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Review

Quantification of circulating 25-hydroxyvitamin D by liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

Hypovitaminosis D is a highly prevalent condition and quantification of serum 25-hydroxyvitamin D3 is accepted to be the most useful marker for the assessment of the individual vitamin D status. Due to the increasing awareness of the prevalence and potential health consequences of hypovitaminosis D, the request numbers for 25-hydroxyvitamin D quantification are growing rapidly in many countries. Automated protein binding assays (based on the use of vitamin D-binding protein or antibodies) for the quantification of 25-hydroxyvitamin D3 are available which enable convenient high-throughput analyses in a routine setting; there is, however, substantial concern about accuracy and analytical reliability of these assays. Several LC–MS/MS methods for the quantification of 25-hydroxyvitamin D3 in serum have been described and in a growing number of clinical laboratories this technology is used routinely for vitamin D monitoring. It is justified to assume that LC–MS/MS enables more reliable analyses of 25-hydroxyvitamin D concentrations compared to protein binding assays. In particular the ability to co-quantify the naturally occurring 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 which is derived primarily from food fortification is a relevant advantage of LC–MS/MS over protein binding assays. This review describes the background of 25-hydroxyvitamin D measurement, compares published LC–MS/MS methods, discusses problems, strengths and limitations of these assays and compares the application characteristics of LC–MS/MS with those of protein binding assays and HPLC-UV.

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1. Background of 25-hydroxyvitamin D3 measurement

The vitamin D endocrine system plays an essential role in the calcium homeostasis of the body [1,2]. Vitamin D3 (cholecalciferol) is formed from its precursor 7-dehydrocholesterol in the skin by ultraviolet irradiation. In the liver vitamin D3 undergoes hydroxylation to 25-hydroxyvitamin D3, which is further metabolized to the active metabolite 1,25-dihydroxyvitamin D3 in the kidney. Vitamin D3 can also be absorbed from the diet, which is important in case of insufficient sun exposure. Fatty fish naturally contains high amounts of vitamin D3, whereas other foods contain relevant amounts of vitamin D only after fortification. For fortification in many countries vitamin D2 (ergocalciferol) is used, which is derived from plants sources.

Severe deficiency of vitamin D during childhood can cause rickets, a disorder that became prevalent during expansive industrialization and urban migration to "sunless" and polluted cities. During the first half of the 20th century the vitamin D endocrine system was discovered and disturbances of this system were recognized to cause rickets [3]. Eradication of rickets by vitamin D supplementation to children was an essential achievement of modern medicine.

During the following decades, vitamin D deficiency and the vitamin D endocrine system in general was perceived to be predom-

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inantly linked with the health of the skeletal system in humans. The vitamin D system controls the absorption of calcium from the diet, but only severe hypovitaminosis D is associated with decreased serum calcium. Already latent hypocalcemia, however, leads to increased blood concentrations of the parathyroid hormone (PTH). Consequently, increased PTH can be indicative of milder normocalcaemic forms of vitamin D deficiency. Since increased PTH concentrations are also found in primary hyperparathyroidisms (albeit associated with hypercalcemia) and since PTH shows a poor sample stability, this analyte is not routinely used to assess the vitamin D status. Nevertheless, hypovitaminosis D can be defined as a condition where supplementation of exogenous vitamin D leads to a decrease of PTH concentrations. Measurement of the active vitamin D metabolite 1,25-dihydroxyvitamin D3 in serum, on the other hand, has been found to be not useful in the assessment of the vitamin D status: paradoxically – probably due to secondary hyperparathyroidism – even increased concentrations may be found in hypovitaminosis D [4]. Moreover the concentrations of 1,25-dihydroxyvitamin D3 are extremely low (ng/L range) and measurement is very demanding. For these reasons, quantification of 25-hydroxyvitamin D3 in serum became accepted to be the most useful marker of the vitamin D status. The analyte is highly stable in serum [5].

Laboratory methods applicable to assess the vitamin D system in the setting of routine clinical chemistry became available with the introduction of ligand binding assays for the quantification of 25-hydroxyvitamin D in the 1970s [6,7]. The first competitive protein binding assay (PBA) employing vitamin D-binding protein for analyte binding was introduced 1971. The use of HPLC with UV detection was described in 1977; and a protein binding assay based on antibodies was introduced in 1985.

The constantly increasing use of these routine tests in clinical medicine disclosed a very high prevalence of hypovitaminosis D [2,8]. This also applies to many sunny countries [9–11], either due to traditional local clothing or due to very intense use of sunscreen in the context of prevention of melanoma [12,13] in combination with a sedentary lifestyle. It is assumed that approximately half of the world's elderly have insufficient vitamin D status [14], in particular during autumn and winter.

During the 1990s, research from various areas suggested a significant role of the vitamin D system for health beyond the bone. Since vitamin D receptors were found in a wide variety of tissues, pleiotropic actions of this endocrine system can be assumed. These findings were in line with a growing number of epidemiological studies, linking hypovitaminosis D with diseases such as diabetes, hypertension, arteriosclerosis, cancer, multiple sclerosis, and others [2,15].

Due to the increasing awareness of potential effects of hypovitaminosis D on health, the number of requested analyses again increased substantially and high-throughput analytical methods became desirable. In 2001 the first automated 25-hydroxyvitamin D test was introduced [7]. At present two automated tests are available implemented on multi-channel-random access analyzers (Roche and DiaSorin) and several companies of in vitro diagnostics will soon follow to introduce automated methods. At present besides automated tests still manual immunoassays with radioactive and non-radioactive labels (ELISA) are in use, as well as HPLC-UV methods.

The rapidly growing awareness of the (more or less global) problem of hypovitaminosis D is certainly an important achievement of the past decade in medical research and was based on convenient analytical methods which provide large epidemiological data sets. It is, however, uncertain if widespread measurement of serum 25-hydroxyvitamin D3 is a useful answer to this issue: hypovitaminosis D is reliably avoided by the administration of 1000–2000 IU of vitamin D per day. If strict protection from sunlight is applied aiming to reduce the risk of skin cancer, such vitamin D supplementation may also be necessary during summer [16,17]. Most reliably vitamin D3 (cholecalciferol) is used for this inexpensive supplementation (<3€ per month), which can probably be recommended to all adults at least during the dark months on the northern hemisphere (with dose adaptation in children and adolescents) [15,18]. Given uncompromised gut absorption, measurement of serum 25-hydroxyvitamin D does not seem useful with such standard does of vitamin D [19]. It must be noted that the safe and efficient substitution of 25 μg cholecalciferol for 1 year causes cost per person in the range of a single measurement of 25-hydroxyvitamin D3. Even in otherwise healthy patients with established osteoporosis, measurement of 25-hydroxyvitamin D is generally not assumed to be useful if the recommended supplementation with 25 μg vitamin D daily is reliably administered. Measurement of serum 25-hydroxyvitamin D3 can be useful in case of severe and etiological uncertain osteopenia and in patients with potentially impaired gut absorption (e.g. cystic fibrosis, chronic inflammatory bowl diseases). Furthermore analyses are useful in patients with increased PTH concentrations to differentiate mild hyperparathyroidism from hypovitaminosis D as the cause of increased PTH. Certainly in the context of epidemiological studies quantification of 25-hydroxyvitamin D is of major scientific and public health importance. Optimum vitamin status is currently assumed for serum 25-hydroxyvitamin D3 concentrations above 30 μg/L[1] [20,21]; concentrations below 10 μg/L are considered as severe hypovitaminosis D. Vitamin D-intoxication with concentrations above 150 ng/mL which can be associated with hypercalcemia is found extremely rarely [2].

Regardless of these above considerations, 25-hydroxyvitamin D screening of healthy individuals was promoted intensively during recent years by many physicians and commercial laboratories mainly in the USA. Consequently, 25-hydroxyvitamin D testing became one of the economically most interesting assays for laboratory test providers.

It was clearly recognized from the 1970s on that quantification of 25-hydroxyvitamin D3 in serum is a particular analytical challenge due to several issues [6,7,22]: the analyte is bound to vitamin D-binding protein with high avidity, and a complete release from this bond is essential for reliable results. This is best achieved by serum precipitation with organic solvents as acetonitrile. In fully automated assays the complete release of 25-hydroxyvitamin D from its bonds is difficult to obtain since organic solvents are incompatible with analytical antibodies. In general, competitive protein binding assays are rather sensitive to matrix effects, much more than applies for double-antibody sandwich immunoassays which can be applied for analytes with a larger number of potential epitopes. Furthermore, differential cross-reactivity of ligand binders to naturally occurring 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 of pharmaceutical origin was recognized as a potential confounder of 25-hydroxyvitamin D results in regions where vitamin D2 is used [23].

Whereas GC–MS reference methods had been developed for the validation and in the context of standardisation of clinically used routine assays for steroid hormone quantification (e.g. testosterone, cortisol), the implementation of GC–MS reference methods for the standardisation of 25-hydroxyvitamin D was found to be extremely complex and was not widely used in assay development or in quality assurance programs. This sub-optimal standardisation and validation of 25-hydroxyvitamin D measurement by ligand binding assays became more and more perceived from 2000 on when automated assays became available. Among

[1] Conversion of unit: 25-hydroxyvitamin D3: [μg/L] × 2.5 = [nmol/L].

other reports, data from Binkley et al. [21,24] discredited the quantification of 25-hydroxyvitamin D in general due to very substantial interlaboratory variability in results, and differential reactivity with 25-hydroxyvitamin D2 [25]. It was also suspected that epidemiological findings and considerations about optimum 25-hydroxyvitamin D serum levels were flawed by analytical shortcomings and poor standardisation [26].

2. Introduction of LC–MS methods for the measurement of 25-hydroxyvitamin D

The advent of the LC–MS/MS technology in clinical chemistry [27–29] promised to overcome these essential limitations of protein binding assays used for serum 25-hydroxyvitamin D measurement. LC–MS/MS enables highly specific analyses since detection is based on molecular mass filtration of the ionized target analyte and of a fragment ion which is generated by collision with gas molecules. Thus detection includes twofold mass selection incorporating the molecular fragmentation patterns of the target analyte. Furthermore, the principle of isotope dilution internal standardisation is applicable in quantiative LC–MS/MS methods allowing to compensate for matrix dependent variances in sample extraction and ionisation. LC–MS/MS typically allows straightforward development and implementation of new methods. Typically – in contrast to GC–MS – derivatisation is not required as sample preparation for LC–MS/MS; the requirements for sample clean-up are limited and the analytical run time is much shorter compared to GC–MS. Whereas GC–MS did not find its way into common routine clinical laboratories, LC–MS/MS is much more compatible with the workflow of modern clinical laboratories due to these features and is used in a constantly growing number of clinical laboratories now.

Indeed, the development of LC–MS/MS methods for the quantification of 25-hydroxyvitamin D in serum was found to be feasible and relatively simple, which is in sharp contrast to attempts with GC–MS [30].

Higashi et al. [31] described for the first time a LC–MS method for the quantification of 25-hydroxyvitamin D3 and D2 in human serum. Atmospheric pressure chemical ionisation (APCI) was used for ionisation with a ThermoQuest LCQ ion trap-mass spectrometer. Although this instrument allows tandem fragmentation of selected ions, for quantification mere molecular mass selective detection was used. 25-Hydroxyvitamin D4 was used as the internal standard. Sample preparation involved a two-step liquid extraction with a Cookson-type reagent derivatisation. Due to the laborious sample preparation this method did not find widespread use. In 2008 [32] the authors reported an adaptation of their derivatisation method to the analysis of saliva for 25-hydroxyvitamin D3, a diagnostic approach that has not found widespread interest.

The first LC–MS method employing MS/MS detection for the quantification of 25-hydroxyvitamin D3 in serum was described in 2004 [33]; the method employed on-line solid phase extraction after protein precipitation. All subsequently published LC–MS/MS methods have also incorporated the simultaneous quantification of 25-hydroxyvitamin D2 together with 25-hydroxyvitamin D3 (Table 1). Aronov et al. [40] have described a research method for the sophisticated profiling of a variety of vitamin D metabolites.

Published methods for the quantification of 25-hydroxyvitamin D use electrospray ionisation (ESI) as well as atmospheric pressure chemical ionisation (APCI). The latter technology is often superior to ESI for the analysis of rather apolar compounds but the performance characteristics for specific compounds are largely instrument dependent.

The detection limit of published methods is typically in the range of 1 μg/L, with a severe hypovitaminosis being characterized by concentrations of less than 10 μg/L. Probably most LC–MS/MS instruments used to date in clinical laboratories are sufficiently sensitive to enable useful quantification of 25-hydroxyvitamin D in serum; high-end instruments are not a prerequisite, as applies, e.g. for the quantification of plasma metanephrins.

Most LC–MS/MS methods describe a standard sample volume of 100 μL for the quantification of 25-hydroxyvitamin D; this volume is rather high with respect to application in paediatrics, but substantial down-scaling is probably possible with most instruments for such analyses.

Eyles et al. [41] have adapted a LC–MS/MS method to the quantification of 25-hydroxyvitamin D in dried blood spots, which may be an interesting tool in epidemiological studies, since venipuncture is avoided and even self-sampling by study participants seems feasible.

In all LC–MS/MS methods described for the quantification of 25-hydroxyvitamin D in serum, analyte detection is based on the singly protonated molecular ion of 25-hydroxyvitamin D3 with a molecular mass of 401; no adducts are described. The fragment ion species used for quantification differ between the methods (Table 1). The efficacy in the formation of particular fragment ion species is often very instrument specific and there is at present no data available that would favour one certain transition with respect to analytical reliability. However, a typical chromatogram given by Chen et al. [37] suggest that the mass transition of 407.7 > 389.7 is more prone to isobaric interference than applies for other transitions. Simultaneous acquisition of more than one mass transition of the target analyte (according to the quantifier–qualifier principle) has so far not been realized in the quantification of 25-hydroxyvitamin D.

The simplest and most straightforward technique of sample preparation for LC–MS/MS analyses is mere protein precipitation with dilution. Since residual matrix components such as salts and phospholipids can impair ionisation, more sensitive methods, however, still have to apply solvent or solid phase extraction protocols. Solvent extraction typically results in very clean extracts and minimized matrix effects, but is cumbersome and hardly applicable for large series in routine use [35,36]. Solid phase extraction as the sample preparation for quantification of 25-hydroxyvitamin D by LC–MS/MS has been described by Tsugawa [34] in a manual off-line approach. This principle typically results in clean extracts but is laborious. Knox et al. [38] reported an automated off-line solid phase extraction method applied after protein precipitation using a high-end autosampler. Calton et al. [39] have reported for the first time an automated off-line solid phase extraction procedure which does not include prior protein precipitation; this method holds without doubt attractive perspectives for the routine application with large series. On-line solid phase extraction after protein precipitation for 25-hydroxyvitamin D measurement has first been described by Vogeser et al. [33]. The first and very recently introduced commercially available kit solution for 25-hydroxyvitamin D measurement by LC–MS/MS (Chromsystems, Munich, Germany) also relies on this very efficient semi-automated approach.

Systematic investigation of matrix effects on ionisation in order to investigate the efficiency of sample preparation and chromatographic fractionation of extracts was systematically assed only in recent methods [37,38]. Despite the very specific detection technique of LC–MS/MS, an appropriate degree of chromatographic sample fractionation is indeed essential; this aims to separate the target analyte from early eluting matrix components such as salts and amino acids and from potentially late eluting compounds such as phospholipids which both may interfere with analyte ionisation. Furthermore a general requirement of chromatography in LC–MS/MS is the separation of the target analyte from its conjugate derivatives which might be disintegrated to the target analyte during ionisation ("in-source transformation"). Sufficient chromatographic fractionation also reduces the risk of interfer-

Table 1
Published methods for the quantification of 25-hydroxyvitamin D3 in serum by LC–MS/MS.

	MS/MS instrument	Ionisation mode	Internal standard	Calibration material	Mass transitions	Sample preparation	Chromatography	Chrom. run time	Lower limit of detection	Calibration range
Vogeser et al. [33]	Waters Quattro LC	ESI pos	Self-synthesised (3xdeut. + C13)	Pure solution in methanol/water	401 > 159 (IS: 405 > 159)	Addition of sodium hydroxide, protein precipitation, on-line SPE (Waters Oasis HLB)	Isocratic elution, C18	Approx. 9 min	n.d.	1-point calibration (25 ng/mL)
Tsugawa et al. [34]	API 3000	APCI	6-fold deuterated, self-synthesised	10–200 ng/mL in ethanol	401.4 > 257.0 (IS: 407.4 > 263.4)	Prot. prec. and off-line SPE	Isocratic elution, C18	11	1 ng/mL	5–100 ng/mL
Manusell et al. [35]	API 3000	APCI	6-fold deuterated (Vitas, commercial source)	Pure solution in ethanol/water (8-point, 4–250 nmol/L)	401.8 > 383.5 (IS: 407.2 > 389.4)	Solvent extraxtion with hexane	C8, gradient	8 min	<1.6 ng/mL	1.6–100 ng/mL
Saenger et al. [36]	Waters Quattro	ESI pos	Deut. Δ^9-THC-D3	Pure solution in ethanol	401.2 > 365.3 (IS: 318 > 196)	Solvent extraction with n-heptane	C18, isocratic elution	6.0 min	0.09 ng/mL	1–100 ng/mL
Chen et al. [37]	Waters Quattro micro	APCI pos	6-fold deuterated (Synthetica, Oslo, Norway, commercial source)	In 4% albumin in PBS (5–100 ng/mL)	401.4 > 383.4 (IS: 407.7 > 389.7)	Protein prec. and on-line solid phase extraction	Isocratic elution, C18	7 min	0.49 ng/mL	5–100 ng/mL
Knox et al. [38]	Waters TQD	ESI pos	6-fold deuterated (Synthetica, Oslo, Norway, commercial source)	Serum based (Chromsys-tems)	401.35 > 159 (IS: 407.4 > 159)	Protein precipitation and automated off-line SPE	UPLC, C8, isocratic elution	5 min	1 ng/mL	1–100 ng/mL
Calton et al. [39] (abstract)	Waters TQD		do	Horse serum based		Automated off-line SPE			n.d.	

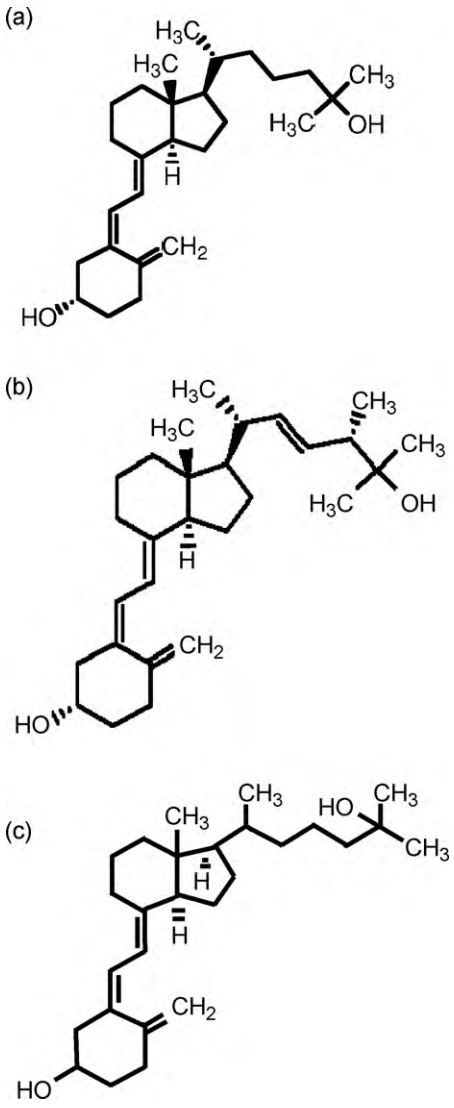

Fig. 1. Structure of 25-hydroxyvitamin D3 (a), 25-hydroxyvitamin D2 (b), and 3-epi-25-hydroxyvitamin D3 (c).

ence by endogenous or xenobiotic compounds present in serum which share identical mass transitions with the target analyte or its internal standard compound. Most LC–MS/MS methods for the quantification of 25-hydroxyvitamin D3 employ isocratic elution and C18 materials, resulting in retention times of few minutes and total run times of 5–10 min (Table 1 and Fig. 1).

With one exception [36] all so far described LC–MS/MS methods for the quantification of 25-hydroxyvitamin D3 involve isotope dilution internal standardisation. While in the first reported LC–MS/MS method for 25-hydroxyvitamin D measurement [33] a fourfold labelled internal standard compound had to be synthesised by the authors, such compounds became available later on from two companies (Synthetica, Oslo, Norway, and Sigma–Aldrich); today typically hexa-deuterated internal standards are used. Indeed, the commercial availability of appropriate internal standards was an important prerequisite for a widespread application of LC–MS/MS for the quantification of 25-hydroxyvitamin D.

The methods described up to 2008 [33–36] were based on pure analyte solutions used for calibration; this approach is typically used in GC–MS methods since this technology is assumed to be practically matrix independent. It must however be recognized that the physico-chemical properties of native and stable isotope labelled compounds can differ to a certain degree ("isotope effects"); it was found that atmospheric pressure ionisation can indeed be subject to substantial isotope effects [42], which is in contrast to the ionisation processes involved in GC–MS. Since the process of ionisation in LC–MS/MS is highly matrix depended and because differential modulation of the ionisation efficacy between target analyte and internal standard compound can lead to inaccuracy, it was tried to introduce calibration materials which are as closely related to serum as possible to the quantification of 25-hydroxyvitamin D. Whereas in drug analyses blank serum can easily be used to prepare spiked calibration materials, the availability of analyte free serum materials for the preparation of calibrators for endogenous compounds is by principle a problem and requires interventions like "analyt stripping".

Chen et al. [37] used for the first time albumin containing calibration materials. Recently, serum based calibration and quality control materials for the measurement of serum 25-hydroxyvitamin D2 and D3 have become commercially available (Chromsystems, Munich, Germany), which were used by Knox et al. [38] and Calton et al. [39]. The availability of such materials can help to increase the reliability of results by diminishing the bias caused by matrix effects which have different impact on target analyte and calibrators; but probably it is even more important that a industrially manufactured and multicentrically validated common calibration material substantially improves the interlaboratory agreement of LC–MS/MS results in the quantification of 25-hydroxyvitamin D [43,44].

In order to enable the long term and world wide standardisation of 25-hydroxyvitamin D measurement, the US National Institute of Standards and Technology (NIST) has very recently introduced serum based reference materials in four different concentrations covering the analytical range (SRM 972 NIH; http://www.nist.gov/cstl/analytical/organic/vitamindinserum.cfm). These materials are supposed to be used as master calibration samples in the industrial production of working calibration materials available for the end customer.

3. Problems in the measurement of 25-hydroxyvitamin D by LC–MS/MS

Despite the sophisticated principle of analyte detection in LC–MS/MS it is important to consider issues which may compromise the reliability of such analyses.

Since 25-hydroxyvitamin D3 is very polar, potential interference from late eluting lipophilic compound has to be considered. Endogenous lipids are a very heterogeneous group of compounds and it is possible that some of them share mass transitions with 25-hydroxyvitamin D3 when using LC–MS/MS. If a compound is present in much higher concentrations compared to the target analyte in a sample also a less favoured mass transition of this compound can potentially interfere substantially with the quantification of the target analyte in low concentration ranges.

Manusell et al. [35] performed a computer search for compounds which are (obviously) isobaric to the singly protonated ion of 25-hydroxyvitamin D3; 56 compounds were identified as potential interferers. The majority of these were not compounds of pharmaceutical or metabolic interest. A few naturally occurring and pharmaceutical compounds of sterols and fatty acid derivatives were identified; of most potential relevance were 1α-hydroxyvitamin D3 (Alfacalcidol), a pharmaceutical compound, and 7α-hydroxy-4-cholestene-3-one, a bile acid precursor, whose plasma concentration has been suggested as a marker of bile acid malabsorption. It must be concluded that these compounds should be separated chromatographically from 25-hydroxyvitamin D3. Other monohydroxy metabolites were not tested. The reported concentrations of these metabolites are extremely low in patients treated with small daily doses of vita-

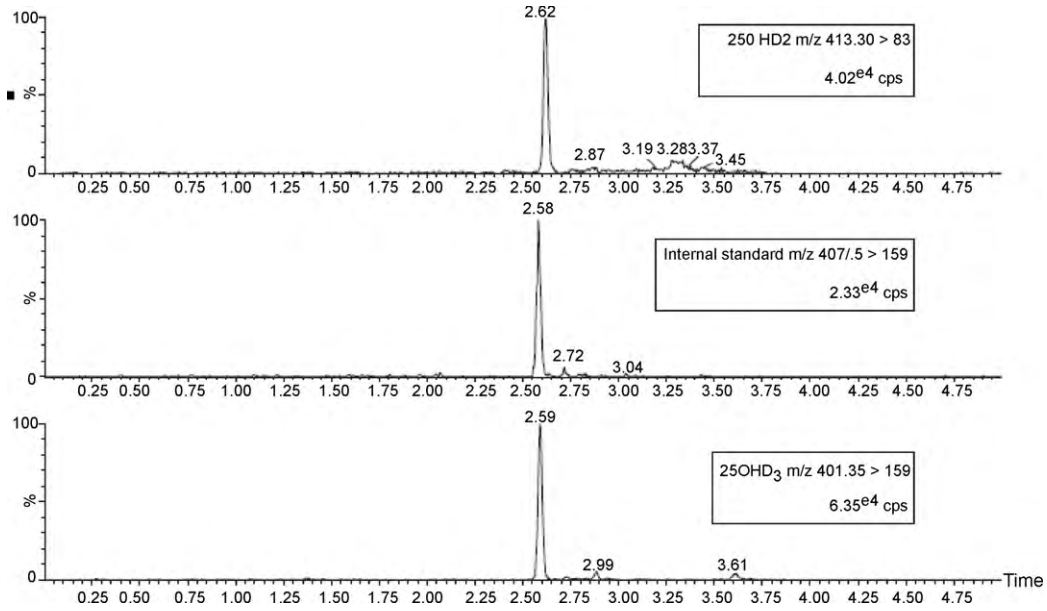

Fig. 2. Quantification of 25-hydroxyvitamin D3 and D2 in serum by LC–MS/MS; representative chromatogram (from [38]).

min D2, but can increase to 70 nmol/L in individuals treated with large daily doses [45].

It also has to be considered that in LC–MS/MS interference might arise from several charged compounds with a higher molecular mass than the target analyte if both share one mass-to-charge ratio. Moreover, clusters of unrelated compounds with e.g. solvent contaminations can have the same molecular mass as the target analyte. Despite these general considerations there are no reports published which would indeed demonstrate false 25-hydroxyvitamin D results obtained by LC–MS/MS due to unrelated isobaric interferents. Nevertheless, general minimum chromatographic requirements for reliable LC–MS/MS analyses (such as separation of target analytes from their conjugate metabolites, separation from early and late eluting compounds which compromise ionization) should not be omitted in an attempt to shorten analytical run times and to increase sample throughput.

Rather little attention has so far been paid to the role of 3-epi-25-hydroxydroxy-vitamin d3 which shares the exact mass with 25-hydroxyvitamin D3 [46] (Fig. 2c). The downstream metabolite 3-epi-1,25-dihydroxydroxy-vitamin d3 is nearly as potent as 1,25-dihydroxyvitamin D3 in suppressing PTH secretion but has significantly reduced calcemic effects [47]. In infants significant concentrations of 3-epi-25-hydroxydroxyvitamin d3 have been demonstrated by Singh et al. [48] by a modified LC–MS/MS method. Most LC–MS/MS methods as well as HPLC-UV methods probably overestimate true 25-hydroxyvitamin D3 concentrations due to co-elution with the epimere; this may be of some relevance in paediatric samples. 3-epi-25-Hydroxyvitamin D3 is commercially available as a pure compound (Sigma–Aldrich) and should considered in further method validation studies.

In-source transformation of 25-hydroxyvitamin D3 sulfate [49] and potentially other conjugate metabolites to 25-hydroxyvitamin D3 during atmospheric pressure ionisation may be a potential pitfall if very limited chromatographic separation is applied; however, no studies have so far addressed this issue.

Insufficient standardisation and quality assurance of 25-hydroxyvitamin D measurements from LC–MS/MS probably caused one of the biggest scandals in laboratory medicine when a large US commercial laboratory had to admit that thousands of incorrect 25-hydroxyvitamin D results had been reported [50]. This scandal has probably discredited the application of LC–MS/MS in laboratory medicine in general; however, there is no reason to assume that this affair might have been caused by actual limitations of this analytical technology. In fact this scandal highlighted that an assay may only be as good as its standardisation materials. Due to its high specificity – in particular with the potential to simultaneously quantify 25-hydroxyvitamin D2 and D3 – LC–MS/MS, if available, can nevertheless be looked upon as the assay of choice. Essential progress in standardisation has been made through the recent introduction of commercially available calibration and QC materials. Also the availability of international standardisation materials for manufacturers is of utmost importance in order to enable long term stability of results; this in turn is essential for all epidemiological surveys and studies.

LC–MS/MS still requires very skilled technicians and typically there is a large number of potential gross errors in the quantification using this technique. For example manual sample preparation protocols incorporate the risk of confusion of samples and manual transfer of results to a laboratory information system is prone to mistakes. Shifting retention times may lead to incorrect peak integration. Today, such gross errors are more likely to occur in LC–MS/MS compared to fully automated protein binding assays used for 25-hydroxyvitamin D quantification. However, with respect to the over-all analytical reliability, the potentially lower error rate of automated competitive protein binding assays is probably offset by the lack of specificity in these methods [51]. Nevertheless, development and introduction of safe and convenient automated sample management and preparation front end modules for LC–MS/MS is highly desirable for the future in order to minimize the risk of such gross errors [29].

4. Current use of LC–MS/MS in the quantification of 25-hydroxyvitamin D

The first LC–MS/MS method for the quantification of 25-hydroxyvitamin D3 was developed in order to validate an automated protein binding assay and to optimize the standardisation during the development of a now widely used high-throughput test [33]. Still currently one major role for the quantification of 25-hydroxyvitamin D3 by LC–MS/MS is such quality assessment of routine protein binding assays. However, since the technol-

ogy of LC–MS/MS has become available in a constantly growing number of clinical laboratories, the implementation of LC–MS/MS methods for routine quantification of 25-hydroxyvitamin D has become feasible for many laboratories worldwide now. Indeed, at present quantification of 25-hydroxyvitamin D is one of the most widely used LC–MS/MS applications at all, besides therapeutic drug monitoring of immunosuppressants and neonatal screening for inherited metabolic diseases. In some commercial laboratories now one or several LC–MS/MS instrument are permanently and exclusively used for 25-hydroxyvitamin D quantification, whereas in a larger number of smaller laboratories quantification of 25-hydroxyvitamin D is one assay among several others implemented on an instrument.

The choice of technology for routine 25-hydroxyvitamin D quantification in a specific laboratory today is between manual protein binding assays (RIA, ELISA), automated protein binding assays (Diasorin, Roche), HPLC-UV, and LC–MS/MS. Decisions will be based on a number of aspects, predominantly including the equipment of a laboratory as well as on considerations of analytical quality and costs.

Also if already equipped with one or more LC–MS/MS instruments, introduction of an additional method in a routine laboratory can cause problems and requires substantial human resources since methods have to be developed in-house and still experts are required also for daily application. Typically the running costs for LC–MS/MS analyses are moderate (extraction materials, vials, mobile phases, wearout of separation columns) while the instrument costs and the expenses for instrument maintenance are still very high. With protein binding assays, in contrast, the reagent costs per determination are often substantial. For less frequently requested tests, reagent costs per analyses can be above 20€, which is about the tenfold amount of the running costs of LC–MS/MS analyses. Consequently, if the utilisation of an existing LC–MS/MS instrument can be increased by implementing a 25-hydroxyvitamin D method there is a perspective of some cost saving for a routine laboratory. However, with the constantly increasing number of vitamin D protein binding assay tests sold per year and with an increasing number of competitors in this attractive market, the protein binding assay reagent costs for 25-hydroxyvitamin D are decreasing and the potential of economisation by using mass spectrometry becomes more and more moderate. Latest automated LC–MS/MS methods for the quantification of 25-hydroxyvitamin D enable the analysis of about 200 samples per day [39], however, requiring full occupation of one 200,000€-instrument. With automated protein binding assays typically a sample throughput of about 100 analyses *per hour* is possible in a very convenient multi-assay, random access, walk-away workflow. Automated protein binding analyzers can be run by less trained technicians while LC–MS/MS still requires a high degree of allocation of highly trained personnel. Indeed, the practicability of LC–MS/MS in the setting of high-throughput clinical laboratories is still very much inferior to automated standard protein binding analyzers.

More evident than economical considerations is the degree of analytical quality: LC–MS/MS methods can overcome the essential limitations of 25-hydroxyvitamin D protein binding assays, namely the inconstant co-quantification of 25-hydroxyvitamin D2, the incomplete release of the analyte(s) from their tight protein bond and substantial and variable matrix effects on analyte binding and potentially on signal generation. Consequently, LC–MS/MS is looked upon as the golden standard for 25-hydroxyvitamin D quantification. However, availability and use of certified calibration materials, thorough validation of individual methods (which are typically in-house developed) as well as sustained and thorough internal and external quality assurance are of utmost importance. The potentially superior accuracy and reliability of LC–MS/MS compared to protein binding assays has clearly been demonstrated for androgen analyses [52] and this obviously seems to be paralleled with 25-hydroxyvitamin D measurement.

Limited reliability of protein binding assays for the quantification of 25-hydroxyvitamin D3 was suspected and demonstrated in the "pre-LC–MS/MS" era based on divergent results obtained from different clinical laboratories for identical samples [24]. These observations were corroborated after the introduction of LC–MS/MS [21,51,53,54]. The so far most comprehensive comparison between 25-hydorxyvitamin D results obtained by several ligand binding assays and LC–MS/MS has been reported by Roth et al. [51]; substantial slopes (up to 0.62), statistically significant deviation from linearity and poor correlation (ρ as low as 0.90) were observed, indicating both systematic bias and non-systematic scatter in ligand binding assays.

In contrast to the older technology of GC–MS, LC–MS/MS enabled for the first time – due to its good practicability and its high sample throughput – to perform method comparison studies which investigate hundreds of samples. Thus – apart from potential routine applications – the introduction of LC–MS/MS to vitamin D monitoring was of essential scientific importance.

In particular automated 25-hydroxyvitamin D tests are of questionable performance with substantial bias for individual samples [54]. Important scatter can be found around the proposed cut-off concentration of 30 μg/L; this is typically associated with a poor reproducibility of protein binding assays in the low concentration rage in an instrument and lot dependent manner (unpublished data).

Differential reactivity with 25-hydroxyvitamin D2 is an essential problem of protein binding assays. If tests are rather specific for 25-hydroxyvitamin D3 (as applies for the Roche Cobas assay) they cannot be used to monitor supplementation with ergocalciferol (vitamin D2) which is widely used in the USA. Typically tests are preferred which detect 25-hydroxyvitamin D2 and D3 in an "equimolar" manner [53]. This is widely accepted in the USA, however, analytically not really satisfactory, since biological equivalence of vitamin D2 and vitamin D3 has never been demonstrated [55]. Using LC–MS/MS, serum concentrations of both analytes can be reported separately, however, no evidence based target concentration can be given in case of vitamin D2 supplementation and correct interpretation of results may be a problem [56,57].

It is uncertain if the availability of LC–MS/MS for clinical laboratories will increase in the nearer future; a precondition for this would be the development of convenient random access, multichannel, walk-away analyzers based on LC–MS/MS which offer the same standards of user friendliness as applies for contemporary immunoanalyzers and which can be handled completely by regularly trained technicians. If this becomes reality at all it will take several years and probably also in the years to come most 25-hydroxyvitamin D analyses worldwide will be performed by protein binding assays. These tests are probably useful to detect severe hypovitaminosis D in individual patients with acceptable reliability; for the assessment and evaluation of optimal target concentrations of 25-hydroxyvitamin D in long term epidemiological surveys, however, the use of these automated protein binding assays is questionable and LC–MS/MS should clearly be preferred.

With respect to accuracy, conventional HPLC with UV detection is probably not substantially inferior to LC–MS/MS for the quantification of 25-hydroxyvitamin D in serum [58]. Kit solutions with either solid phase extraction (Chromsystems, Munich, Germany) or with simple protein precipitation (Recipe, Munich, Germany) are commercially available. A good degree of chromatographic separation may overcome the poor specificity of UV detection. The instrument costs for this technology are a fraction of those required for LC–MS/MS. However, due to the lack of sensitivity with UV

detection, large sample volumes (250–500 μL) are required. Furthermore, the required analytical run times of HPLC-UV methods are about twice that of LC–MS/MS methods. Nevertheless, HPLC-UV may represent a good alternative to both protein binding assays and LC–MS/MS.

In summary, LC–MS/MS methods allow the quantification of 25-hydroxyvitamin D3 and D2 on a reference method level of accuracy also in a routine setting. The methods are moderately demanding and perform equally well as for example immunosuppressant quantification by LC–MS/MS in daily routine. Due to the high accuracy of LC–MS/MS methods their use is to be recommended in particular for epidemiological studies and for the continuous cross-validation of protein binding assays.

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