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Review

Measurement of 25-hydroxyvitamin D in the clinical laboratory: Current procedures, performance characteristics and limitations

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ABSTRACT

In this review we describe procedures, performance characteristics and limitations of methods available for the measurement of 25-hydroxyvitamin (25OHD) since the year 2000. The two main types of methods are competitive immunoassay and those based on chromatographic separation followed by non-immunological direct detection (HPLC, LC–MS/MS). Lack of a reference standard for 25OHD has, until recently, been a major issue resulting in poor between-method comparability. Fortunately this should soon improve due to the recent introduction of a standard reference material in human serum (SRM 972) from the National Institute of Standards and Technology (NIST). For immunoassay, specificity can be an issue especially in relation to the proportion of 25OHD2 that is quantified whereas HPLC and LC–MS/MS methods are able to measure the two major vitamin D metabolites 25OHD2 and 25OHD3 independently. HPLC and LC–MS/MS require more expensive equipment and expert staff but this can be offset against lower reagent costs. Increasingly procedures are being developed to semi-automate or automate HPLC and LC–MS/MS but run times remain considerably longer than for immunoassays especially if performed on automated platforms. For most HPLC and LC–MS/MS methods extraction and procedural losses are corrected for by the inclusion of an internal standard which, in part, may account for higher results compared to immunoassay. In general precision of immunoassay, HPLC and LC–MS/MS are comparable and all have the required sensitivity to identify severe vitamin D deficiency. Looking to the future it is hoped that the imminent introduction of a standard reference method (or methods) for 25OHD will further accelerate improvements in between method comparability.

Contents

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1. Introduction

1.1. History

It is widely acknowledged that circulating 25-hydroxyvitamin D (25OHD) is the best indicator of vitamin D status [1]. There are two major vitamin D metabolites in the circulation, 25-hydroxyvitamin D3 (25OHD3) mainly derived from vitamin D3 produced by sunlight in the skin and 25-hydroxyvitamin D2 (25OHD2) derived from plants in the diet. In addition circulating 25OHD3 and 25OHD2 may be present due to supplementation with vitamin D3 or vitamin D2, respectively. Severe vitamin D deficiency (25OHD < 25 nmol/L) causes rickets in children and osteomalacia in adults [2]. Less severe deficiency, where the 25OHD concentration is between 25 and 50 nmol/L, causes secondary hyperparathyroidism and increases in bone turnover and bone loss [3,4]. Furthermore vitamin D insufficiency has been implicated in an extremely wide range of clinical disorders. Some experts are of the opinion that for optimal health circulating 25OHD concentrations should be maintained above 75 nmol/L [5,6].

The first methods for measuring 25OHD were described in the early 1970s being based on competitive protein binding after solvent extraction. The binding agent in these assays was vitamin D binding protein obtained from the serum of vitamin D-deficient rats. Later in the 1970s, methods based on high performance liquid chromatography (HPLC) became available. In 1985 the first radioimmunoassay (RIA) was developed which incorporate a specific 25OHD antibody. To avoid problems related to handling of radioactivity and the limited shelf-life of radioactive labels these have now been largely, but not completely, superseded in immunoassays by labels employing chemiluminescent substances (CLIA) or enzymes (EIA). Advances in tandem mass spectrometry towards the end of the last century enabled the introduction in 2004 of routine procedures based on LC–MS/MS for measuring vitamin D metabolites and the use of this methodology is increasing.

1.2. Today

1.2.1. Immunoassays

Current commercial immunoassays are supplied as kits which can be run manually or on platforms. With increasing clinical demand for 25OHD assays, fast automated platforms are attractive. The first automated procedure was a chemiluminescent competitive protein binding assay supplied by Nichols for their 'Advantage' platform. Unfortunately Nichols had to withdraw the assay at the end of 2005 as it overestimated total 25OHD concentrations and did not fully detect 25OHD2.

In 2004 Diasorin introduced a chemiluminescent immunoassay for use on their 'Liaison' automated immunoassay platform. In 2007 Diasorin updated and replaced this assay to improve sensitivity and precision and renamed it Liaison Total. All three Diasorin assays (RIA, Liaison and Liaison Total) use the same antibody. Recently Roche marketed an electrochemiluminescent immunoassay for the 'Elecys' and 'Cobas E' platforms which is specific for 25OHD3. The IDS EIA method is a manual assay which can also be performed on standard automated ELISA platforms (NEXgen, Triturus). IDS have recently released a new assay which uses a chemiluminescent label on an automated platform (iSYS). All three IDS methods (RIA, EIA and iSYS) use the same antibody. Detailed procedural and performance information on each immunoassay is described later.

1.2.2. Physical detection methods

Two methods employing non-immunological direct detection are currently available: HPLC and LC–MS/MS. Vitamin D metabolite measurement by high performance liquid chromatography (HPLC) incorporates a chromatographic separation followed by a variety of detection procedures. The term HPLC is usually restricted to procedures that have a ultra-violet (UV) or electrochemical detector. If HPLC is linked to mass detectors, the procedure is commonly termed LC–MS/MS or tandem mass spectrometry. Prior to chromatographic separation, an initial purification step is required. The simplest is extraction into an organic solvent (liquid/liquid extrac-

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tion) or alternatively a simple protein crash procedure followed by solid phase extraction (SPE). Extraction and chromatographic separation will inevitably lead to loss of analyte which can be corrected by the inclusion of an internal standard. The internal standard should be indistinguishable from the analyte during the process of extraction and purification and choice is to some extent dictated by the quantitation procedure. For example, for procedures employing mass spectrometry deuterated internal standards are ideal, being chemically identical yet detectable by virtue of increased mass. This procedure generally compensates for any matrix related effects and is commonly termed isotope dilution mass spectrometry. When other types of detection systems such as light absorption and electrochemical properties are used, an internal standard is usually selected that has similar chemical properties to the analyte but is not present in biological samples. Detailed procedural and performance information on HPLC and LC–MS/MS methods is given later.

To summarise there are currently two main types of measurement used routinely for measuring the main circulating metabolites of vitamin D, 25OHD3 and 25OHD2. These are competitive immunoassay and methods based on chromatographic separation followed by non-immunological direct detection (HPLC, LC–MS/MS). Currently immunoassay is the most popular method with the majority using an automated platform. Recently there has been a steady increase in the use of LC–MS/MS.

2. Data source

A search of the literature was carried out on April 30th 2009 using the Pub Med database to identify all publications relating to a comparison of 25OHD measurement methods published since 2000, including letters and editorials. This date was chosen to ensure that the methods discussed were reasonably up-to-date. The search terms used and the number of papers identified were "25-hydroxyvitamin D and measurement (178 papers)", "25-hydroxyvitamin D and measurement assay (149 papers)" and "25OHD and measurement assay (17 papers)". All papers that compared two or more methods of measurement in the abstract were considered potentially relevant. A cross-check was also made for related papers. This process yielded 73 relevant publications for which the full paper was obtained. Four papers were subsequently found not to be relevant, leaving 69 relevant publications. References were abstracted into a bibliographic database (ENDNOTE v8, Adept Scientific plc, Herts, UK) and full papers obtained from libraries, journals or by request direct from authors. Papers were checked for relevance and details entered into an Excel spreadsheet noting the methods compared, conclusions and any quantitative performance data. For papers which reviewed or commented on the results of other papers, expressed opinions were noted. They were classified into weaknesses identified, strengths identified, solutions proposed and conclusions or recommendations drawn. For commercial methods information was also obtained from the manufacturer's kit insert data sheet (Table 1).

2.1. External quality assessment

To assess comparative performances we used much information made available by the vitamin D External Quality Assessment Scheme (DEQAS). This scheme is organised by Dr. Graham Carter, Imperial College Healthcare NHS Trust, Oncology/Endocrinology Laboratory, Charing Cross Hospital, Fulham Palace Rd, London W6 8RF. Full details can be obtained at www.DEQAS.org.uk.

3. Method details

The methods detailed below are summarised in Table 1.

Table 1
Methods for measuring 25-hydroxyvitamin D.

Manual immunoassay
Diasorin[a] radioimmunoassay (RIA)
IDS[b] enzyme immunoassay
IDS[b] RIA
Automated immunoassay[c]
Diasorin Liaison (now unavailable)
Diasorin[a] Liaison Total
IDS[b] iSYS
Roche[d]
Direct detection methods
HPLC
LC–MS/MS

[a] DiaSorin Ltd., 1st Floor, Richmond House, Oldbury, Bracknell RG12 8TQ, UK.
[b] Immunodiagnostics Systems Ltd. (IDS Ltd.), 10, Didcot Way, Boldon Business Park, Boldon, Tyne & Wear NE35 9PD, UK.
[c] All four automated immunoassays use a chemiluminescent label.
[d] Roche Diagnostics Limited, Charles Avenue Burgess Hill West, Sussex RH15 9RY, UK.

4. Diasorin RIA

4.1. Assay procedure

The DiaSorin 25OHD RIA assay consists of a two-step procedure. The first step involves a rapid extraction of 25OHD and other hydroxylated metabolites from serum or plasma with acetonitrile. Following extraction, the treated sample is then assayed by competitive RIA using an antibody with specificity to 25OHD. The sample, antibody and tracer are incubated for 90 min at 20–25 °C. Phase separation is accomplished after 20-min incubation at 20–25 °C with a second antibody precipitating complex. To reduce non-specific binding buffer is added after this incubation prior to centrifugation.

4.2. Assay performance

Reporting DEQAS data in 2004, the organisers of the scheme commented that Diasorin RIA had less than 1% bias from the all laboratory trimmed mean (ALTM); however RIA methods made up about 60% of DEQAS returns at the time [7]. From 2004 to 2008 the mean annual bias in DEQAS ranged from −1.1 to −5.4%. Over the same period the mean average between laboratory precision (CV%) ranged between 16 and 20.5%. The method has decreased in popularity, in 2001 it accounted for 60% of DEQAS returns and this dropped to 7% in 2009. No detailed published information was available to substantiate the manufacturer's claim of a detection limit of 4 nmol/L. The insert in the manufacturer's kit suggests recoveries of 100% for 25OHD3 and 25OHD2. Compared with HPLC, Hollis reported that the Diasorin RIA recovered 91–100% of both 25OHD2 and 25OHD3 [8]. Other studies, however, suggest that Diasorin RIA underestimates 25OHD2 relative to HPLC [9,10]. In a DEQAS recovery experiment performed in July 2005, using serum pools to which 25OHD was added, a mean recovery of 82% for 25OHD3 and 83% for 25OHD2 was reported [11]. The manufacturer's kit evaluation information reports within and between-batch precision of <12 and <11%. Precision in this range has been confirmed by a number of studies [8,10,12,13]. Seventeen papers compared Diasorin RIA with other methods, including HPLC ($n = 10$), LC–MS/MS (6) the IDS-RIA (4) IDS-EIA (4) and Diasorin Liaison assay (7). Conclusions varied as to how close agreement is with LC–MS/MS. Some studies found correlations around 0.91 or 0.96 [14,15]. Comparing results in 551 samples from the National Health and Nutrition Examination Study (NHANES), Diasorin RIA gave lower values than LC–MS/MS at low concentrations and higher values at high concentrations, with LCMS giving a mean value 13%

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higher [16]. In a comparison with HPLC Turpeinen et al. found reasonable overall agreement (*r* 0.82, slope 1.02) although several samples showed large differences [13]. Two studies comparing Diasorin RIA and Liaison yielded different conclusions; Souberbielle et al. found Liaison read lower than Diasorin RIA at low concentrations but higher at high concentrations [17], whereas Ibrahim et al. found the reverse [18].

4.3. Immunodiagnostics (IDS) RIA

4.3.1. Assay procedure

The IDS 25OHD RIA kit is a complete assay procedure for the extraction and quantitation of 25OHD and other hydroxylated metabolites in serum or plasma. Addition of two reagents to samples and calibrators causes precipitation of serum proteins and extraction of 25OHD. Following centrifugation, portions of the supernatant are incubated with ^{125}I-labelled 25OHD (tracer) and a highly specific sheep antibody to 25OHD. Separation of antibody-bound tracer from free is achieved by a short incubation with Sac-Cel® (antisheep IgG cellulose) followed by centrifugation and decanting. Bound radioactivity is inversely proportional to the concentration of 25OHD.

4.3.2. Assay performance

From 2004 to 2008 the mean annual DEQAS bias ranged from −10.5 to +8.6%. Over the same period the mean average laboratory precision (CV%) ranged between 13.1 and 15.2%. In 2009 this method accounted for only 5% of DEQAS returns. No detailed published information was available to substantiate the claim of a detection limit of 3 nmol/L. The manufacturer's kit insert suggest a recovery of 100% for 25OHD3 and 75% for 25OHD2. In DEQAS recovery experiments performed in July 2005 a recovery of 54% for 25OHD3 and 29% for 25OHD2 was recorded [11]. Since recovery from exogenously prepared pools has been called into question for immunoassay (see Section 12) these results should be treated with caution. Hollis reported recovery of 92–95% for 25OHD3 and 21–29% for 25OHD2 [8]. The manufacturer's kit evaluation information reports within and between-batch precision of <6.1% and <8.2%. Other studies report between assay CVs of 7–8% [19], <10% [8] and 12% [10].

Roth et al. reported a bias of −15% compared with LCMS [19], while Carter et al. reported a bias of −5% compared with ALTM in DEQAS in 2004 [7]. Glendenning et al. suggest weak correlation with HPLC (*r* 0.6) and slope of 0.64 with positive bias at low concentration and negative bias at high concentrations.

5. IDS enzyme immunoassay (EIA)

5.1. Assay procedure

The IDS 25-hydroxy vitamin D EIA kit is an enzyme immunoassay for the quantitation of 25OHD and other hydroxylated metabolites in serum or plasma. Calibrators, controls and samples are diluted with 25OHD labelled with biotin. A propriety buffer reagent is used for dissociating 25OHD from its binding proteins. The diluted samples are incubated in microtitre wells which are coated with a highly specific sheep 25OHD antibody for 2 h at room temperature before aspiration and washing. Enzyme (horseradish peroxidase) labelled avidin, is added and binds selectively to complexed biotin, and, following a further wash step, colour is developed using a chromogenic substrate (TMB). The reaction is stopped by the addition of hydrochloric acid and the absorbance read in a microtitre plate reader, colour intensity developed being inversely proportional to the concentration of 25OHD.

5.2. Assay performance

From 2004 to 2006 the mean annual DEQAS bias has ranged from +5.7 to +23%. The assay was re-calibrated in 2006 with a significant reduction in positive bias to around 5%. Between 2004 and 2008 the mean average between laboratory precision (CV%) ranged between 14.1 and 18.4%. In 2009 this method accounted for 19% of DEQAS returns. No detailed published information was available to substantiate the manufacturer's claim of a detection limit of 5 nmol/L. Results from DEQAS indicate moderately low recovery for this assay (56% for 25OHD2, 79% for 25OHD3) using exogenously prepared pools [11]. Since recovery from exogenously prepared pools has been called into question for immunoassay (see Section 12) these results should be treated with caution. According to Hyppönen et al., within-assay CVs ranged from 5.3 to 7.4% and between-assay CVs from 5.1 to 11.7% depending on concentration, with lower CVs at high concentrations [20]. According to Hyppönen et al. the IDS EIA gave lower values (13–15 nmol/L) than either Diasorin RIA or DEQAS ALTM with a bias that increased with concentration. Kimball and Vieth reported that the IDS EIA method run on the NEXgenis platform is simpler, faster and safer than the RIA, which was reasonably correlated (*r* −0.8) [21]. The study by Roth et al. reported a significant negative bias for the EIA relative to LC–MS/MS (−21%) [19]. Using a different platform (Triturus), Knox et al. reported that IDS EIA compared well with LC–MS/MS (*r* 0.96) although the correlation was poorer at higher concentrations of 25OHD (>125 nmol/L) where the immunoassay had a positive bias [22].

6. Diasorin Liaison automated immunoassay

6.1. Assay procedure

The method for quantitative determination of 25OHD is a direct, competitive chemiluminescence immunoassay (CLIA) on an automated platform. Specific antibody to vitamin D is used for coating magnetic particles (solid phase) and vitamin D is linked to an isoluminol derivative. During the incubation, 25OHD is dissociated from its binding protein, and competes with labelled vitamin D for binding sites on the antibody. After the incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as relative light units (RLU) and is inversely proportional to the concentration of 25OH vitamin D present in calibrators, controls, or samples. This method was phased out during 2008 and has been replaced by the DiaSorin Liaison Total assay.

6.2. Assay performance

From 2004 to 2008 the mean annual DEQAS bias ranged from −16.9 to −7.9%. Over the same period the mean average between laboratory precision (CV%) ranged between 17.6 and 21.6%. The manufacturer claim that the within and between-batch precision are <11.3 and <14.6% respectively are significantly lower than published values. Within assay CV ranged from 8 to 21% and between assay from 8 to 34% [12,17–19]. Eight studies compared the Diasorin Liaison with other methods. According to DEQAS, recoveries using Diasorin Liaison methods were good (81% for 25OHD3 and 89% for 25OHD2 at a concentration of 36 nmol/L) [11]. Since recovery from exogenously prepared pools has been called into question for immunoassays (see Section 12) these results should be treated with caution. Two studies compared Diasorin Liaison with Diasorin RIA. Souberbielle et al. reported [17] that both methods yielded comparable results, although Liaison tended to read lower at low concentrations and higher at high concentrations. Ibrahim

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et al. reported that the Liaison method gave higher results at both extremes. Ibrahim et al. gave values for the between-assay CV of 15% at 30 nmol/L and 5% at 117 nmol/L [18]. Kimball and Vieth compared a pre-2007 Liaison with the IDS EIA and found a moderate correlation (r 0.77) [21].

7. Diasorin Liaison Total automated immunoassay

7.1. Assay procedure

The Liaison Total 25OHD assay is a direct competitive chemiluminescence immunoassay (CLIA) for quantitative determination of total 25OHD in serum or plasma on an automated platform. This is a reformulation of the Diasorin Liaison method. The same antibody is used but now in a two-step incubation procedure. During the first incubation, 25OHD is dissociated from its binding protein and binds to the specific antibody on the solid phase. After 10 min the tracer (vitamin D linked to an isoluminol derivative) is added. After the second 10 min incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added to initiate a flash chemiluminescent reaction. The light signal is measured by a photomultiplier as relative light units (RLU) and is inversely proportional to the concentration of 25OHD present in calibrators, controls, or samples.

7.2. Assay performance

This method replaced the Diasorin Liaison method from 2007. The mean annual DEQAS bias in 2008 was −9% and between-laboratory precision (%CV) was 15.5. The method is extremely popular and in 2009 accounted for 36% of DEQAS returns. The between-batch precision of <12.2% claimed by the manufacturer was verified in the study of Roth et al. who quote between assay CV of 8–10% [19]. These authors also performed comparative studies between Diasorin Liaison and the newer Liaison Total against LC–MS/MS [19]. Compared with original Liaison, Roth et al. reported lower CVs (8–10% vs. 13–15%), a higher correlation with LC–MS/MS (0.95 vs. 0.90) and less bias (−8% vs. −21%) for the Liaison Total. Diasorin Liaison Total is currently the most popular method within DEQAS [19]. Compared with original Liaison, Roth et al. reported the Liaison Total to have lower CVs, a higher correlation with LC–MS/MS (r 0.95) and less bias (−8%) [19].

8. Roche Elecsys automated immunoassay

8.1. Assay procedure

A competitive immunoassay format is used based on the streptavidin–biotin technology. The assay employs a polyclonal sheep antibody against 25OHD3, which is ruthenium labelled. The vitamin D in the sample competes for binding with biotinylated 25OHD antigen which is bound to streptavidin coated microparticles. The test is intended for use on Elecsys and Cobas E automated immunoassay analysers. The literature search identified two papers that specifically examined the new Roche assay, Elecsys 25-OH-D(3), developed for use on Roche analysers [19,23].

8.2. Assay performance

This procedure came onto the market in 2008. Currently there are 32(6%) users who participate in DEQAS. The mean annual DEQAS bias in 2008 was +7.6 nmol/L and the mean between laboratory annual precision (%CV) was 16.7%. The manufacturers claim a between-batch precision of <10% which is similar to that in published data [19,23]. Overall, Leino et al. reported that the method was in good overall agreement with results determined by LC–MS/MS and RIA, although large between-method variation was observed in individual patient samples [23]. At concentrations of 48, 76, and 124 nmol/L, within-run CVs were 5.1, 3.1, and 7.1% and total CVs were 12.1, 7.4, and 10.6%, respectively. Results from plasma samples were markedly higher than those in serum. The method is specific for 25OHD3 and does not detect 25OHD2.

9. IDS iSYS automated immunoassay

9.1. Assay performance

This method was introduced early in 2009. The assay is based on chemiluminescence technology performed on an automated platform. Samples are subjected to a pre-treatment step to denature the vitamin D binding protein. The treated samples are then neutralised in assay buffer and a specific, anti-25OHD antibody labelled with acridinium is added. Following an incubation step, magnetic particles linked to 25OHD are added. After a further incubation step, the magnetic particles are "captured" using a magnet. After washing and addition of trigger reagents, the light emitted by the acridinium label is inversely proportional to the concentration of 25OHD in the original sample.

9.2. Assay procedure

This procedure has only recently come onto the market. DEQAS information is currently limited to only six users. To date there is no published detail on assay performance apart from detail in the manufacturer's kit insert (Table 2).

HPLC and LC–MS/MS

HPLC and LC–MS/MS methods for measuring 25OHD in serum begin with deproteinisation followed by extraction and purification. Chromatographic separation is invariably effected on a reverse-phase HPLC analytical column (usually containing C18 particles). In HPLC procedures a UV detection system at a wavelength of 265 nm is employed. In LC/MS/MS a mass detector is used. To correct for procedural losses many of these methods include an internal standard (see above). All methods described have been developed in specialist laboratories and all have slight adaptations.

10. HPLC

10.1. Assay procedure

We identified seven publications (since 2000) with detailed information on the HPLC procedure [10,13,24–26]. Sample volumes ranged between 0.5 and 1 ml. Deproteinisation was achieved by acetonitrile [27,10], ethanol [24,26] an ethanol:acetonitrile mixture [25] or a methanol:isopropanol mixture [13]. Three [19,25,26] used an off-line solid phased extraction procedure and one used on automated on-line procedure [27]. Two procedures employed straight solvent extraction into either hexane [13] or a hexane/dichloromethane mixture.

Three of the published methods include detail on the internal standard used to correct for procedural losses, Glendenning et al. used trans-vitamin D3, Alvarez and De Mazancourt used 1α-hydroxyvitamin D3 and, in a less detailed description, Lensmeyer et al. used laurophenone as internal standard [10,24,28].

10.2. Assay performance

From 2004 to 2008 the mean annual DEQAS bias ranged from −8.6 to +9%. Over the same period the mean average between-

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Table 2
Performance information detailed in kit inserts for commercial immunoassays.

Supplier	Method	Detection limit (nmol/L)	Functional sensitivity (nmol/L)	Measuring range (nmol/L)	Sample size	Specificity	Within-batch precision (CV%)	Between-batch precision (CV%)
Manual assays kits								
DiaSorin	RIA	4	Not given	4–250	2× 50 μL	25OHD3 100% 25OHD2 100% 24,25diOHD3 100%	<12.5%	<11%
IDS	RIA	3	Not given	3–300	2× 50 μL	25OHD3 100% 25OHD2 75% 24,25diOHD3 ≥100%	<6.1%	<8.2%
IDS	EIA	5	Not given	5–250	2× 25 μL	25OHD3 100% 25OHD2 75% 24,25diOHD3 ≥100%	<6.7%	<8.7%
Assays on automated platforms								
DiaSorin Liaison	CLIA	Not given	17.5	17.5–375	25 μL (250 μL (for 1st specimen and 25ul for additional measurement)	25OHD3 100% 25OHD2 100% 24,25diOHD 100% 25,26diOHD 100%	<11.3%	<14.6%
DiaSorin Liaison Total	CLIA	Not given	10	10–375	25 μL (250 μL (for 1st specimen and 25 μL for additional measurement)	25OHD3 100% 25OHD2 104% 3-epi-25OHD3 <1% no info supplied on 24,25diOHD	<4.8%	<12.2%
IDS iSYS	CLIA	9	14	14–350	20 μL (150 μL for 1st specimen and 25 μL for additional measurement)	25OHD3 100% 25OHD2 ≥70% 24,25diOHD3 ≥100%	<7.3%	<8.9%
Roche Elecys	CLIA	10	Not given	10–250	35 μL (no info supplied on dead volume)	25OHD3 100% 25OHD2 <20% 24,25diOHD <20%	<5.7%	<9.9%

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batch precision (CV%) ranged between 21.2 and.8%. In 2009 this method accounted for <5% of DEQAS returns. The lower limit of detection ranged between 3 and 7.5 nmol/L while the lower limit of quantitation ranged between 10 and 17.5 nmol/L (Table 3). Detailed 25OHD3 recovery experiments have been conducted in six of the method papers [10,13,24–26] and 25OHD2 in two [10,24]. Recovery of 25OHD3 ranged from 85 to 102% and 25OHD2 from 81.5 to 97%. Most descriptions provide precision details for 25OHD3. Within-batch and between-batch precision ranged from 0.83 to <10% and 1.9 to <12% respectively. Mata-Granados et al. and Alvarez and De Mazancourt also include data on 25OHD2 with within- and between-batch precision ranging from 0.92 to 6% and 1.9 to <11.85 respectively [24,27]. Several papers compared HPLC with other methods. Agreement with LC–MS/MS was close (HPLC = 1.01 × LCMS − 12.2 nmol/L) [28] and in the study of seven methods by Roth et al., HPLC was the only method giving values for 25OHD that were comparable with LC–MS/MS with a mean ratio concentration of 0.99 (95% CI: 0.98, 1.00) [19]. HPLC was also in close agreement with DEQAS ALTM (HPLC = 0.98 × ALTM + 2.15 nmol/L (r 0.96))[7]. Turpeinen et al. reported the correlation between HPLC and Diasorin Liaison and RIA assays to be moderate (r 0.73 and 0.83 respectively) and concluded that HPLC was preferred in terms of accuracy and precision and also cost [13].

11. LC–MS/MS

11.1. Assay procedure

We identified seven publications (since 2000) containing detailed information on the LC–MS/MS method used [14–16,22,29–31]. Sample volumes were either 100 μl (n = 2) or 200 μl (n = 4). Deproteinisation was achieved by acetonitrile [16], acetonitrile plus sodium hydroxide [31], methanol [22,30] or a methanol:propanol mixture [14]. Two used a liquid/liquid extraction with either n-heptane [15] or hexane [14]. The remaining five [16,22,30,31] used SPE with either C8 or C18 or equivalent solid phases. Two [16,31] used an on-line SPE step, one [22] used an automated off-line procedure. One method compared liquid/liquid with SPE extraction [29] in a method that incorporated a Diels–Alder derivatitisation step which improved the analytical signal 100 fold. During mass detection molecules were ionised by either atmospheric pressure chemical ionization [16,30] or electrospray atmospheric pressure ionization [14,15,22,31]. Chromatographic separation was achieved on either a reverse-phased C8 or C18 or equivalent analytical HPLC or UPLC analytical columns. Five methods used hexadeuterated 25OHD3 [14,16,22,29,30], one used one deuterated and ^{13}C 25OHD$_3$ [31] and one used deuterated tetrahydrocannabinol [15] as internal standard. In only three methods were stock standards reported as being checked by molar extinction [14,16,26]. Of the sixteen methods only two used a serum-based standard. Knox et al. used a human serum commercial preparation (Chromsystems, München, Germany) both neat and diluted in horse serum and Lensmeyer et al. made calibrators up in 'drug-free serum'. Two prepared working standards in ethanol [24,30], two in phosphate-buffered saline with [32] or without [16] bovine serum albumin and two used a methanol water mixture [14,31].

11.2. Assay performance

From 2005 to 2008 the mean DEQAS annual bias ranged from −1.3 to 9.5 and the mean annual precision (CV%) ranged between 9 and 21.3%. Lower limit of detection ranged between 0.2 and 7.5 nmol/L while lower limit of quantitation ranged from 0.75 to 7.5 nmol/L. Saenger et al. also quote a functional sensitivity (2 nmol/L) [15]. All published methods detail recovery experiments. The recovery ranged from 89 to 110% for 25OHD3 and 86–108% for 25OHD2. All but one method describes both 25OHD3 and 25OHD2 precision data. Vogeser et al. only describe between-batch precision for 25OHD3 [31]. Within-batch precision ranged from 4.5 to 10% for 25OHD3 and from 4.5 to 11% for 25OHD2. Between-batch precision ranged from 2.5 to 12% for 25OHD3 and 5.1–16% for 25OHD2. Many comparative studies used LCMS as the reference. Maunsell et al. described "good agreement (r 0.91) with Diasorin RIA [14] and Saenger et al. found that Deming regression of LCMS on Diasorin RIA yielded a slope of 0.97 and y intercept of −5 nmol/L with r 0.96 [15]. Chen et al. reported a similar high correlation (r 0.96) using log-transformed values [16]. Correlation with the IDS EIA was also reported to be good at concentrations below 125 nmol/L, above this, the IDS EIA tended to overestimate 25OHD. The simplified automated LC–MS/MS method described by these authors also had a high throughput of >300 samples per day [22]. It has been clearly demonstrated that inter-lab CV is reduced using a common calibrator [33] or standard, from about 16 to 10% [34].

12. Discussion

12.1. Assay standardisation

There are a number of significant limitations of current methods for measuring 25OHD. Analysts have been aware of many of these problems for a number of years [7,10,35,36] but it was a publication of Binkley et al. that first drew attention of the wider clinical community to the large variability in 25OHD results, both between methods and between laboratories [37]. They compared the values reported for samples from healthy individuals sent to six laboratories using different methodologies and found a two-fold difference in mean values reported from 42.8 to 89 nmol/L (17.1–35.6 ng/mL). For half the samples, whether the individual was classified as having insufficient or normal vitamin D status depended solely on the laboratory used. These authors concluded that if the medical community was to make progress in correcting widespread hypovitaminosis D, the measurement of 25OHD must be standardised. Currently there are a range of procedures used to prepare and assess the accuracy of 25OHD standards. Stock standard solutions have been calibrated by either gravimetric analysis or UV spectrometry. It has been suggested that the concentration and purity of stock 25OHD standard solutions is best established using a double-beam scanning spectrophotometer. Vitamin D metabolites have a well-defined absorbance spectrum with an absorbance maximum at 265 nm and minimum at 228 nm. There is, however, some uncertainty over the molar absorption coefficient used to calculate the 25OHD concentration which could result in small differences in calculated concentration [34]. A number of HPLC and LC–MS/MS procedures use a commercial standard in human serum (Chromsystems, München, Germany) but no information is available on how values have been attributed to these standards. A number also use working standards in buffer or solvent rather than serum which introduces matrix differences which could be problematic and will be discussed in detail later. For many of the commercial immunoassays detail on standardisation and standard matrix is not available. The lack, until recently, of a recognised calibrator has been debated extensively [34,37–47]. Two studies have clearly demonstrated that inter-laboratory precision for LC–MS/MS assays can be improved by use of a common calibrator [34]. In a recent editorial it was concluded that it was of prime importance that an internationally agreed standard material for both 25OHD2 and 25OHD3 is required that can be utilized worldwide to improve not only LC–MS/MS consistency but also immunoassay comparability [47]. To this end the National Institute of Standards and

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Table 3
Summary of published performance detail on HPLC and LC–MS/MS procedures.

Reference	Mean recovery	Lower limit of detection (nmol/L)	Lower limit of quantification (nmol/L)	Range (nmol/L)	Within-batch (CV%)	Between-batch (CV%)
HPLC						
Mata-Granados [27]	99–100.2%	5.2	17.5		0.83 25OHD3 0.92 25OHD2	1.8 25OHD3 1.9 25OHD2
Jakobsen [26]	90.3%	4		4–200	3.8	5.7
Roth [19]						2.3–6.5% 25OHD3
Glendenning et al. [10]	88–104% 25OHD3 86–97% 25OHD2	7		7–250	<10%	<12%
Brunetto [25]	91–98%	7.5		7.5–80	<3%	<3%
Turpeinen [13]	85–105% 25OHD3	3	10	15–200		
Alvarez [24]	88 ± 5.1% 25OHD3 81.5 ± 4.7% 25OHD3	4			<7.7 25OHD3 <6 25OHD2	<10.8 25OHD3 <11.8 25OHD2
LC–MS/MS						
Knox [22]	89–104%		4 25OHD3 7.5 25OHD2	4–300	<10D2 + D3	<10 D2 + D3
Chen [16]	99% 25OHD3 95% 25OHD2	1.2 25OHD3 4.6 25OHD2		1–250	<7% 25OHD3 < 11% 25OHD2	<9%25OHD3 <16% 25OHD2
Saenger [15]	93–103% 25OHD3 86–92% 25OHD2	0.2	0.75	2–250	<8% 25OHD3 <8.8% 25OHD2	<10% 25OHD3 <11.5 25OHD2
Tsugawa [30]	104% 25OHD3 99% 25OHD2	7.5			5.7% 25OHD3 4.5% 25OHD2	2.5% 25OHD3 5.1% 25OHD2
Maunsell [14]	91–110% 25OHD3 94–108% 25OHD2		<5 25OHD3 <5 25OHD2	4–250	5.7% 25OHD3 4.5% 25OHD2	2.5% 25OHD3 5.1% 25OHD2
Vogeser [31]	91–95% 25OHD3					<12% 25OHD3

Technology, working with the National Institute of Health Office of Dietary Supplements (NIH/ODS), the US Department of Agriculture (USDA) and the Centers for Disease Control and Prevention have developed a serum-based reference material (SRM) for 25OHD2 and 25OHD3. This material (SRM 972) has recently been made available and consists of four standards. Level 1 of SRM 972 was prepared from "normal" human serum and has not been altered. Level 2 was prepared by diluting Level 1 with horse serum to achieve a lower 25OHD concentration. Level 3 contains "normal" human serum that has been fortified with 25OHD2, and Level 4 contains "normal" human serum that has been fortified with 3-epi-25-hydroxyvitamin D3 (http://www.nist.gov/srm). Although the hope was that these standards would improve between assay comparability initial indications highlighted in poster presentations at the 14th vitamin D workshop (Bruges, October 2009) are disappointing for SRM 972 pools 2 and 3. Significant under-recovery in immunoassay, but not HPLC or LC–MS/MS, in the standard diluted with horse serum (Level 2) and the standard containing added 25OHD2 (Level 3) were demonstrated. Comparability was, however, better, in the endogenous Level 1 pool [48,49]. It is unclear why endogenous and exogenous pools differ in this respect but sample matrix may be a factor. The consequence of these findings is that only the SRM 972 Level 1 pool should be used for standardisation purposes in immunoassays.

12.2. Assay specificity

Further causes of poor between method comparability may be related to the proportion of 25OHD2 that is measured. In many individuals not receiving vitamin D2 the circulating 25OHD2 will approach the sensitivity of most assay procedures and measurement of 25OHD3 will suffice. Supplements or pharmaceuticals may contain either vitamin D2 (ergocalciferol) or vitamin D3 (cholecalciferol). If an individual receives vitamin D2 rather than vitamin D3, both metabolites need to be measured and reported as total 25OHD. In the United Kingdom vitamin D3 is the preferred pharmaceutical preparation but vitamin D2 is often used in situations where a high replacement dose is required. In the USA the only pharmaceutical vitamin D preparation approved by the FDA is vitamin D2 and in some parts of Europe vitamin D2 supplementation is common. Furthermore intake of irradiated commercial mushroom, a source of high concentrations of vitamin D2, may increase as demands from the vegan population increase [50]. Some wild edible mushrooms also contain high concentrations of vitamin D2 [51,52].

For most immunoassays antisera have been incorporated that detect 25OHD3 and a significant proportion of 25OHD2. For IDS immunoassays the proportion of 25OHD2 measured is claimed to be around 75% but in early DEQAS recovery experiments the amount measured was closer to 50% but did increase following a more recent recalibration by the manufacturers [11]. Diasorin claim that their immunoassays measure all 25OHD2 but DEQAS recovery experiments suggest the amount measured may be closer to 80% [11]. Wootton in a review of 25OHD assays, reported that some authors had found that the antibody in the Diasorin RIA did not recognise 25OHD2 and 25OHD3 equally but underestimated 25OHD2 while others had found that the antibody in the Diasorin Liaison assay, although said to be the same as that in the RIA assay, overestimated 25OHD2 compared to 25OHD3 [39]. Zerwekh, however, in a more recent review, stated that the antiserum in the Liaison assay had equal affinity for both forms of the vitamin [53] and Hollis stated that it is co-specific for both 25OHD2 and 25OHD3 [54]. Rather surprisingly the Roche immunoassay is specific for only 25OHD3 and does not measure 25OHD2, which restricts its use to those individuals known not to be receiving vitamin D2. The clinical dangers of adopting this approach have been recently highlighted [55,56]. HPLC and LC–MS/MS procedures have the advantage of being able to measure both 25OHD2 and 25OHD3 independently. Reporting separate results for 25OHD2 and 25OHD3, however, can confuse the requesting clinician and it is clearer to report results as the sum of 25OHD2 plus 25OHD3. If 25OHD2 is present in significant amounts (>25 nmol/L) an additional comment can be appended to the report to alert the clinician.

A separate issue relating to the specificity of different methods is the possibility of cross-reactivity with other metabolites of 25OHD, leading to apparently higher concentrations of total 25OHD. For example, most immunoassays significantly cross-react with 24,25-(OH)2D3, 25,26-(OH)2D3, and 25OHD3-26,23-lactone (Table 2). Although it has been suggested, by some, that these interferences are clinically irrelevant [57] it should, however, be borne in mind that 24,25-dihydroxyvitamin D metabolites do circulate at about 10–15% of the 25OHD concentration and their presence could slightly increase the '25OHD' concentration as measured by immunoassay and cannot be ignored completely. The observation that infants under 1 year have relatively high levels of the C-3 epimers of 25OHD and that these could either cross-react with the antibodies used in immunoassays or show similar chromatography and give rise to the same MS/MS ion pairs in LC–MS/MS methods is of greater concern. It has also been shown that the Diasorin RIA assay did not cross-react with either the 3-epi-25OHD2 or the 3-epi-25OHD3 [57]. It is therefore important that when measuring serum 25OHD in children below 1 year of age an assay is used that either does not cross-react with 3-epi-25OHD or allows unequivocal separation of 3-epi-25OHD from 25OHD. The recent availability of a standard material containing 3-epi-25OHD3 (SRM 972 level3) now allows laboratories to check whether or not their method suffers from interference from this metabolite. At risk HPLC and LC–MS/MS procedures can be adapted by replacing the standard C-18 analytical column with a 5-dinitrobenzoyl-(R)-phenylglycine column and extending run times to permit the separation of the isomers [58].

12.3. Matrix interferences

Matrix effects are known to be a problem with immunoassays and can lead to spuriously high results [56]. The most important type of matrix effect is any that occurs between the matrix in calibrants and patient samples. The lipophilic nature of 25OHD makes it particularly vulnerable to the presence of other lipids in the serum or plasma sample which change the ability of the binding agent to associate with 25OHD in the sample and the standard in an equal fashion [36]. A further problem is that 25OHD cannot be accurately measured unless it is released from its specific binding protein. Many of the earlier competitive protein binding and immunoassay procedures incorporated a solvent extraction step to release 25OHD and remove the binding protein. HPLC and LC–MS/MS use either solvent or solid phase extraction. Many modern commercial immunoassays employ a denaturing agent 'in situ'. Few details are available, due to commercial secrecy, on exactly what is used and the effectiveness of extraction but both ethanol and sodium hydroxide have been implicated.

There is evidence that the earlier vitamin D binding protein assays were more susceptible to matrix effects than assays which use antibodies [36]. Others, however, argue that current immunoassays are also prone to matrix effects particularly those on automated platforms [42] and point to evidence suggesting that the success of the block-and-displace approach used by automated assays is limited. Spuriously high 25OHD results in individual patient samples and higher than expected imprecision can occur, for example, the all-method mean CV for 20 recent specimens distributed through the DEQAS was 18.7% (range: 16.6–20.2%), and the CV for some individual immunoassay methods was greater than this all-method mean [42].

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Table 4
Comparison of methods available for measuring 25-hydroxtvitamin D in the circulation.

	Supplier	Strengths	Limitations[a]
Manual immunoassays			
RIA after solvent extraction	Diasorin	Solvent extraction minimises matrix effects and interferences	More labour intensive than direct immunoassays
			Generates radioactive waste
		Relatively inexpensive	Possible between lot variability in product
RIA after solvent extraction	IDS	Technically simple	Susceptible to matrix effects
		Relatively inexpensive	Possible between lot variability in product
			Generates radioactive waste
Direct EIA	IDS	Technically simple	Susceptible to matrix effects
		Relatively inexpensive	Possible between lot variability in product
			Under-recovers 25-hydroxyvitamin D2
Automated immunoassay			
Liaison Total	Diasorin	Extensively used	Susceptible to matrix effects
		Technically simple	Possible between lot variability in product
		High throughput	
iSYS	IDS	Technically simple	Susceptible to matrix effects
		High throughput	Under-recovers 25-hydroxyvitamin D2
			Possible between lot variability in product
Elecys	Roche	Technically simple	Susceptible to matrix effects
		High throughput	Only detects 25-hydroxyvitamin D3
			Possible between lot variability in product
Direct detection methods			
HPLC	Usually developed or adapted 'in-house'	Solvent or solid phase extraction followed by and interferences	Requires specialised staff
		Process can be automated or semi-automated	Some procedures require large sample volume.
		Separate simultaneous measurement of 25OHD2 and 25OHD3	Lower sample throughput and relatively longer turnaround time compared to immunoassay
		User able to control standardisation	Possible interference from C3-25OHD epimer
		Low reagent costs	
LC/MS/MS	Usually developed or adapted 'in-house'	Solvent or solid phase extraction followed by chromatography minimises matrix effects and interferences	Equipment is expensive
		Process can be automated or semi-automated	Requires specialised staff
		User able to control standardisation	Lower sample throughput and relatively longer turnaround time compared to immunoassay
		Separate simultaneous measurement of 25OHD2 and 25OHD3	Susceptible to ion suppression interference
		Highly accurate and precise when properly validated	Possible interference from C3-25OHD epimer

[a] Prior to mid-2009 the lack of a standard reference material has been a limitation for all methods.

Competitive protein binding procedures are not alone in exhibiting matrix related problems. For LC–MS/MS ion suppression can reduce the performance of the mass detector. Ion suppression can be caused by the presence of non-volatile compounds such as salts, ion-pairing agents, endogenous compounds and drugs or metabolites. A matrix effect related to collecting samples in gel tubes to separate serum from red cells has been recently documented for a LC–MS/MS procedure [59]. Matrix problems can usually be minimised by modification of reagents or chromatographic conditions or choice of ion transitions for measurement [59,60].

12.4. Assay sensitivity

One of the aims of assessing vitamin D status is to identify individuals who are vitamin D deficient. A circulating 25OHD concentration of less than 25nmol/L indicates severe vitamin D deficiency [62]. The functional sensitivity (immunoassays) or limit of quantitation (HPLC and LC–MS/MS) should therefore be below 25 nmol/L. Surprisingly little information is available on functional sensitivities for manual 25OHD immunoassays although detection limits of 5 nmol/L or less imply a functional sensitivity of <10 nmol/L. Functional sensitivities for automated immunoassays are reported by the manufactures as being 17.5 nmol/L or less. All immunoassays are designed to use a small sample volume of 50 μl or less but in automated procedures the 'dead volume' required within the instrument can be considerably higher. It is clear from this survey that LC–MS/MS is significantly more sensitive than HPLC. Reported limits of quantitation were <5 nmol/L (25OHD3) and <8 nmol/L (25OHD2) for LC–MS/MS compared to <17.5 nmol/L (25OHD3) for HPLC methods. This is despite the fact that most HPLC methods use a sample volume of at least 500 μl compared to 200 μl or less for LC–MS/MS. Manual RIA's and LC–MS/MS procedures appear to be the most sensitive but all assays appear to have the required sensitivity to detect identify severe vitamin D deficiency.

12.5. Procedural loss correction (HPLC and LC–MS/MS only)

A significant advantage of HPLC and LC–MS/MS is the potential for correction of procedural losses. Three of the HPLC methods described incorporate an internal standard for this purpose. In one

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HPLC method [27] it is claimed that extraction efficiency is consistently 100% negating the need for a recovery procedure. All the LC–MS/MS procedures use a deuterated (usually hexadeuterated) 25OHD3, for correction of both 25OHD2 and 25OHD3 by isotope dilution. In part the higher values and higher recoveries obtained in DEQAS by LC–MS/MS users may be explained by the correction for procedural losses. Theoretically a separate deuterated 25OHD2 standard should be used for correction of 25OHD2 losses but in practice many laboratories use the 25OHD3 internal standard for this purpose.

12.6. Method comparability

Given the range of methodologies available for the measurement of 25OHD the choice of which is the best method for an individual laboratory is challenging especially given the dramatic increase, over the last few years, of both routine clinical and research use of the measurement. It is clear that there are advantages and limitations for each method and these are summarised in Table 4.

A number of commentators point to the strengths of immunoassays running on automated platforms in terms of convenience and high throughput, especially for laboratories analysing large numbers of samples regularly [39,54,61]. Wootton concluded that measurement of 25OHD by immunoassay would remain the method of choice for reasons of convenience, speed, turnaround and cost [38]. Hollis and Horst suggested that for laboratories needing higher throughput, one of the commercially available RIA, ELISA or instrumentation methods would be more appropriate, although they also noted that commercial kits give more variable results when performed by inexperienced users [63]. Zerwekh recommended that although establishing an in-house assay might be the most cost-effective means of performing 25OHD assays, without the appropriate equipment and expertise, such an undertaking could be formidable [53]. Others, however, argue that the simpler, more convenient methods have sacrificed analytical rigor on the altar of expediency [34] and that they are only able to provide data to indicate relative vitamin D status in semi-quantitative terms. Used in the right context, the kit-based assays are valuable tools for the clinician, but at the moment, most cannot be expected to perform at the level of the HPLC or LC–MS/MS assays which do require more expensive equipment and expert staff [62]. Holick noted recently that these kit-based assays are fraught with technical difficulties, especially if they are not run routinely [6].

Assessing method comparability is not helped by the lack of a recommended reference method. A method based on gas chromatography mass spectrometry (GCMS) is currently under development [34]. Such a method would be too labour intensive to run routinely but would be extremely valuable for comparative purposes. In addition the Center for Disease Control in the USA is currently attempting to obtain reference method status for the LC–MS/MS procedure developed to quantitate the NIST 25OHD standard reference material (SRM 972). In the meantime the between method comparability should show significant improvement over the next year as assays become standardised against SRM 972.

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