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Review

The coming of age of liquid chromatography coupled to tandem mass spectrometry in the endocrinology laboratory☆

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ABSTRACT

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has been rapidly incorporated in the routine of the endocrinology laboratory. Most endocrinologists are aware of the benefits afforded by this technique and tandem mass spectrometers are clearly no longer a mere research method but an important tool widely used for diagnosis. In the last 15 years, LC–MS/MS has replaced techniques such as immunoassay and HPLC for the analysis of hormones because it provides higher specificity and good sensitivity. Also, it permits simultaneous measurement of several analytes and sample preparation and acquisition are fast and simple. Although several strategies based on LC–MS/MS have been described in the last 15 years, there is still room for improvement. The impact of matrix effects and isobaric interferences have been addressed by only a few studies, and standardization with reference materials is available for a limited number of analytes. This review summarizes the application of LC–MS/MS in analyzing three classes of hormones: steroids, derivatives of the aromatic amino acids, and peptides and proteins. The benefits and current limitations of LC–MS/MS will be discussed for these hormone categories.

Contents

1. Introduction

Laboratory testing plays a decisive role in the practice of endocrinology. The measurement of hormones and/or their metabolite levels in biological fluids such as blood and urine allows the clinician to determine in which gland(s) abnormal levels are being produced and trace a strategy for treatment. However, hormone quantification is among the most troublesome determinations in the clinical laboratory. One of the main reasons is the minute concentrations at which hormones are found in blood. For instance, important analytes such as parathyroid hormone, free thyroxine, and estradiol are present in the picomolar range (Fig. 1).

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic; APPI, atmospheric pressure photoionization; APCI, positive atmospheric pressure chemical ionization; DHEA, dehydroepiadrosterone; DHT, dihydrotestosterone; HAb, heterophilic antibodies; MRM, multiple reaction monitoring; PTH, parathyroid hormone; RIA, radioimmunoassay assays; T3, triiodothyronine or 3,3′,5-triiodo-L-thyronine; T4, thyroxine or 3,3′,5,5′-tetraiodo-L-thyronine.

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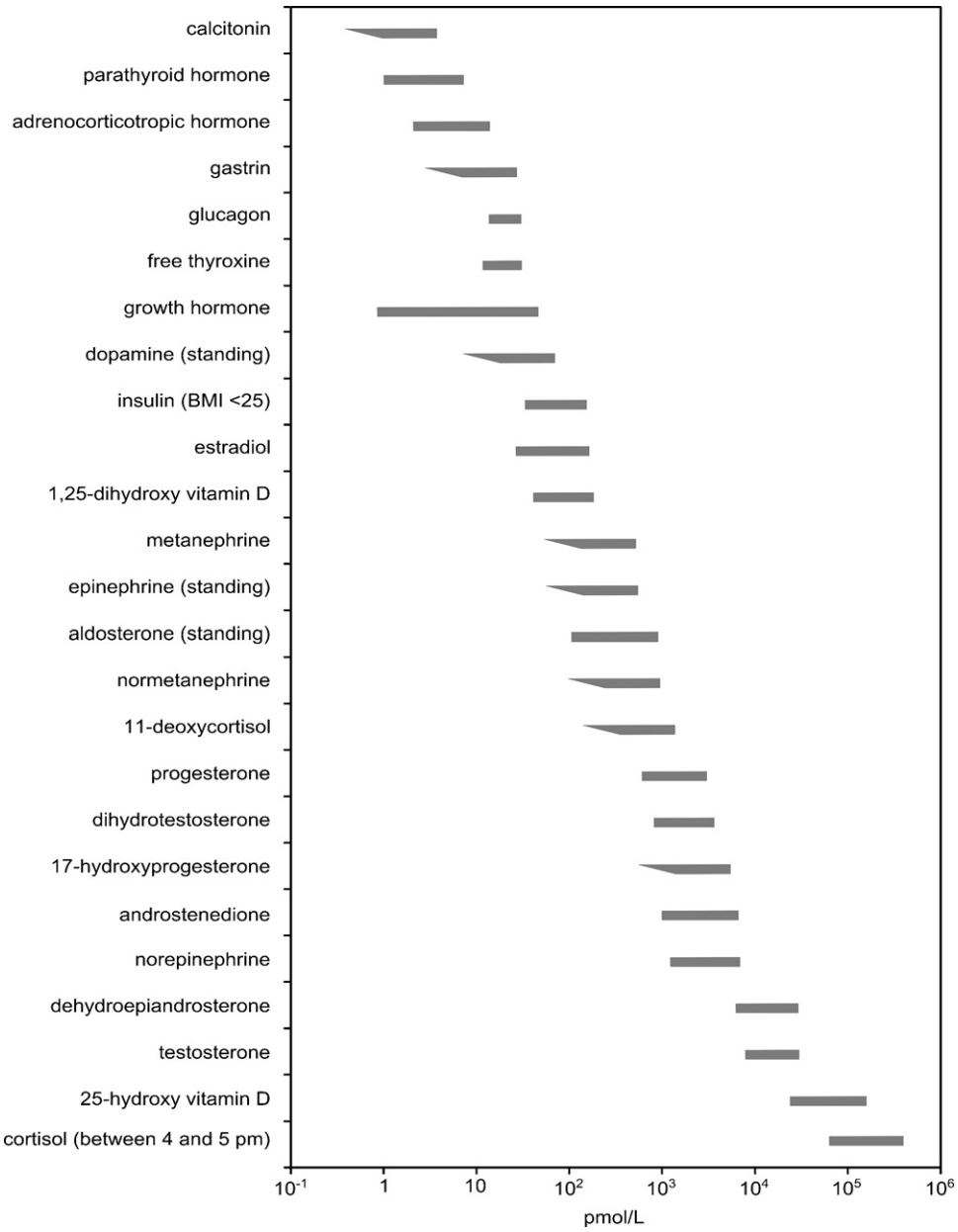

Fig. 1. Reference intervals for some human hormones in serum of adult males (based on Fleury Test Catalogue [159]). The left vertex indicates that the inferior limit is not defined or equals 0.

Detection of minute amounts within very complex matrices poses a tremendous analytical challenge.

The determination of low concentrations of hormones was made possible by the development of immunoassays. The basis of radioimmunoassay assays (RIA) postulated by Yalow and Berson in 1959 [1] turned endocrinology into a real quantitative science. Because antibodies can be produced to bind to virtually any analyte, RIA methods became highly successful as they allowed the determination of not only hormones but also a wide range of analytes of clinical importance. Still important for steroid determination, RIA produces reliable results when used in combination with previous extraction steps required for the elimination of interfering compounds [2,3]. However, laborious sample preparation, the need for radioactive reagents, and difficulties in achieving automation have limited its application in clinical laboratories.

In the late 1970s, the development of new approaches such as non-competitive assay [4], two antibody systems [5,6], chemiluminescent and fluorescent labels, as well as monoclonal antibodies [7] resulted in a new generation of immunoassays. These new techniques were successfully adapted into commercial kits to be used in automated platforms. Their low cost, simplicity, and speed led most clinical laboratories to acquire them.

However, recent studies have indicated that automated immunoassays are far from ideal and often fail to provide accurate results. For example, Taieb et al. [8] showed that among 10 commercially available immunoassays none was reliable enough for the investigation of testosterone levels usually found in children and women. The low accuracy obtained with the use of direct immunoassays could be explained by the limited specificity of the antibody, a frequent situation in the case of small molecules. Additionally, direct immunoassays overestimate the levels of estrone and estradiol in post-menopausal women due to cross-reaction with estrone sulfate, which is found in relatively higher levels [9].

Another source of growing concern regarding immunoassay accuracy is the potential interference of heterophilic antibodies (HAb), which are human antibodies that can bind to animal antibodies. In two-site immunoassays they can form a bridge between the capture and detection antibodies, leading to false-positive results or false increase in the analyte concentration [10–12]. False-negative results are also expected if the HAb reacts with only one of the assay antibody components, preventing binding with the analyte. Initially seen as rare findings controllable by the addition of animal protein or animal serum, the problems caused by this type of interference on several immunoassays are becoming increasingly obvious to clinicians and researchers. Examples of interference from HAb are well described in assays for determination of human chorionic gonadotrophin [13], calcitonin [14], thyroglobulin [15], and gastrin [16]. Indeed, there are descriptions of unneeded surgeries and chemotherapic treatments that have been performed because of a misdiagnosis caused by interference from anti-animal antibodies [17].

These limitations revealed a great need for developing alternative techniques for hormone determination. Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has been rapidly finding a place in the endocrinology laboratory. The tandem mass spectrometer has some unique features as a detector. These include its good sensitivity, high selectivity, and the ability to provide structural information. As for sensitivity, LC–MS/MS is still surpassed by some immunoassays but development of mass spectrometry instruments seems to be far from reaching its limit. Therefore, it is expected to equal or surpass immunoassays in the next years.

Selectivity is an important and sought-after attribute in LC–MS/MS. Indeed, LC–MS/MS has a great potential to be used as a selective hormone detection method although it also has some limitations. In fact, in the first years of its application in the endocrinology laboratory, the quality of the results obtained was overestimated whereas the technical requirements were underestimated [18].

More recently, matrix effects have been recognized as important factors that might adversely impact the quantitative performance of LC–MS/MS [19]. Due to the complexity of biological matrices, a number of endogenous compounds, including phospholipids and salts, can impact the ionization efficiency [20]. The specificity achieved by LC–MS/MS led to a limited application of the liquid chromatography in the first LC–MS/MS protocols. At the time, the need for the analytical column was thought to be limited to loading the sample into the detection system [20]. The resolving power of LC–MS/MS is not only important to reduce matrix effects but also to determine isobars that are frequently found in endocrinology, as shown further in this review.

Another challenge in the replacement of immunoassays by LC–MS/MS is the establishment of new reference intervals that are essential for interpreting the results. Among clinical laboratory tests, the determination of reference intervals for hormones is especially complicated given that hormone levels change over time in response to chronobiological and/or external stimuli. Therefore, it is necessary to determine reference intervals for several categories such as age, gender, body mass index, time of the day, and body position during sample collection. One of the main difficulties in determining reference intervals is the selection of "healthy populations." Reference intervals for immunoassays were determined over nearly 50 years of studies; consequently, it will take time to solidify the new reference intervals for LC–MS/MS methods among endocrinologists.

One of the greatest powers of mass spectrometry is the possibility to include stable-isotope-labeled internal standards. Isotopic analogues are added in the first step of the analytical method to compensate for errors during all stages of the analysis. Stable isotope analogues are available for most hormones. This text includes only isotopic dilution methods; therefore, LC–MS/MS is here assumed as a synonym for isotope dilution LC–MS/MS.

This review intends to cover the status of the application of LC–MS/MS in the routine of laboratorial endocrinology. Not only the advantages over competing methodologies are discussed but also important challenges that most clinical laboratories face in replacing traditional methodologies by LC–MS/MS. The following sections describe applications used for three classes of hormones: steroids, derivatives of the aromatic amino acids, and peptides and proteins.

2. Steroids

LC–MS/MS was rapidly embraced as the method of choice for steroid analysis. Currently, most endocrinologists are already aware of the advantages afforded by this technique compared to immunoassays. These include better specificity and the possibility of performing simultaneous measurement of several analytes.

Although gas chromatography–mass spectrometry (GC–MS) with isotope dilution internal standardization methods are still considered by some as the gold standard for steroid analysis [21–23] and are still very important for studies of complete metabolic pathways [24], several reference methods based on isotope dilution and LC–MS/MS have been proposed [25–29]. This turned the LC–MS/MS-determined steroids into one of the best-established techniques in the clinical laboratory. Recent reviews focusing mostly on steroids have covered other issues such as basic principles of ionization techniques and detection by tandem mass spectrometry and strategies for analyte extraction and separation [30–34].

Although well established in clinical laboratories, the use of LC–MS/MS for steroid analysis is still challenging. One of the main complicating factors is the presence of isobaric interferences, mainly in the case of isomers [18]. Most steroids of clinical interest present endogenous and/or pharmaceutical isomeric interferents such as testosterone and epitestosterone [35], cortisone and prednisolone [26,36,37], and 17-hydroxyprogesterone and 11-desoxycosticosterone [38]. Isomeric discrimination by mass spectrometry can be achieved in exceptional situations where one of the isomers undergoes distinct ionization routes producing an exclusive ion [39], but in most cases mass spectrometry is a poor technique for isomer discrimination. While techniques such as travelling-wave ion mobility, which are promising as a complement to mass spectrometry for the analysis of isomers [40], are not available commercially in triple quadrupoles, chromatography is essential for accurate determination of steroids. The acquisition of two or more mass transitions for each analyte is an important aspect in the identification of co-elution with isobaric interferences [41]. Unfortunately, the use of "ion ratios" or "branching ratios" as a critical criterion for LC–MS/MS analysis has not been mandatory in clinical routine.

Through the 15 years of the application of LC–MS/MS for steroid analysis, an abuse of the use of fast chromatography has been observed. The most common stationary phases used in LC–MS/MS have limited capability to provide isomer resolution in short analysis. With the gain in sensitivity of new instruments it is very likely that natural isomers will emerge from unresolved chromatographic peaks.

2.1. Corticosteroids

The determination of cortisol in serum and urine was one of the first well established endocrinology routines using LC–MS/MS [42]. Cortisol is an important marker for the diagnosis of

Cushing syndrome, for the evaluation of apparent mineralocorticoid excess, congenital adrenal hyperplasia, and adrenal insufficiency. Cortisol is extremely abundant in serum, with levels in the order of hundreds of nmol/L, therefore it is easily detected even with low-performance LC–MS/MS instruments. Besides its high concentration in serum, like other 3-oxo-4-ene (Δ^4)-structure steroids, detection of cortisol is favored by its relatively high proton affinity. Therefore, good response can be achieved using positive electrospray [37,43], positive atmospheric pressure chemical ionization (APCI) [38], and photoionization [44,45]. The analysis of cortisol in urine collected over 24-h periods is also very important in endocrinology routine and several applications have been described using strategies similar to those used for serum [42,46–52]. Recently, cortisol has also been used as a biomarker of stress. However, serum and urine are not good matrices for this purpose since blood sampling may induce stress and urine represents a delayed and averaged response to stress. Saliva is an excellent alternative matrix to assess stress by cortisol levels because of the good correlation between salivary cortisol and unbound serum cortisol [53]. Additionally, its collection is non-invasive and thus stress-free. Unbound (free) hormones are gaining importance in endocrinology because they reflect the biologically active fraction. Salivary cortisol, as is the case with other free hormones, is found in lower levels compared to the total fraction and requires more sensitive methods for its detection [52,54–56].

The quantification of precursors and metabolites of cortisol is also of great interest for the diagnosis of several adrenal disorders as the determination of the ratios between precursor and metabolite allows the evaluation of the activity of enzymes involved in cortisol metabolism. Consequently, the application of LC–MS/MS is very useful as it allows simultaneous quantification of multiple metabolic intermediates. Cortisol metabolites have been efficiently determined by LC–MS/MS, such as cortisone [38,46,49,52], 6β-hydroxycortisol [47], and tetrahydrocortisols [49]. The determination of 11-desoxycortisol and 21-desoxycortisol, immediate cortisol precursors, is also important in the evaluation of 11β- and 21-hydroxylases, respectively [57,58]. Recent LC–MS/MS methods allowed accurate quantification of low levels of 11-desoxycortisol and 21-desoxycortisol [38,59].

The measurement of 17α-hydroxyprogesterone by LC–MS/MS is widely employed among clinical laboratories. According to the most recent College of American Pathologists survey, 35% of the participants were using LC–MS/MS for 17α-hydroxyprogesterone analysis. The 17α-hydroxyprogesterone is a very important steroid used for the diagnosis of congenital adrenal hyperplasia, an inherited disease with relatively high incidence. Congenital adrenal hyperplasia includes defects of several enzymes from steroid biosynthesis pathways, but about 95% of the cases are related to 21-hydroxylase deficiency, which results in 17α-hydroxyprogesterone accumulation. With endogenous levels in the order of nmol per liter, the quantification of 17α-hydroxyprogesterone has been successfully achieved by many groups using positive electrospray [60–63], positive APCI [38,64], and atmospheric pressure photoionization (APPI) [45].

Two precursors of the 17α-hydroxyprogesterone, namely pregnenolone and 17α-hydroxypregnenolone, are important to elucidate other defective enzymes related to congenital adrenal hyperplasia. However, due to their 3β-hydroxy-5-ene (Δ^5)-structure, they have low proton affinities, resulting in poor responses using either electrospray or APCI [65]. Therefore, derivatization has been required to improve sensitivity through the introduction of a group with higher proton affinity. The formation of oxime derivatives by the reaction of hydroxylamine with keto groups is a simple derivatization procedure and it is very effective in achieving the sensitivity required to detect endogenous levels of pregnenolone and 17α-hydroxypregnenolone [62,66].

The application of LC–MS/MS for aldosterone determination is also growing. Aldosterone is the most potent regulator of electrolyte excretion and its determination is important for the diagnosis of primary aldosteronism, which accounts for 5% of hypertensive patients. This hormone has an unusual structure among corticosteroids, consisting of an equilibrium of three structural isomers: 18-aldehyde, 11β,18-oxide, and 18-acetal-20-hemiketal [67]. Due to this structural feature, the deprotonated molecule is more efficiently detected in negative mode by APCI [68], electrospray [69,70], and APPI [45]. The physiological concentrations of aldosterone are very low, starting from tens of pmol per liter, thus requiring very sensitive methods.

2.2. Androgens

Testosterone testing is one of the most important tests routinely done in endocrinology. It is used for the evaluation of puberty progression and hypogonadism in men whereas in women it diagnoses several conditions such as hyperandrogenism, oligo- or amenorrhea, virilization, acne, and infertility. Due to very well documented weaknesses of immunoassays regarding accuracy, precision, specificity, and sensitivity, LC–MS/MS was a long-awaited alternative to improve the clinical use of testosterone. In response to this need, several methods based on LC–MS/MS have been published in the last 8 years [29,45,64,71–81].

A conference held a few years ago by the Endocrine Society, Centers for Disease Control and Prevention, and other representatives of professional societies, government, and industry aimed to reach a consensus about testosterone assay standardization. The need for an inter-laboratorial study to assess the measurement variability among laboratories using mass spectrometry was one of the main recommendations. Thus, an inter-laboratory study was conducted by eight research and commercial laboratories [82]. The study showed that data produced by different LC–MS/MS assays are more comparable than data generated by other immunoassays.

However, problems related to LC–MS/MS precision and accuracy were detected. Most of the assays studied were not able to meet the precision criterion based on biological variability at low concentrations, indicating the need for improvement. Individual assays showed significant differences in slopes and intercepts. These findings reinforce the need to standardize different LC–MS/MS methods against reference measurement procedures [29,83]. Lessons can be learned from this inter-laboratory study [82] and might usefully be extended to most of the analytes presently assayed by LC–MS/MS. Although LC–MS/MS has surpassed other competing analytical techniques for hormone determination, it still requires standardization to reach maturity.

Dihydrotestosterone (DHT) is produced by reduction of testosterone by 5α-reductase and due to its greater affinity for androgen receptors it has 3–10 times greater androgenic potency. The DHT measurement is important in the monitoring of patients receiving 5-alpha-reductase inhibitor therapy and in the evaluation of 5-alpha-reductase deficiency. The DHT determination is more challenging than is the case for testosterone due to its lower endogenous levels. In addition, its saturated 3-oxosteroid structure has lower proton affinity and some groups reported the need for derivatization to enable adequate detection [84–86]. The use of new tandem mass spectrometers with improved sensitivity seems to be allowing the analysis of underivatized DHT, as reported in previous studies [87–89].

Dehydroepiadrosterone (DHEA) is the precursor or prohormone for the sex steroids, and is produced mainly by the adrenal gland. The 3-sulfoconjugate (DHEA-S) form is the most abundant steroid in blood. It is found in a concentration of μmol per liter and therefore is efficiently determined by automated direct immunoassays. The non-sulfated form is also useful in endocrinology for the

evaluation of acute adrenal responses to stimuli or depression. However DHEA, like other steroids of 3β-hydroxy-5-ene (Δ^5)-structure, presents low ionization efficiency, thus direct detection is not favored. Derivatization has been necessary to provide the sensitivity required for accurately quantifying endogenous levels of this hormone. The application of 2-hydrazino-1-methylpyridine, a derivatization reagent having a permanently charged moiety, for example, improved by 1600 times the detection of DHEA and allowed its determination in saliva using small sample volumes [79,90]. The conversion to oxime derivatives has also been applied successfully to improve limits of quantification of DHEA [80].

Androstenedione is another intermediate in the biosynthesis of androgens and estrogens and its measurement is useful for the diagnosis of hyperandrogenism and detection of androgen-producing adrenal or gonadal tumors. As with other 3-oxo-4-ene (Δ^4)-structure steroids, detection of androstenedione is straightforward by positive-mode electrospray [63,76,80,81], APCI [91], and atmospheric pressure photoionization (APPI) [45].

2.3. Estrogens

Estrogens are responsible for the development and maintenance of the female phenotype, for germ cell maturation, and for pregnancy. The determination of estradiol in serum is one of the most important common tests in the routine of any clinical laboratory, as this parameter is used for important evaluations such as female reproductive function, infertility, and menopausal status. Testing estrone levels is less frequent. The test is used to evaluate post-menopausal estrogen levels produced by peripheral aromatization of androstenedione. The low sensitivity of immunoassays for the evaluation of estrogens in the sera of children and men led to the development of numerous assays based on LC–MS/MS in the last decade [27,89,92–99]. Although estrogen's aromatic structure favors its ionization by negative mode, many assays have incorporated a derivatization step to improve its detectability [92–97,99], including a reference method [27]. The combination of liquid-liquid extraction and derivatization results in excellent sensitivity but also in very labor-intensive procedures. Although gain in analytical quality is unquestionable, for high-volume testing such as that done routinely for estradiol, the shift from fully automated immunoassays to highly manual methodology has to be carefully examined in terms of logistics. Recently, high-performance tandem mass spectrometers have allowed estrogen quantification without derivatization [89,98]. Simplification in sample preparation, without compromising quality, is an important step towards the incorporation of LC–MS/MS for high-throughput steroid testing, but it is still far from the desired full automation.

2.4. Vitamin D

Vitamin D_3 is produced in the skin through a photocleavage of 7-dehydrocholesterol promoted by ultraviolet radiation. Vitamin D_2 is acquired in the diet, usually as a vitamin supplement. Both vitamins are prohormone and are activated sequentially in the liver producing 25-hydroxyvitamin D and finally in the kidneys and most tissues into the real hormone, 1,25-dihydroxyvitamin D. The 25-hydroxy vitamins D_3 and D_2 are the most important circulating forms and therefore they are used as markers for vitamin D status. Together with parathyroid hormone, vitamin D promotes elevation of calcium concentration in serum by increasing intestinal absorption and release from bones. It has been demonstrated that vitamin D transcends its endocrinal function in calcium homeostasis and evidence of its protective role against cancer and infectious diseases has been found. Vitamin D deficiency is widely spread across different populations around the world and it has been recognized as a deficiency of our modern times due to lack of adequate sun exposure.

All these factors have resulted in a large demand for vitamin D testing in most clinical laboratories around the world. As described previously for other steroids, LC–MS/MS is well suited for vitamin D quantification and many applications are available in the literature. In fact, the quantification of 25-hydroxyvitamin D is one of the more important applications of LC–MS/MS in the clinical laboratory. Recently, an excellent in-depth review covered the most important analytical aspects of vitamin D quantification by LC–MS/MS [100].

A recent episode regarding problems with the quantification of 25-hydroxyvitamin D in a large US commercial laboratory resulted in negative publicity for LC–MS/MS and raised questions about the reliability of this technique in clinical laboratories [101]. Although large interlaboratory studies have not yet been published, the standardization of 25-hydroxyvitamin D quantification by LC–MS/MS is being conducted worldwide and candidate reference methods have been described [102–104]. The application of the serum-based reference materials recently made available by the US National Institute of Standards and Technology (SRM 972) will contribute significantly to harmonize different LC–MS/MS approaches to 25-hydroxyvitamin D quantification.

3. Amino acid-derived hormones

3.1. Thyroid hormones

Thyroxine (T4 or 3,3′,5,5′-tetraiodo-L-thyronine) and triiodothyronine (T3 or 3,3′,5-triiodo-L-thyronine) are tyrosine-derived hormones with relevant physiological roles such as regulation of cellular metabolism and cell differentiation. T4 is secreted by the thyroid gland and converted in the tissues by deiodinases into the more active form T3. Determining T4 and T3 levels is fundamental in the evaluation of the thyroid gland function and the diagnosis and follow-up of hyper and hypothyroidism. The first determinations of T4 by LC–MS/MS were made just after the first commercial triple quadrupole instruments became available [105], as an alternative reference to GC/MS. This early interest in LC–MS/MS was due to the difficulties associated with GC/MS analysis such as multiple steps for analyte extraction and derivatization, resulting in low recovery and poor precision and accuracy. Later, other candidate reference methods with straightforward procedures for sample preparation were proposed, such as solid phase extraction [106,107]. The determination of T4 is easy, as it is present in high concentrations in blood (in the order of 100 nmol/L) and it has ionizable groups. On the other hand, analysis of T3 is more complicated because its concentration in blood is 100 times lower and it can be artifactually produced from T4 during sample preparation [108,109]. As reference methods, these first-generation assays were not meant for the daily clinical routine of T3 and T4 determination.

Recently, most endocrinologists have turned their attention to the concentration of free hormones. Only 0.04% of T3 and 0.4% of T4 are not bound to carrier proteins such as thyroxine binding protein, transthyretin, and albumin. The assessment of thyroid activity by the determination of total T3 and T4 is often unreliable because the binding protein concentration can fluctuate in function of several factors such as health status, pregnancy, and the presence of congenital diseases. Therefore, it is now widely accepted that only this small fraction of unbound hormones are able to exert their physiological effects. However direct determination of the free T3 and T4 is extremely difficult because they are present in the low picomolar range in the serum. The most recent generation of high-performance triple quadrupoles have been able to provide adequate sensitivity to achieve accurate

determination of low concentrations of free T3 and T4 [110–114]. Another challenge is to obtain their physical separation from binding proteins without disturbing the subtle equilibrium between bound and unbound hormones. In fact, the process for physical separation of the free fraction from the protein-bound hormones has been the subject of a current debate on the analytical aspects of determining free T3 and T4. Equilibrium dialysis and ultrafiltration are appropriate to separate the free hormone prior to analysis [110–114]. Ultra-filtration has the advantage of being significantly faster than equilibrium dialysis [110,112,115]. However some groups have opted for equilibrium dialysis after detecting some technical drawbacks in ultra-filtration such as the need for centrifuge preheating and accurate thermostatic control during centrifugation, as well as the observation of device- or batch-dependent protein leakage [111].

3.2. Catecholamines and metabolites

Catecholamines are hormones and neurotransmitters derived from tyrosine that have broad effects over most tissues. They comprise epinephrine, norepinephrine, and dopamine and are synthesized mostly in the adrenal gland and nervous tissue. Epinephrine, norepinephrine, and dopamine have short half-lives in plasma and are rapidly inactivated to, respectively, metanephrine, normetanephrine, and 3-methoxythyramine by catechol-O-methyltransferase. Metanephrine and normetanephrine are further catabolized to vanillylmandelic acid, and 3-methoxytyramine to homovanillic acid. In endocrinology, the catecholamines and their metabolites are useful markers for the diagnosis of the neuroendocrine tumors pheochromocytoma, paranganglioma, and neuroblastoma. The use of LC–MS/MS for quantification of catecholamines and metabolites has been reviewed by de Jong et al. [116].

Due to its ease of use, analysis of catecholamines in urine is one of the most popular tests for diagnosis of neuroendocrine tumors. Quantification of urinary catecholamines is acceptably achieved by HPLC combined with electrochemical detection after previous analyte extraction [117,118]. The introduction of LC–MS/MS for quantification of urinary catecholamines a decade ago reduced interference from co-eluting compounds such as drugs and reduced acquisition times [119–121]. Analyte extraction procedures have been adapted from those used in conjunction with HPLC with electrochemical detection, such as ion exchange [119], interaction with phenylboronic acid [120,121], or interaction with alumina [122].

The determination of plasma catecholamines is much more complicated than is the case for urinary catecholamines, not only because of lower concentrations but because of the great effect of several pre-analytical factors (reviewed by [123]). The blood sample is affected by the body position at the time of collection, the time of day collection is made, and whether or not the individual is a smoker or has drunk coffee. Many procedures to increase the stability of catecholamines in plasma have been suggested, such as the addition of antioxidants and sample storage at −80 °C. The ideal pre-analytical conditions are hard to achieve in most clinical laboratories and even sensitive and specific LC–MS/MS might not compensate for errors introduced in collection and sample preparation. Nevertheless, some assays have been developed to quantify plasma catecholamines by LC–MS/MS [124,125].

The quantification of plasma metanephrines (metanephrine and normetanephrine) has gained favor with endocrinologists since its superior discrimination value in the diagnosis of pheochromocytoma was accepted [126]. In addition, plasma metanephrines are less susceptible to pre-analytical variability including collection and storage prior to analysis. Like catecholamines, plasma metanephrines have been initially analyzed by HPLC with electrochemical detection [127]. However due to limitations in the sensitivity and specificity of electrochemical detectors, the procedure is susceptible to interference from co-eluting drugs and requires extensive sample preparation and lengthy acquisition times. The application of LC–MS/MS considerably reduced the acquisition times by four- to five-fold, and sensitivity was improved by 10-fold [128–130]. Sample preparation was dramatically simplified by the introduction of online solid-phase extraction coupled to LC–MS/MS [129]. In addition, a recent application incorporated 3-methoxytyramine to the quantification of metanephrines [129], which is useful for detection of dopamine-producing tumors such as paragangliomas and neuroblastomas.

Vanilmandelic and homovanillic acids are usually measured in urine together with 5-hydroxyindoleacetic acid by HPLC with electrochemical detection [131–133]. As previously discussed for other catecholamines and metabolites, the advantage of using LC–MS/MS over electrochemical detection is the reduced acquisition times and simpler sample preparation procedures. As vanilmandelic, homovanillic, and 5-hydroxyindoleacetic acids are found in elevated levels in urine, even dilute and shoot assays have been employed for their determination. However, matrix effects have not been investigated [134].

3.3. Serotonin

Serotonin is a tryptophan-derived neurotransmitter and an important mediator of the gastrointestinal physiology. Although it is usually associated with depression and other mental disorders, the most important clinical application of serotonin measurement is the detection and follow-up of carcinoid tumors. Actually, blood levels of serotonin do not reflect brain levels because serotonin does not cross the blood–brain barrier. Most serotonin is produced and stored in the duodenum by enterochromaffin cells, and then is taken up and transported by platelets. In healthy individuals, 99% of circulatory serotonin is stored in platelets. The high sensitivity provided by LC–MS/MS is very important for the determination of free serotonin in platelet-poor plasma. Online solid-phase extraction employing strong cation exchange [135] or weak cation exchange [136] has been used for first column, and reverse phase [135] or hydrophilic interaction chromatography has been used for second column [136].

Although less sensitive than serotonin, urinary 5-hydroxyindoleacetic (5-HIAA), the major metabolite of serotonin, is also used for the detection of carcinoid tumors. The quantification of urinary 5-HIAA is easily accomplished by HPLC with electrochemical detection [132,133,137]. The application of LC–MS/MS for 5-HIAA quantification in urine noticeably simplified sample preparation, and approaches based on direct injection [138] and online extraction [136,139,140] have been described.

4. Peptide and protein hormones

Endocrinology has been revolutionized by the unmatched capabilities of LC–MS/MS to quantify small molecules. Nonetheless, the contribution of this technique to endocrinology is far from having reached its limit. One of the most promising applications of LC–MS/MS not only to endocrinology but also to other specialties in clinical chemistry is the quantification of peptides and proteins. In addition to previously mentioned sources of interference with immunoassays such as heterophilic antibodies, a particular antibody employed in an immunoassay recognizes only a small portion of the antigen molecule. Consequently an immunoassay may not detect important structural variations occurring in other regions of the antigen molecule such as post-translational modifications. This fact can explain part of the variability found among different immunoassays [141,142]. The quantification of peptide and

protein hormones by LC–MS/MS will not only eliminate some limitations associated to current immunoassays, it will also provide valuable new information about structural variability and physiological meaning.

The first report of protein quantification employing stable isotope dilution and liquid chromatography and mass spectrometry dates from the pioneer work of Barr et al. in 1996 [143]. However, the first applications to hormone quantification are reported more than a decade later [144,145]. These methods were not meant for the clinical routine but for standardization of immunoassays. Although considerable progress has been recently observed in terms of analytical strategies for quantification of proteins by LC–MS/MS, the principles are still based on the strategy described by Barr et al. [143]. Protein digestion releases peptides that are selected as surrogate for the whole protein. Surrogate peptides are then detected by selected reaction monitoring and quantified by isotope dilution similarly to small molecules. Due to the extreme dynamic range of proteins in plasma, with peptide and protein hormones being found at the very low concentration end, it becomes necessary to apply an enrichment step. Immunoaffinity purification is the most efficient process for hormone enrichment and it can be achieved before [146] or after protein digestion [147].

The quantification of serum thyroglobulin is a good illustration of some of the limitations of immunoassays and the potential of LC–MS/MS to overcome these limitations. Thyroglobulin is the thyroid prohormone and one of the serum tumor markers best validated. Due to its clinical relevance, several immunoassays for thyroglobulin are commercially available but great variability has been detected among them [148,149]. The difficulties in thyroglobulin quantification can be explained by its low levels in serum and its extremely complex structure with a molecular mass of 660 kDa and a diversity of post-translational modifications such as glycosylation, iodination, phosphorilation, and sulphation [150–153]. Recently, the application of the method termed “stable isotope standards and capture by antipeptide antibodies” (SISCAPA) [147] allowed the detection of 4 pmol/L of thyroglobulin in human serum by LC–MS/MS [154].

Another advantage of LC–MS/MS over immunossays is the possibility to perform multiplex assays. Lopez et al. [155] described an application for simultaneously monitoring full-length parathyroid hormone (PTH1-84) and a truncated variant (PTH7-84). Unlike thyroglobulin, PTH is a small protein with a relatively well-characterized structure and several peptide fragments described. These fragments exert biologic effects distinct from those of the intact form, and immunoassays often present cross-reaction between variants [156,157]. A variety of different mass spectrometry techniques have been applied to the characterization of new structural variants [158]. Quantitative LC–MS/MS methods [155] have been fully validated and presented a sensitivity lower than 1 pmol/L, which is close to the sensitivity reached by immunoassays.

5. Conclusions

The introduction of LC–MS/MS into the routine of the endocrinology laboratory is one of the most significant advances since the development of immunoassays. This technique has increased the value of several hormone tests and has become the gold standard, in many cases in a relatively short period of time. However, only a few applications have been rigorously standardized and a number of problems and limitations of the technique have been noticed, such as matrix effects, interference from isobaric compounds, and poor comparability among different assays. These might be explained by the recent introduction of LC–MS/MS and also by false assumptions about its infallibility.

Inter-laboratory studies and the availability of a wider range of reference materials will be essential to achieve standardization. Because many hormones are presently processed by fully automated immunoassay systems, the introduction of LC–MS/MS can initially negatively affect laboratory logistics because of the frequent manual steps involved in the analytical procedure. Manual sample preparation is more susceptible to errors, and delays results. Therefore improvements in automation of LC–MS/MS are essential for a wider application of this technique among endocrinology laboratories. The potential of LC–MS/MS is still enormous and it is likely to be very important for the analysis of most peptide and protein hormones, as it has been for small molecule hormones.

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