LC-MS as a method for non-invasive measurement of steroid hormones and their metabolites in urine and faeces of animals

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Summary

This review presents the technique of liquid chromatography-mass spectrometry (LC-MS). It briefly outlines how LC-MS works, its different functions, the different types of instruments available, the applications with which it is mainly used and the costs involved. In particular, it highlights the effectiveness of LC-MS in measuring steroids and emphasizes its usefulness in measuring steroids non-invasively in animals, from matrices such as urine and faeces. The advantages and disadvantages of LC-MS in comparison to other methods of steroid analysis such as immunoassays and gas chromatography-mass spectrometry (GC-MS) are discussed.

Introduction

LC-MS is an instrumental method that combines the separation capabilities of a high performance liquid chromatography (HPLC) system with those of a conjoined mass spectrometer.

Applications of LC-MS

LC-MS has a broad range of applications across many different fields, including the petroleum industry (HSU et al., 2000), metabolomics (KOTLOWSKA, 2012), proteomics (AEBERSOLD and MANN, 2003), pesticide control (MATTERN et al., 1991), food safety (NUÑEZ et al., 2005), clinical and forensic toxicology (MAURER, 2005, 2007), clinical diagnosis (RASHED, 2001; SHACKLETON, 2010), doping control (THEVIS and SCHÄNZER, 2007; SHEN et al., 2009) and pharmacokinetics (BEAUDRY et al., 1998).

The usefulness of LC-MS in (steroid) hormone analysis of animals has become very well known over the past decade. For example, LC-MS is widely used in the livestock industry for the detection of illegal growth hormones such as clenbuterol and other beta agonists, which contribute to health risks in consumers (BLANCA et al., 2005). Other studies describe methods developed for LC-MS to determine levels of synthetic corticosteroids in bovine urine (VAN POUCKE and VAN PETEGHEM, 2002; TÖLGYESI et al., 2001; SHACKLETON, 2010), doping control (THEVIS and SCHÄNZER, 2007; SHEN et al., 2009) and pharmacokinetics (BEAUDRY et al., 1998).

Abbreviations:

APCI = atmospheric pressure and chemical ionization; EIA = enzyme immunoassay; ESI = electro-spray ionization; GC-MS = gas chromatography - mass spectrometry; HPLC = high performance/pressure liquid chromatography; HRMS = high resolution mass spectrometry; LC-MS = liquid chromatography - mass spectrometry; MS/MS = tandem mass spectrometry; OQ/PQ = operational qualification/performance qualification; RIA = radioimmunoassay; SPE = solid phase extraction; ToF = time of flight; UPLC = ultra performance liquid chromatography
Performance-enhancing drugs in race horses and other equine athletes can be detected with a method for measuring anabolic steroids in the serum of horses (LIU et al., 2011). Misuse of testosterone for doping purposes in human athletes can be determined by the simultaneous measurement of testosterone and its isomer epitestosterone in urine with LC-MS (THEVIS and SCHÄNZER, 2007; DANACEAU et al., 2008). LC-MS became the method of choice in these research areas as it has the highest specificity.

**Principle of LC-MS**

HPLC separates compounds according to their polarity, resulting in different retention times on a stationary phase such as a silica-packed column (MEYER, 2010). In brief, the compounds are eluted from the column via a liquid (mobile) phase. Different compounds vary in their affinities to the stationary phase of the column and the mobile phase leading to different retention times (Fig. 1). Compounds differing in polarity elute at different times, with unpolar substances eluting first when silica-packed columns are used. If elution is carried out with an isocratic system, consisting of only one mobile phase, elution times can be unacceptably long, complicating the detection of later compounds as peaks become smaller and broader. Alternatively, a gradient elution can be applied whereby a second mobile phase of opposite polarity is added to the system. The main purpose of gradient elution is to move strongly retained components of the mixture faster by slowly mixing the mobile phases, changing the eluent from polar to non-polar or vice versa. In the case of a reversed-phase column, as on an isocratic system, substances elute in order of decreasing polarity when the mobile phase is gradually changed from polar to less polar. Using elution gradients can improve peak form as well as shorten run times.

The compounds are then transferred via a flow of solvent to the MS, where they are further separated according to their mass/charge ratio (DE HOFFMANN, 2005). The solvent is vapourized in a heated spray chamber as it enters the MS. Vapour is transported through the chamber via a flow of carrier gas (e.g. nitrogen) and then bombarded by an electron beam, which ionizes the analytes within. Ions are created either by transferring an electron to an analyte (forming a negatively charged ion) or by causing one to be lost (forming a positively charged ion). The ions are transported through a vacuum to the mass analyser, where electro-magnetic forces differentiate them by their mass and charge state. The information is passed into a detector and a mass spectrum is produced. Ions are eventually neutralized, by colliding with the MS walls and collecting electrons, and removed from the instrument by a vacuum pump. The technique is very sensitive and has a high dynamic range of measurement, up to four orders of magnitude. In combination, HPLC and MS separation mechanisms offer a very high compound specificity and enable the simultaneous measurement of a large number of substances.

Using MS/MS (tandem mass spectrometry) further increases the specificity of a method. Not only is the target analyte measured but in addition its precursor ion is transferred to a chamber in the MS where it is bombarded with a collision gas (e.g. argon, nitrogen or xenon) and fragmented into product ions (Fig. 2), the mass of which is then read in the detector. This allows the identification of structural isomers that display similar chromatographic behaviour in the HPLC column and that produce similar ions in the MS, as different target analytes give different product ions when fragmented.

For example, the cortisol metabolite tetrahydrocortisol and its isomer allotetrahydrocortisol share a common retention time on a HPLC column. They also share a common molar mass and therefore the same precursor ion mass when ionized. With MS/MS, the precursor ion of each of the isomers fragments into...
different product ions, which allows them to be distinguished from each other. The most abundant product ion mass for tetrahydrocortisol is 301.15 m/z, whereas for allotetrahydrocortisol it is 331.15 m/z (Fig. 3).

**Instrumentation**

In recent years, there has been a tendency to replace standard HPLC instruments with ultra performance liquid chromatography (UPLC) systems that, like HPLC, can be conjoined to all types of MS (SWARTZ, 2005). In contrast to HPLC, these systems are designed to work with microbore® columns, which can withstand higher pressures leading to faster flow rates and elution times and shorter run times. Faster separations reduce solvent amounts and running costs. However, most standard HPLC instruments are also capable of attaining the higher pressures and flow rates required for microbore® columns.

Similarly, MS interfaces that are responsible for the desolvation and ionization of the analytes have become increasingly sophisticated. There are two main types of source interface:

1. electro-spray ionization (ESI) is more commonly used and is better suited to ionizing polar compounds such as steroid hormones (DEVENTER et al., 2006).
2. atmospheric pressure chemical ionization (APCI) is used to measure thermally stable, less polar substances such as some hydrocarbons (MARVIN et al., 1999).

MS instruments can be divided into two main types: MS (e.g. quadrupoles and ion traps) and high resolution mass spectrometry (HRMS, e.g. LC-MS time of flight (ToF) and orbitrap). The principle difference between these instruments is that MS separates ions via electro-magnetic forces in a mass analyser, whereas HRMS separates ions via their acceleration through a flight tube. For example, LC-MS ToF measures the mass/charge ratio of an ion via the time it takes to reach the detector. MS instruments can only scan for a target range of masses, whereas HRMS instruments can scan for masses of both target and non-target substances. MS instruments such as quadrupoles are currently more sensitive and therefore mainly used for quantitative analysis but HRMS instruments, which are mainly used for qualitative purposes, are catching up. There are already HRMS instruments available that allow for higher resolution and sensitivity (WU et al., 2012).

**Steroid hormones and LC-MS**

Measuring steroid hormones is of huge importance to investigating stress levels in the fields of animal welfare (MÖSTL and PALME, 2002) and behavioural ecology, where steroid hormones influence animal behaviour and vice versa (DUFTY et al., 2002; GODWIN et al., 2002; WINGFIELD et al., 2006; ANESTIS, 2011).

Steroids comprise a wide group of natural and synthetic organic compounds that share a similar chemical structure of 17 carbon atoms arranged in a four-ring system. They are largely produced in specific glands such as the ovaries, testes and the adrenal cortex (BORON and BOULPAEP, 2009). They are involved in a variety of endocrinological functions ranging from metabolism and digestion to the development and functioning of the sexual organs and include the sterols, bile acids, corticosteroids (glucocorticoids and mineralcorticoids) and the sex steroids (progestogens, androgens and oestrogens;
GOMES et al., 2008). Steroid hormones are present in their active form mainly in the blood, metabolized in the liver and kidney and excreted via urine and faeces in a multitude of modified, inactive forms that can be conjugated to substances such as glucuronides and sulphates. However, the metabolites of a steroid hormone in urine or faeces can originate from different sources. For example, while testosterone is largely produced in the testes, the testosterone metabolites that are abundant in urine can also derive from the adrenal glands. Therefore, if researchers are primarily interested in testicular function it is unwise to rely on the concentration of metabolites that also originate in the adrenal glands (HAUSER et al., 2011).

An LC-MS can be used to develop methods to measure either conjugated or non-conjugated forms of steroid hormone metabolites, as required. A large number of steroid conjugates are possible. Often it is preferable to measure the de-conjugated form than to measure all possible conjugated forms. The measurement of conjugated steroids can be more complex due to the different ionization properties of conjugated steroids. In-source dissociation of glucuronides from steroids, for example, can potentially interfere with accurate determinations (KAKLAMANOS et al., 2009). Furthermore, reference standards used to identify and quantify the target analyte are not always available for some conjugated forms. Care must be taken to ensure that chemical de-conjugation does not lead to degradation and/or transformation of the target analyte (HAUSER et al., 2008a).

Non-invasive measurement of hormones in animals

Blood collection from animals, especially wild animals, is highly invasive and difficult to perform and can be very stressful for the animal (WILSON et al., 1978; BEERDA, 1996). It has become increasingly important to develop methods to measure steroid hormones in samples obtained non-invasively. Over the last few decades there has been increased research into enzyme immunoassay (EIA)-, radio immunoassay (RIA)- and HPLC-based techniques (e.g. fraction collection) to measure hormones in samples from humans and domestic, wild and captive animals. Examples include steroid hormone analysis in the urine and faeces from livestock (PALME et al., 1996), faecal steroid analysis for monitoring reproductive status in farm, wild and zoo animals (SCHWARZENBERGER et al., 1996), analysis of salivary cortisol in squirrel monkeys (FUCHS et al., 1997), pregnancy determination in faecal samples from felids (DEHNHARD and JEWGENOW, 2013) and measurement of urinary reproductive hormones in captive apes (SHIMIZU et al., 2003). In addition, methods to analyse non-steroidal hormones such as oxytocin in the urine of chimpanzees (CROCKFORD et al., 2013) and thyroid hormones in urine and faecal samples from birds and mammals have been described (WASSER et al., 2010). In wild chimpanzees, for example, urine samples (MULLER and LIPSON, 2003; EMERY THOMPSON, 2005; SOBOLEWSKI et al., 2012) and faecal samples (EMERY THOMPSON, 2005; MUEHLENBEIN, 2006) can easily be collected for the purpose of non-invasive study.

In addition to these immunoassay-based methods of quantification, a number of LC-MS methods have been developed to measure steroid hormones and their metabolites in matrices obtained non-invasively, such as urine and faeces. This has been very beneficial in clinical contexts (KRONE et al., 2010; LUTZ et al., 2006) as well as in the field of primatology (HAGEY and CZEKALA, 2003; HAUSER et al., 2008a; WELTRING et al., 2012).

Immuoassays versus LC/GC-MS

Immunoassays are based on the binding of a specific antibody to an antigen of the target analyte (RAEM and RAUCH, 2007). The specificity of any given assay depends on how exclusively the antibody binds to the target analyte. Cross-reactivity occurs when the antibody of an assay also binds to other substances that are similar in their structure to the target analyte. Many potentially cross-reactive substances must be tested during the development of an assay. Most commercial assays are developed for a specific compound in serum. As the concentration of potentially confounding metabolites and/or degradation products found in serum is generally rather low and target analytes such as testosterone and cortisol are highly abundant, cross-reactivity does not play such an important part in these assays. However, in matrices such as urine or faeces the metabolites are more abundant than the target analytes and so the potential confounding effects of cross-reactivities are dramatically increased (PREIS et al., 2011; WELTRING et al., 2012). Successful attempts to derive meaningful measurements of steroid hormones and their metabolites in matrices such as urine and faeces, despite the confounding effects of cross-reactive substances, have led to the use of validated group-specific assays (MÖSTL et al., 2005; PALME, 2005; HEISTERMANN et al., 2006) or a chromatographic separation of sample extracts prior to assay (ZIEGLER, 1996; MÖHLE et al., 2002).

LC-MS techniques do not suffer from problems related to cross-reactivity. A high compound specificity is achieved by the combined separation based on specific characteristics in terms of polarity during liquid chromatography separation, as well as the mass/charge ratio of the parent ion in combination with the mass/charge ratio of fragments of the analyte during MS and MS/MS separation steps. A growing number of studies point out the advantages of LC-MS compared to techniques such as EIA and
RIA (ROSNER et al., 2006; FANELLI et al., 2011; KOAL et al., 2012).

Although they are less accurate and versatile, immunoassays have the benefit of being faster and cheaper than LC-MS (RAUH, 2009; THIENPONT et al., 2008). An additional advantage is that there is no need to develop methods, as many commercial assays for many different hormones already exist. However, the application of an assay in a species or matrix other than the one for which it was developed necessitates careful validation (PALME, 2005). LC-MS can be used to estimate the degree of interference by cross-reactivity and therefore can have an important role in the analytical validation of EIA or RIA for specific hormones in a variety of matrices (KUTSUJapan et al., 2009; BEHRINGER et al., 2012; PREIS et al., 2011). LC-MS can be used to ensure that assays reliably measure the target analyte. Whatever method is used, a thorough physiological validation is mandatory to ensure that the measured steroids reflect something biologically meaningful (PALME, 2005).

LC-MS allows the simultaneous measurement of several hormones and metabolites in any given sample, leading to high throughput capabilities. This renders the method particularly interesting for studies where a number of metabolites are of interest (HAUSER et al., 2011; KOAL et al., 2012; KUTSUJapan et al., 2009; PREIS et al., 2011; SURBEC et al., 2012a,b). Particularly interesting for behavioural endocrinology research is a method that allows the simultaneous measurement of 23 steroid hormones and their metabolites in small volumes of urine collected non-invasively from wild primates (HAUSER et al., 2008a).

Another method of chromatographic separation in connection with mass spectrometry is GC-MS. The detection principle is similar to that of LC-MS, using the mass fragments that result from ionization of the molecules. Before analysis, analytes of interest must be extracted from the matrix into a liquid solvent phase. The extract is injected into the GC where it is swept onto a separation column by an inert carrier gas. The analytes in the mixture are carried through the column by the carrier gas and separated from one another by virtue of their interaction with the coating (stationary phase) on the inside wall of the column. Each analyte interacts with the stationary phase at different rates, resulting in different retention times. In the case of steroids, derivatization is necessary as GC-MS requires volatile substances. In contrast, LC-MS does not require a lengthy derivatization that could result in analyte losses (GOMES et al., 2009). Nonetheless, GC-MS has the advantage that fragmentation patterns of substances can be reproduced. The patterns are instrument independent, allowing databases and libraries to be created and shared between users (HALKET, 2004). Furthermore, internal standards can be used to monitor efficiency of derivatization so that, once produced, a calibration curve can be used repeatedly. This is in contrast to the situation in LC-MS, where ion suppression can occur with ESI or APCI so a new standard curve has to be created for each sequence of samples (WUDY and HARTMANN, 2004).

Before hormone analysis of bio-fluids with LC-MS, sample clean-up is essential to remove unwanted matrix compounds that could potentially affect the measurements. Often solid phase extraction (SPE) is employed. Depending on whether the study is interested in measuring conjugated or unconjugated forms of metabolites, further steps may also be required, such as hydrolysis or solvolysis procedures (HAUSER et al., 2008b). Although extraction procedures can be time consuming, the sensitivity and reliability of LC-MS instruments is constantly increasing with the development of new instruments and thus it is highly likely that less time will need to be spent on clean-up procedures in the future.

There are a few other disadvantages when it comes to using LC-MS, including the two most common complaints, matrix effects and ionization issues. The matrix effect is interference from molecules in the sample that cannot be removed during the clean-up steps. Unwanted matrix can cause ion suppression or enhancement and ultimately interfere with the quantification of analytes (VAN EECKHAUT et al., 2009). However, this can usually be controlled for with the use of synthetic hormones as internal standards, something that cannot be done with EIA or RIA.

**Costs of measuring hormones via LC-MS**

The cost of new LC-MS instruments ranges from € 100,000 to more than € 600,000. Costs depend on the features, such as whether an HPLC or UPLC system is required, the presence of on-line extractions, the type of MS interface, ionization mode, sensitivity and detection limits. In addition to the initial purchase cost, machines require an annual operational qualification and performance qualification (OQ/PQ) and maintenance work, which are often performed by an external company, usually the vendor of the instrument. Maintenance contracts currently cost roughly € 10,000 per year, including an annual OQ/PQ visit and year-round technical support. Contracts are recommended as there can be frequent troubleshooting involved for instrument upkeep. However, with qualified personnel much of the work can be carried out in-house, greatly reducing the costs. It is estimated in our endocrinology laboratory that analysis of one analyte per urine/faecal sample costs an average of € 15. The figure includes consumables, reagents, extraction costs, hydrolysis (urine only), solvolysis, LC-MS analysis, equipment maintenance contracts and personnel costs. It is evident that LC-MS is only cost-efficient when multiple compounds per sample are analysed.
and when the machine is frequently in operation, i.e. it is used for routine analysis.

The level of training and expertise varies amongst personnel working with LC-MS. System operation and data analysis can be carried out by anyone with a reasonable amount of training. Maintenance of the instruments and troubleshooting requires the technical, mechanical and electronic skills of a scientist/engineer. Interpretation of data and the development of methods requires in-depth knowledge of both organic and inorganic chemistry.

**Conclusion**

LC-MS can be very useful for the measurement of hormones obtained non-invasively in a variety of matrices from many different species. Furthermore, it can be used for the analytical validation of other measurement techniques such as EIA and RIA. The advantages of specificity and the ability to perform simultaneous measurements can outweigh its biggest disadvantage, which is its cost, when multiple substances are routinely analysed per sample. The continual development of instruments hints at an exciting future in metabolite analysis across many fields of research.

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