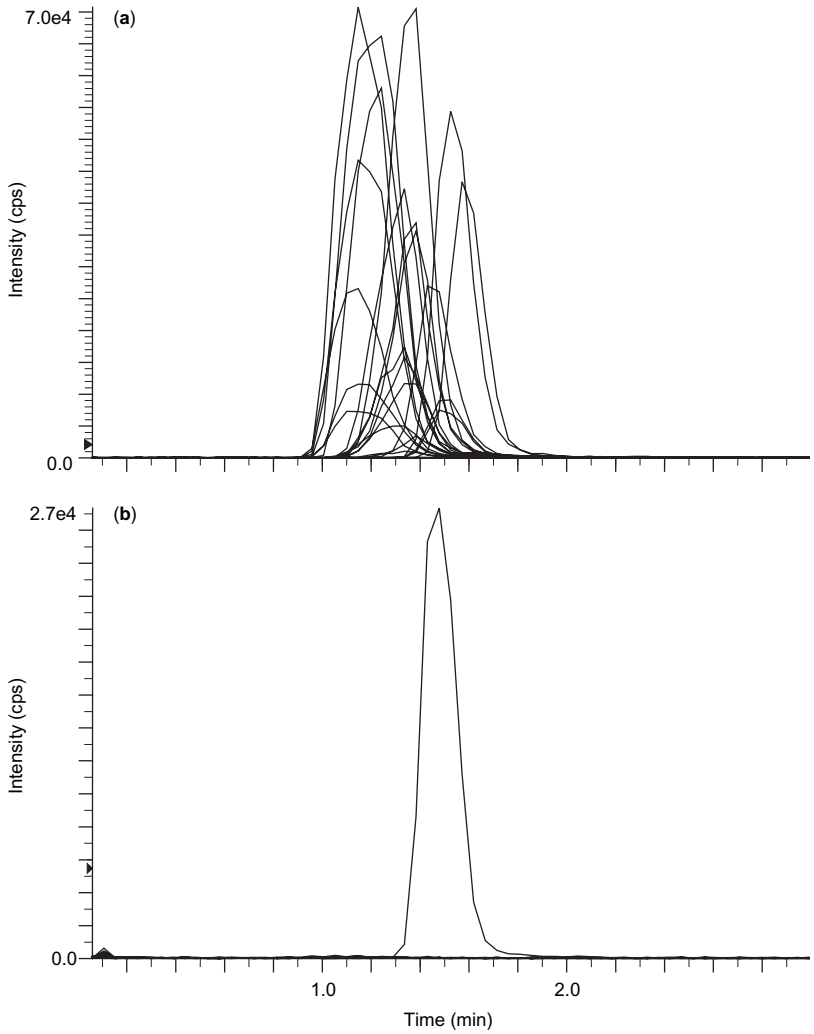


Figure 9.2 Multiple-reaction monitoring (MRM) experiments permit the screening of low amounts of prohibited diuretics in negative-ionisation mode. The turnaround time and sample preparation may be reduced to a minimum due to the outstanding selectivity. A mixture of 20 diuretics extracted from a urine matrix (a) is compared with a blank urine containing mefruside as internal standard (IS) (b).

their protonation and detection in positive mode. According to their high structural similarity, there are group-specific fragmentation reactions for both classes of β-blockers (Table 9.3). Substances with a terminal *t*-butyl group are characterised by loss of isobutene (M – 56), usually in combination with loss of water, while most of isopropyl terminated compounds undergo a loss of an isopropylamino group (M – 77) in addition to the formation of an *N*-isopropyl-propanolamine fragment (mass-to-charge [*m*/*z*] ratio 116) [25].

The proposal of a combined screening of diuretics and β-blockers agents, utilising a scan-to-scan polarity-switching technique [19], is very promising for clinical purposes, because both substance groups are frequently combined in the treatment of hypertension. However, this

Table 9.2 Chemical structures of typical β-agonists

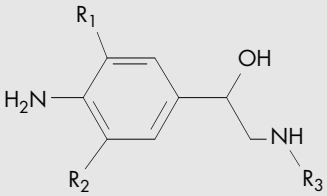
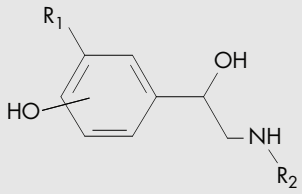
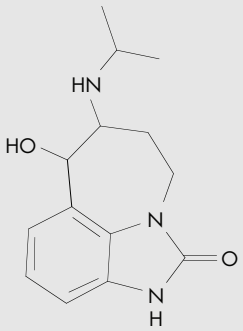
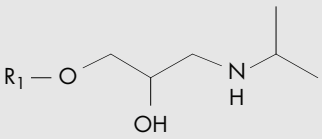
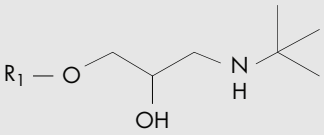
Class	Typical modifications	Examples	Chemical structure
Aniline	R_1, R_2 = Cl, Br, CN, OH R_3 = $C(CH_3)_3$, subst. phenyl	clenbuterol brombuterol fenoterol	
Phenol	R_1 = OH, subst. alkyl R_2 = alkyl, subst. phenyl	salbutamol orciprenaline	
Benzazepinone		zilpaterol	

Table 9.3 Chemical structures of typical β-blocking agents prohibited in particular sports

Class	Modifications	Examples	Chemical structure
Isopropylamine	R_1 = substituted aromatic rings	atenolol acebutolol propranolol	
t-Butylamine	R_1 = substituted aromatic rings	bupranolol carteolol timolol	

approach seems to be less rational in doping control, because the intention of an abuse of both substance classes and the scope of their prohibition (concerning sports, in competition versus out of competition) are different.

Steroids

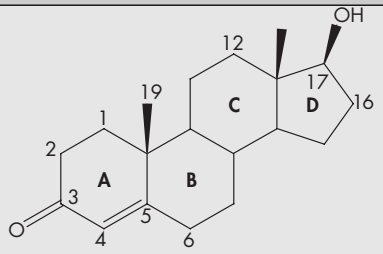
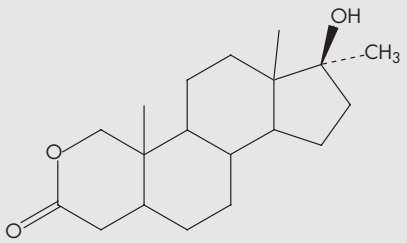
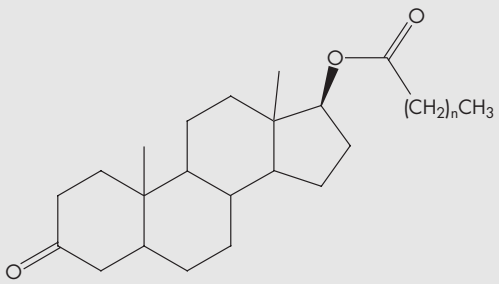
This substance class is characterised by a uniform structure consisting of a modified sterane skeleton (Table 9.4). Due to this apolar structure, the

efficiency of ionisation and suitability of LC-MS mainly depend on molecular substitutions, resulting in tremendous variations of sensitivity among different steroids. Saturated steroid molecules (e.g. by saturation of rings or reduction of keto groups) may hardly be ionised by protonation or formation of ammonium adducts. The presence of conjugated double bonds (3-keto-4-ene, steroids), oxidation of the sterane moiety or other polar ring substitutions (e.g. the pyrazol ring in stanozolol) improves the ionisation rate significantly. Therefore, this apparently uniform substance class needs to be divided into the following groups accounting for their LC-MS properties.

Synthetic anabolic steroids

The group of anabolic steroids still includes a diversity of similar structures, which cannot be systematically separated. The subgroups listed in Table 9.4 (biologically active anabolic steroids, precursors and metabolites) overlap each other from legal as well as biochemical perspectives. These compounds (e.g. androstenedione) may be considered as precursors and metabolites in the biosynthesis of steroids, but they also originate from prohibited application of synthetic analogues (pro-hormones) of endogenous steroids or even from synthetic hormones. The analytical result of a urine analysis does not necessarily allow the differentiation of an approved medication, the abuse of pro-hormones (legally

Table 9.4 Chemical structures of selected endogenous and synthetic steroids

Class	Typical modifications	Example	Chemical structure
Endogenous steroids (including their precursors and metabolites)	oxidation/reduction in positions 3/17, saturation of 4 double bond, 5-α/β isomers	testosterone	
Synthetic steroids	17α alkylation, double bonds at position 1–2, 4–5 or 5–6, A-ring condensation, 4-chlorination, 19-demethylation	oxandrolone	
Esters	acetate, propionate, decanoate	testosterone esters	

tolerated in certain countries) or the application of scheduled prohibited steroids.

The attempts to develop LC-MS methods for the identification of anabolic steroids of the so-called 'free fraction' (i.e. slightly polar steroids which are excreted unconjugated in urine) demonstrated significant diversity of ionisation principles [26]. All anabolic steroids are detected in positive mode. However, the appearance of the most abundant precursor ion depends significantly on structural modifications of the sterane skeleton and is almost unpredictable. Protonated ions $[M + H]^+$, ammonium adducts $[M + NH_4]^+$ and fragments resulting from loss of up to three molecules of water $[M - nH_2O]^+$ were reported as base peaks. The balance between these ions is determined by proton affinity and therefore conjugated double bonds (3 keto-4-ene steroids, pyrazol ring condensation, aromatic rings) are the most obvious structural indicators for an improved protonation. Physical conditions in the ion source appear to represent another sensitive factor of ionisation efficiency. Comparing ESI, APCI and APPI for various anabolic steroids, it turned out that the choice of a precursor depends significantly on the ionisation technique. Oxandrolone (Table 9.4), for example, formed predominantly the $[M + H]^+$ ion in ESI, the adduct $[M + NH_4]^+$ in APCI and the $[M - H_2O]^+$ fragment in APPI [27].

Moreover, there is no general consensus about the preferences of various ionisation techniques for steroid analysis (Figure 9.3). Controversial evaluations of APPI [27, 28] suggest that technical differences between manufacturers' concepts are more significant than physical constraints. The typical acquisition mode of these methods is MRM, including two to three fragmentation reactions per analyte. LC-MS has contributed to the structural elucidation in the case of the new upcoming anabolic steroid tetrahydrogestrinone (THG) (Figure 9.3) [29] and provided an efficient complementary screening method, directed to the identification of polar steroids (e.g. trenbolone, THG, stanozolol).

However, unpredictable formation of precursor ions, relatively low ion abundances, and unspecific fragmentation reactions in combination with the existence of numerous isomeric steroids and metabolites complicate the design of comprehensive and sensitive LC-MS methods for the identification of anabolic steroids. Application in routine steroid analysis is mainly focused on selected polar steroid molecules (Figure 9.4), e.g. stanozolol [26, 30], boldenone [31], trenbolone [32, 33] or THG [29]. These polar steroids often encounter analytical difficulties in GC-MS, due to the formation of instable derivatives and artefacts after silylation. The application of an additional derivatisation (e.g. methoxime derivatives of THG) followed by an extra GC-MS procedure would be required to identify these substance groups. Therefore, LC-MS is a beneficial alternative to the extra effort of additional derivatisations or modified GC techniques.

Endogenous steroids

The problem of endogenous steroids in drug testing human athletes is mainly reduced to the quantitative balance between testosterone and its biochemical byproduct epitestosterone (*see* 'Steroid conjugates'). In addition, there is an upcoming interest in the quantification of endogenous steroids related to the therapeutic administration of steroids (treatment of testosterone deficiency [34]) and to their increasing relevance as lifestyle drugs. Therefore, the quantitative evaluation of endogenous steroid profiles using LC-MS-MS is of increasing importance in clinical chemistry.

The growing number of precursors of endogenous anabolic steroids (so-called prohormones) that are widely available as nutrition supplements and abused in sports and bodybuilding constitutes another analytical challenge. Biochemical precursors of testosterone (e.g. androstenedione, dehydroepiandrosterone) were the first products on the market, but they have been recently replaced by synthetic steroids, representing slight structural modifications of the endogenous compounds (e.g. 1-testosterone, 1,5α-androstenedione, where the location of the double bond is shifted from the 4 to the 1 position). Analytical problems to identify these compounds by LC-MS are comparable to those encountered for their endogenous counterparts [35]. The technique is probably not sufficient for the unambiguous identification of all potentially

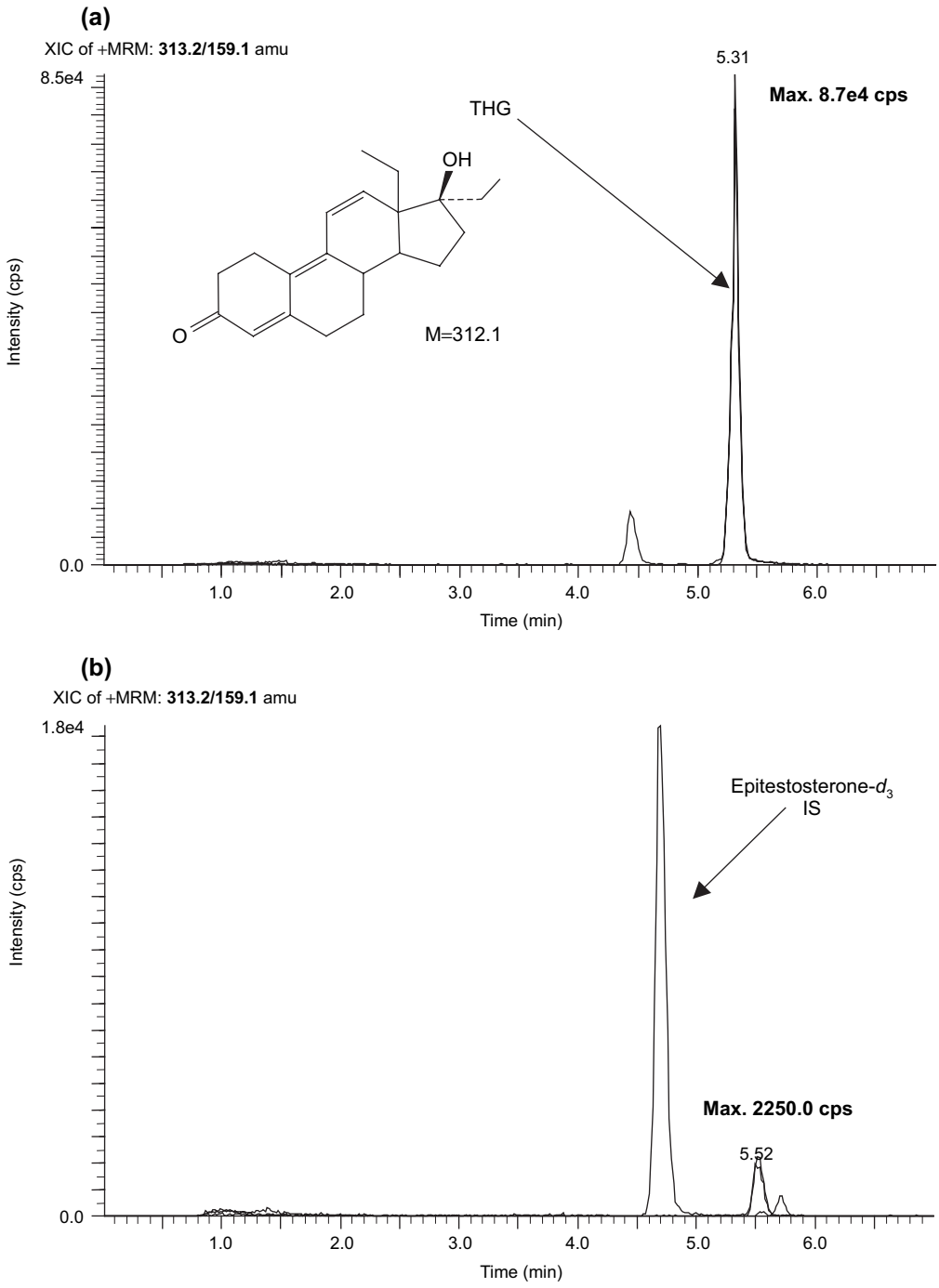


Figure 9.3 Comparison of LC-MS-MS detection of epitestosterone and THG in (a) APPI and (b) ESI. Both chromatograms were run under identical LC conditions. APPI shows an outstanding sensitivity for detection of THG, but is limited to steroids with chromophoric molecular structures, while ESI appears to be the most versatile ionisation technique.

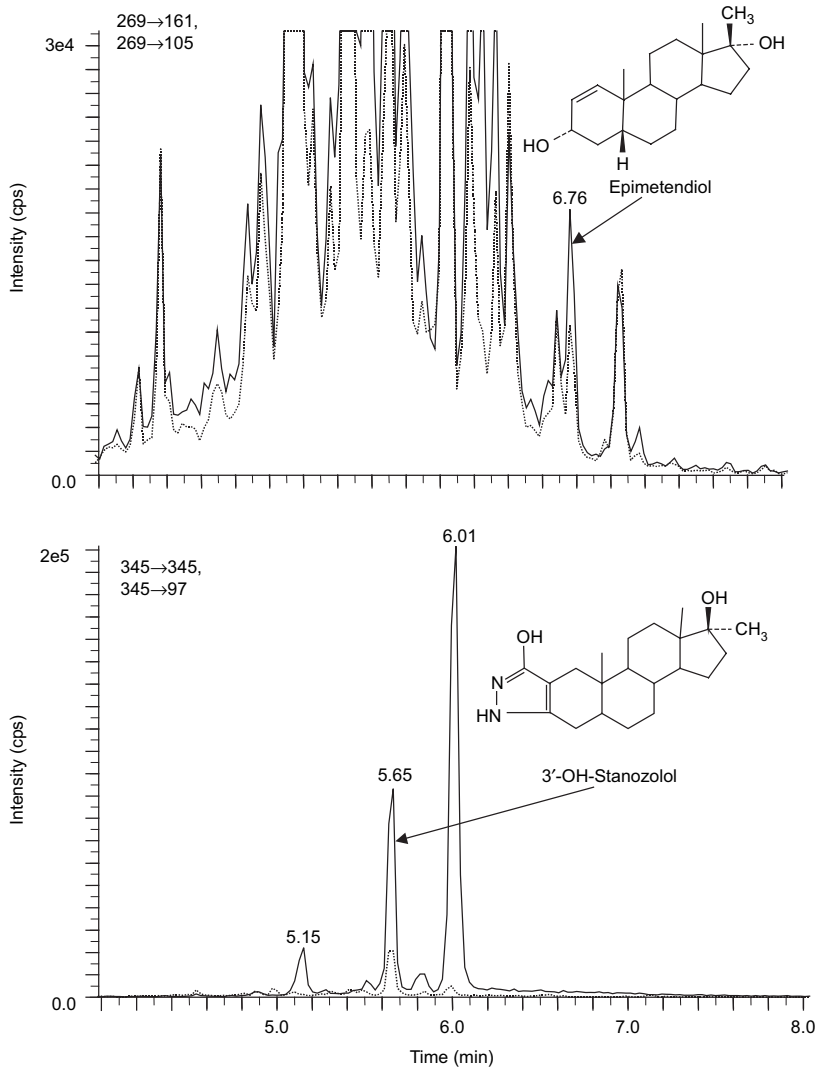


Figure 9.4 Threshold concentrations of 2 µg/L of 3′-OH-stanozolol and epimetendiol in extracts from a urinary matrix. The polar stanozolol metabolite is exceptionally well ionised and permits a sensitive identification, while detection of the medium polar epimetendiol is hampered by a limited intensity and specificity.

relevant steroids, but represents a helpful supplement to GC-MS.

Steroid esters

Steroids are typically administered as fatty acid esters by intramuscular injection. These compounds are stored in adipose tissues, rapidly hydrolysed in blood, not markedly excreted in urine as such and, therefore, not included in routine doping analysis.

The general benefit of the identification of steroid esters is an unequivocal proof of illegal administration of a synthetic compound. Any trace amount of the exogenous esters provides a clear indication for a doping offence, while the corresponding free steroids need to be distinguished from natural endogenous levels.

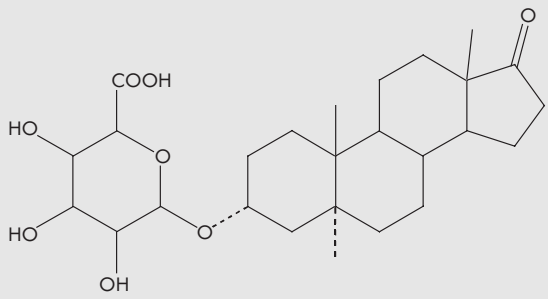
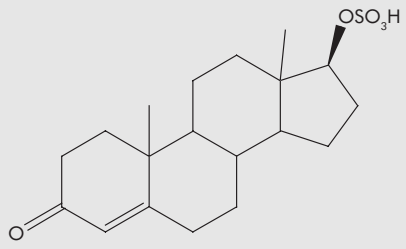
LC-APCI-MS-MS was reported to facilitate a sensitive identification of testosterone [36] or 19-nortestosterone esters [37] in equine plasma. The formation of dominant $[M + H]^+$ precursor ions was reported for all steroid esters.

Steroid conjugates

The detection of the abuse of endogenous steroids (testosterone or its precursors) is based on a quantitative evaluation of the urinary concentrations of testosterone and epitestosterone. The latter steroid is a byproduct of the biosynthesis of steroids and supposed to be suppressed after the administration of steroids. According to WADA criteria, further investigations to exclude physiological deviations are mandatory, if the ratio of testosterone/epitestosterone exceeds a value of 6. This definition is mainly empirical and derived from the conventional analytical GC-MS procedure, which was traditionally based on the quantification of bis-TMS derivatives of both epimers. Hydrolysis of the conjugates (mainly glucuronides and sulphates, Table 9.5) was carried out after hydrolysis with glucuronidase from *Helix pomatia*, which does not cleave the corresponding sulphates. Logically, the testosterone/epitestosterone ratios are by consensus referred to the total amount of free and glucuronidated steroids. It was suggested that elevated relative amounts of epitestosterone sulphate may cause increased testosterone/epitestosterone ratios when measured by conventional GC-MS. A potential racial bias of phase 2 biotransformation was discussed.

The quantification of intact conjugates, which is made possible by LC-MS, seems to be a conclusive option to examine the individual influence of glucurono- and sulpho-conjugation on testosterone/epitestosterone ratios directly and circumvent the uncertainty of hydrolysis recovery [38, 39]. Steroid glucuronides were reported to form different adducts (protonated, ammoniated and sodiated ions) in positive ESI. Depending on the declustering potentials, the $[M + H]^+$ was found to be most suitable due to its high signal-to-noise (S/N) ratios. Reasonably specific fragments (aglycone and its singly or doubly dehydrated fragment ions) were chosen for sensitive MRM experiments. The use of a deuterated IS for each

Table 9.5 Chemical structures of steroid conjugates

Conjugation	Examples	Chemical structure
Glucuronides	androsterone glucuronide	
Sulphates	testosterone sulphate	

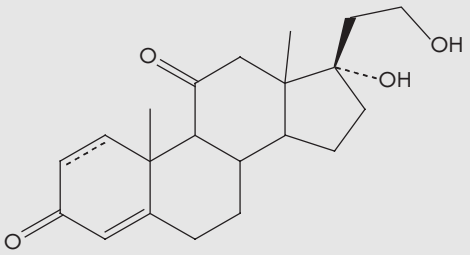
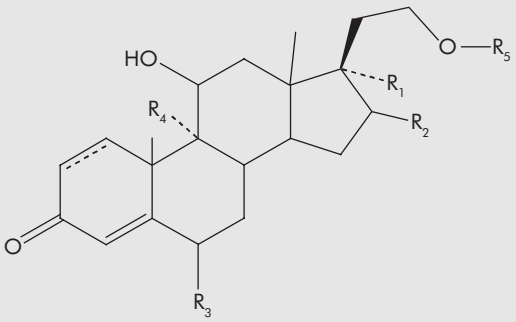
conjugate was found to be mandatory due to the potential-ion suppression in the urinary matrix. The assumed influence of sulphation on elevated testosterone/epitestosterone ratios was not supported by LC-MS-MS examinations [39].

Quantitative uncertainties of conjugate hydrolysis are less crucial in the case of exogenous steroids, because anti-doping legislation does not require a quantitative threshold. Approaches to identify glucuronides of synthetic steroids by LC-MS were directed to structural investigations of steroid glucuronides and automation of the analytical process by application of on-line micro-extraction [40]. Steroid glucuronides proved to exhibit similar ionisation principles to their free analogues. The presence of conjugated double bonds increases the proton affinity, resulting in an elevated abundance of $[M + H]^+$ pseudo-molecular ions, while saturated molecules tend to form ammonium adducts rather than protonated ions. The steroid conformation (5α versus 5β linkage of the A and B rings of the sterane skeleton) was reported to influence the fragmentation of A-ring-saturated steroids; 5β isomers were found to produce considerable higher amounts of the dehydrated aglycone fragments (relative to the deconjugated steroid) than 5α isomers [41].

Corticosteroids

Glucocorticosteroids (Table 9.6) are prohibited when administered systemically (orally or by injection), whereas all other administration routes require medical notification. Due to their influence on protein and carbohydrate metabolism, they are known to be abused as growth promoters in food-producing animals and may reasonably be abused in sports. At the moment, there is no analytical solution for a reliable differentiation of the administration pathway and analytically positive cases may be rejected by the availability of a therapeutic use exemption. ESI in negative mode was consistently reported to be the most efficient ionisation for a corticosteroid screening method [42, 43]. Typical fragmentation reactions are loss of the CH_2OH moiety, water or hydrofluoric acid. The LC-MS assays are carried out either in single-MS mode using diagnostic

Table 9.6 Chemical structures of synthetic corticosteroids

Class	Typical modifications	Examples	Chemical structure
Cortisone	1–2 double bond	prednisone	
Cortisol	1–2 double bond R_1 = H, OH R_2 = H, OH, CH_3 R_1, R_2 = $C(CH_3)_2$ R_3, R_4 = H, F	prednisolone betamethasone triamcinolone	

fragments or in MS-MS mode, where $[M + CH_3COO]^-$ adducts serve as precursor ions due to the absence of $[M - H]^-$ pseudo-molecular ions.

Alternatively, the identification of intact corticosteroid conjugates in bovine urine was evaluated [44]. Base peaks detected in positive ESI mode are sodium adducts, while a predominant $[M - H]^-$ ion is observed in negative mode. Both precursors exhibit a low fragmentation rate. MRM experiments in negative-ionisation mode using a $[M - H]^- \rightarrow [M - H]^-$ pseudo-transition (monitored at elevated collision energy) were found to produce specific signals due to the high ion stability.

Other prohibited substances (stimulants, narcotics, cannabinoids)

There are numerous other prohibited substances in doping control eligible for application of LC-MS, like stimulants or narcotics. Respective analytical procedures are available in clinical, veterinary or forensic toxicology and may certainly be adopted in doping analysis. However, conventional screening procedures based on GC with nitrogen phosphorous-selective detection (NPD) or GC-MS are still state of the art, because the concentration and stability of respective compounds are sufficiently high. Moreover, the list of prohibited stimulants and narcotics has been revised and condensed recently, simplifying the analytical challenges.

Analytical development in these areas is focused on upcoming (e.g. modafinil) or critical substances (e.g. mesocarb). The latter stimulant is thermally instable, forms irreproducible artefacts and is therefore difficult to identify by conventional GC-MS. The long history of attempts to use various ionisation techniques (particle beam, thermospray, ESI, APCI) to detect mesocarb and elucidate its biotransformation [45] demonstrates the analytical difficulties when tackling this substance. Mesocarb does not fit analytically into its native pharmacological group of stimulants and is typically integrated into other LC-MS screening procedures (e.g. diuretics).

Other isolated substances causing GC problems due to their high polarity and/or low thermal stability (e.g. the anti-oestrogenic clomiphene or the cocaine metabolite benzoylecgonine) are comparable candidates for an insertion into LC-MS screening procedures.

The identification of the major urinary metabolite of Δ^9-tetrahydrocannabinol (THC) (the glucuronide of 11-nor-9-carboxy-THC) in doping control does not represent any particular exception compared with forensic urine analysis (*see* Chapter 8). A threshold value of 15 µg/L specifies a potential doping violation; however, there are no uniform sanctions and cannabinoids were downgraded to the group of 'specified substances, which are particularly susceptible to unintentional anti-doping rule violations . . . and which are less likely to be successfully abused as doping agents' [1]. Any eligible LC-MS assay for cannabinoids [46] may be applied in sports drug testing without modifications.

The identification of alkylated xanthine derivatives is no longer essential in human doping analysis after its removal from the list of prohibited substances in 2004, although recent publications have dealt with its identification and quantification by LC-MS in horses [47, 48]. According to the required performance specification of the Association of Official Racing Chemists (AORC), concentrations as low as 100 µg/L need to be detected in equine urine.

Among numerous pharmaceutical substances that are controlled in equine doping control, there are quaternary ammonium anti-cholinergic agents (e.g. isopropamide, glycopyrrolate) that appear to be well suited for LC-MS. According to their ionic structure, quaternary ammonium drugs require special pre-treatment (ion pair formation) to enable conventional liquid–liquid extraction (LLE) or LC separation. Examining the identification of eight quaternary ammonium drugs in horse urine, the application of capillary electrophoresis was found to be more appropriate (enhanced sensitivity and separation power) than LC [49].

Large molecules

Introduction

Several proteins and peptide hormones are included in the list of prohibited substances,

due to their anabolic effect, as in the case of hGH or hCG, or due to an increase in the oxygen transportation capacity of blood, like EPO and haemoglobin-based oxygen carriers (HBOCs). These substances are synthetic or recombinant analogues of endogenous hormones. The possibility to discriminate between an abuse of prohibited substances and endogenous production depends on the structural modifications of the respective exogenous compound. Possible analytical approaches are:

- quantitative evaluations of compounds with a significant concentration difference between basal levels and exogenous administrations (hCG, IGF-1)
- identification of variations of the primary structure (insulin, HBOCs)
- discrimination of mass variants of proteins (20- and 22-kDa isomers of hGH)
- investigation of charge variants resulting from post-translational modifications (e.g. glycosylation of EPO).

In fact, the analytical approach depends on the current availability of pharmaceutical substances which are potentially abused. EPO, for instance, was originally available as recombinant protein with a primary structure identical to the endogenous hormone. The introduction of synthetic variants with modified amino acid sequences required an immediate adaptation of the analytical approach.

The practical application of LC-MS techniques to identify large molecules in routine doping control is still restricted by sensitivity limitations and the requirement of extensive and selective sample clean-up. A shotgun approach based on digestion of proteins followed by HR separation of the digests is most frequently applied. Selective clean-up procedures (e.g. immunoaffinity enrichment) of the resulting complex peptide mixtures need to be applied to reduce ion suppression and to be able to identify relevant proteins at low amounts.

The characterisation of structural particularities based on the identification of intact proteins requires relatively high amounts of sample material and HR-MS for identification of the multiply charged precursor ions. Adequate analytical techniques (e.g. FTICR MS combined with linear ITs) are used for research investigations, but are not available for routine applications.

The identification of macromolecular compounds by LC-MS in routine doping analysis is so far restricted to HBOCs and synthetic insulin.

Human chorionic gonadotrophin

This gonadotrophic hormone stimulates the endogenous production of testosterone and is therefore prohibited in men. Quantification of hCG by two different immunological assays is recognised by WADA as sufficient analytical technique. Different conventional cut-off values of 10 or 25 IU/L were suggested (but not officially adopted) to discriminate normal values and pathological situations or misuse. An LC-MS procedure using an IT MS in positive ESI mode was found to be suitable for a sensitive quantification procedure of hCG [50]. Immunoaffinity extraction was used to enrich the peptide from the urinary matrix prior to the tryptic digestion of the glycoprotein. A residue of the β-subunit containing 17 amino acids was chosen as a significant marker for hCG; in particular, a distinct structural specificity compared with the similar subunit of the luteinising hormone was confirmed. MRM experiments of the doubly charged peptide allowed a quantification down to threshold concentrations of 5 IU/L. Examination of the glycosylation of hCG revealed a considerable micro-heterogeneity [51], which does not affect the evaluation of doping cases because a reliable differentiation is possible based on a wide concentration gap between endogenous and abnormal hCG levels in men.

Human growth hormone

Human GH stimulates the production of IGF-I, leading to a promotion of protein synthesis, increase of muscle mass, reduction of the amount of stored fat and an induction of the growth of long bones. The secretion from pituitary glands occurs in three to five daily pulses, during which the basal serum concentrations (around 3 µg/L) are temporarily greatly exceeded and return rapidly (half-life around 15 min) to normal.

Therefore, hGH serum concentrations are not suitable for the detection of its abuse. Instead, a combined evaluation of concentrations of IGF-I, its binding protein (IGF-BP3) and bone markers was proposed to reveal abuse of growth hormone in human athletes [52].

In horses, a cut-off serum concentration of 700 µg/L of IGF-I was anticipated as the criterion for growth hormone administration [53]. A sensitive LC-ESI-MS quantification procedure (limit of quantification = 30 µg/L IGF-I) was reported to be eligible for routine screening. The use of Arg^3-IGF-I, which is derived from IGF-I by replacement of Glu^3, as IS, appears to be essential, due to its similar properties in the affinity chromatography clean-up. Full-scan MS was applied to detect the intact pseudo-molecular ion ($[M + 7H]^{7+}$, m/z 1093.4) of IGF-I, which is used for quantification in equine serum samples [53].

Another analytical approach is based on the evaluation of qualitative variations of growth hormone. There are several mass variants of the predominant protein containing 191 amino acids corresponding to a molecular mass of 22 kDa. The most interesting one is a 20-kDa molecule, which is derived by deletion of residues 32–46 and exhibits comparable physiological activity. The concentration ratio of both variants does not depend significantly on gender, age, body height and weight [54]. Administration of any hGH isoform suppresses the endogenous secretion of both variants. Accordingly, abuse of commercially available hGH may be detected by an elevated 22/20 kDa ratio, because it contains exclusively the 22-kDa variant. Studies to prove the appropriateness of this approach based on enzyme-linked immunoassays are documented [55]. Current alternative quantification procedures of low-level proteins by LC-MS, based on sophisticated analytical equipment, e.g. FTICR MS, require extensive sample preparation and do not yet achieve the required sensitivity [10, 56]. Identification of two tryptic peptides of hGH (22 kDa), which was obtained from digestion of LC fractionated plasma, provided limits of detection 10-fold higher than relevant clinical plasma concentrations [57]. Another approach was capable of identifying clinically relevant levels (5 µg/L) from unfractionated plasma, applying tagged peptides, isolated by affinity chromatography [10]. The detection was carried out by HR-LC-MS (FTICR), which was coupled to a linear IT.

In another study, qualitative investigations on hGH were carried out using LC-MS-MS (FTICR). Based on the isolation and accurate mass measuring of the $[M + 17H]^{17+}$, a deviation of the molecular mass of 4 Da (compared with non-post-translationally modified hGH) could be detected and attributed to the formation of two disulphide bonds [56].

The identification of structural modifications like disulphide linkage or variations of glycosylation, influencing proper folding of proteins, may be of diagnostic value in doping analysis. Present technical constraints (relatively high cut-off values and/or high amounts of sample material, requirement of high mass ranges and MS resolution, and high MS accumulation times that are incompatible with typical LC peak widths and prohibit on-line coupling) seem to impede the application of LC-MS to the identification of hGH abuse in the near future.

Erythropoietin

EPO is a 34-kDa glycoprotein hormone controlling red blood cell production and is therefore a potent doping agent to enhance the oxygen transport capacity of blood. Recombinant human EPO (rhEPO) has been available since 1989 and cannot be directly distinguished from endogenous EPO due to its identical amino acid sequence. Indirect methods (e.g. haematological parameters) served as an indicator for a potential abuse. The variable glycosylation is determined by post-translational modification, which depends on the availability of enzymes in the respective cells, resulting in a wide diversity of carbohydrates, typically terminated by one or two sialic acid residues.

The number and location of sialic acid residues per molecule (Figure 9.5) determine the formation of quaternary isoforms and influence the plasma half-life of EPO. Removal of sialic acid groups leads to a total inactivity, due to a rapid clearance from blood circulation, while new EPO variants (i.e. darbepoetin) provide a prolonged activity, triggered by the inclusion of additional oligosaccharides, which are terminated by sialic acid.

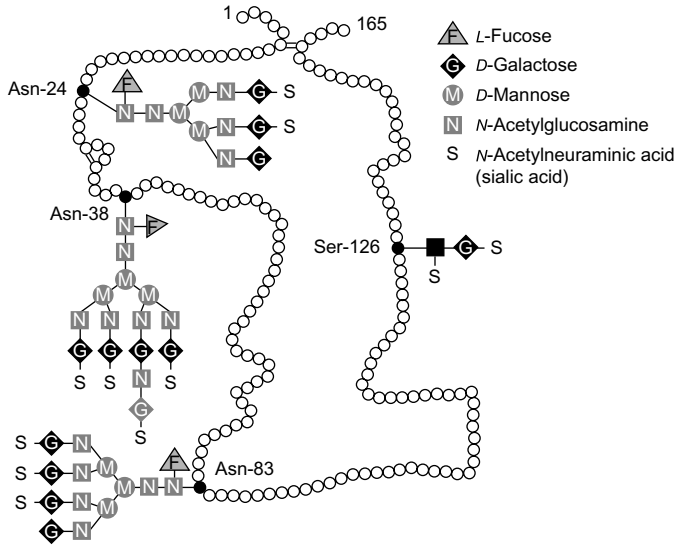


Figure 9.5 Chemical structure of EPO.

This alteration is based on a modification of five amino acids of the polypeptide and respective substances may be distinguished from natural EPO by analysis of the primary structure. Another variant of EPO (SEPO) consists of a synthetic peptide that is conjugated with a polymer, aiming for a reduction of the biological diversity.

The identification of EPO variants in doping control urine samples is to date based on a chemiluminescence detection of its isoforms after immunological enrichment and electrophoretic separation [58]. The logical MS approaches to an identification in combination with LC or capillary electrophoresis are hampered by the limited sensitivity.

Application of capillary electrophoresis- and LC-IT MS enabled the quantitative characterisation of EPO reference material after cleavage and derivatisation. The relative amount of sialic acid per molecule of rhEPO was found to be 17.6 mol/mol. This was supposed to provide a potential parameter to differentiate recombinant from endogenous EPO [59]. Alternatively, a characteristic sulphation of *N*-linked oligosaccharides in EPO molecules from different cell lines was detected, based on MS examinations (negative-ionisation mode) after LC separation of the protein digest using graphitised carbon columns [60]. An LC-ESI-MS investigation of various commercially available EPO forms (epoetin α, epoetin β, darbepoetin) resulted in astonishingly good LC separation. The full-scan MS of the three variants shows characteristic particularities in ESI (on-line micro LC coupling) as well as in matrix-assisted laser desorption ionisation (MALDI) mode (examination of the LC fractions) [61]. The injected quantity of protein was about 100 ng and, hence, far beyond the amount available in routine analyses. The limited MS sensitivity is due to the heterogeneity of glycosylation, because the ion abundance is spread over a large number of individual molecules. A deglycosylation of the EPO molecule leads to a reduction of diversity and increase of MS sensitivity. Analyses of deglycosylated EPO (rhEPO and darbepoetin) by MALDI showed a good match of its molecular mass with the assumed structure [62]. LC-ESI-MS experiments allowed a sensitive identification of $[M + nH]^{n+}$ pseudo-molecular ions (n = 5–8) of rhEPO after application of 250 fmol on-column. The additional identification of peptide, obtained from endoprotease Glu-C digestion, provides complementary structural information and is supposed to permit a sufficient identification of rhEPO and darbepoetin in race horses and greyhounds [62].

Insulin

The presumptive abuse of insulin among athletes and body-builders is due to its stimulation of endogenous protein synthesis. Several cases of hypoglycaemia (i.e. significantly reduced levels of blood sugar) were observed in fatal incidents in body-building, indicating its high popularity in this field. Insulin (Figure 9.6) belongs to the group of prohibited peptide hormones according to the recent WADA definition [1]. There are various medications available, based on structural alterations of insulin. The main purpose of these structural variations consists in the regulation of its bioavailability. Insulin tends to self-association to biologically inactive hexamers, which can be suppressed by structural modifications like switching of the positions of lysine and proline (B28 versus B29, Humalog) or replacement of proline with an aspartic acid residue (B29, Novolog). Alternatively, long-acting insulin was synthesised by elevation of its isoelectric point (modification in A and B chains, Lantus).

The identification of intact insulin derivatives, carried out on a QTOF instrument equipped with a nano-ESI ion source, revealed the predominant formation of corresponding multiply charged molecules (z = 5–7). This enables a mass-specific differentiation of the synthetic derivatives from human insulin [63] (Figure 9.7) after LC separation. Alternatively, the identification of the B chains can be applied after reduction of the disulphide bonds.

Haemoglobin-based oxygen carriers

Similar to the administration of EPO or synthetic perfluorocarbons, the intention of an abuse of HBOCs in sports is an elevation of the oxygen transportation capacity of blood. Various cross-linked bovine or human haemoglobin preparations (e.g. Hemopure, Hemolink, Oxyglobin) are approved for the treatment of animals or humans. The intact oxyglobin was reported to be inadequate for an efficient confirmation of haemoglobin by LC-MS in blood samples, because of the formation of one single dominant fragment that was interfered with by impurities from haemolysed plasma. Sufficient variations between the primary structures of human and bovine haemoglobin permit the differentiation of both proteins, based on the identification of diagnostic tryptic peptides. Specific peptides resulting from α and β chains of haemoglobin were found to be suitable for a differentiation of both species [64, 65]. Subunits of the α (residue 69–90, 2367 Da) and the β chain (residue 40–58, 2090 Da) were commonly used as markers for bovine haemoglobin. Human haemoglobin was identified by recording of a diagnostic peptide

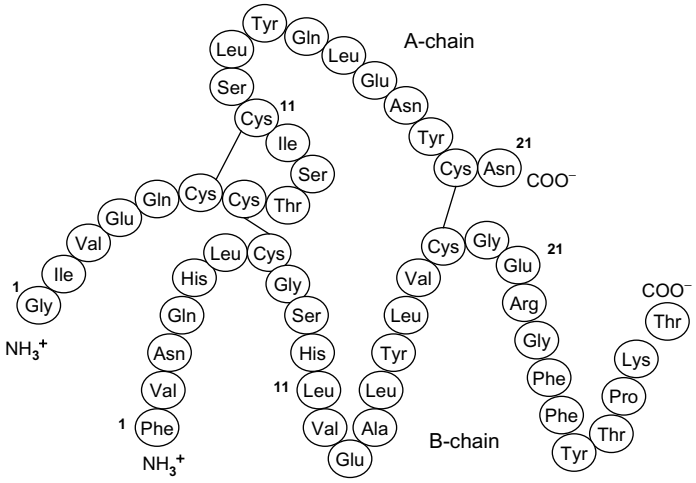


Figure 9.6 Chemical structure of insulin.

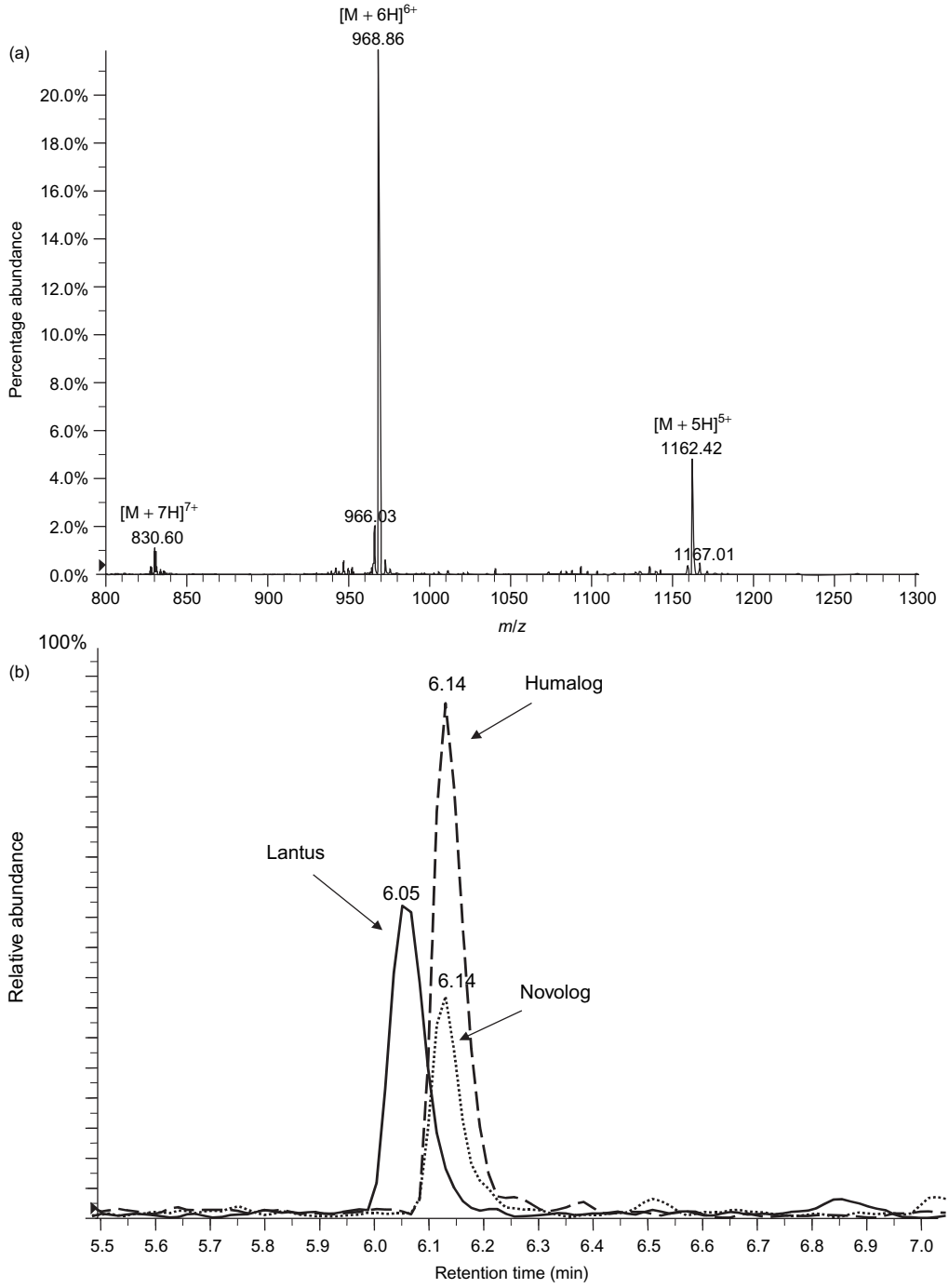


Figure 9.7 ESI full-scan spectrum of human insulin (a) generating the multiply charged molecules $[M + 5H]^{5+}$, $[M + 6H]^{6+}$ and $[M + 7H]^{7+}$ at *m/z* 1162.4, 968.9 and 830.6, respectively. Extracted ion chromatogram (b) of a plasma sample fortified with Humalog (*m/z* 1162), Novolog (*m/z* 1166) and Lantus (*m/z* 1213) at 10 pmol/mL. Insulins were analysed as intact proteins. (Reproduced from Thevis *et al.* [63] with permission.)

from the β chain (residue 42–60, 2059 Da). Doubly or triply charged ions of these peptides are suitable for LC-ESI-MS-MS screening. The assay is able to identify, confirm and quantify HBOCs in human and equine plasma samples. Respective assays were established using either QTOF or triple-quadrupole (QqQ) MS [64, 65]. Sample preparation requires a laborious combination of SPE, filtration of macromolecules and enzymatic digestion.

Summary

LC-MS has been introduced in different branches of doping analysis.

Qualitative progress in the analysis of highly polar substances (diuretics) could be achieved in combination with a significant reduction of sample preparation. Other low-mass pharmaceutical substances (stimulants, narcotics, β-blockers) may certainly be analysed by LC-MS, but well-established and validated GC assays are still dominant.

Progress in steroid analysis is mainly focused on substances with high proton affinity (polar substituents, conjugated double bonds, corticosteroids, steroid conjugates). The identification of relevant steroids by LC-MS has considerably advanced the classic GC-MS screening for anabolic steroids, but an extensive replacement of the conventional surveys is not likely.

Finally, there is increasing application of LC-MS to the analysis of doping-relevant proteins. Synthetic (e.g. insulin) or non-human (e.g. haemoglobin) peptides may be positively discriminated from endogenous analogues, whereas recent approaches to an unequivocal identification of abuse of recombinant EPO or growth hormone are promising, but so far insufficiently sensitive and reliable for routine application.

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