Journal of Chromatography B, 878 (2010) 1654–1661

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Review

# Advances in determination of vitamin D related compounds in biological samples using liquid chromatography–mass spectrometry: A review☆

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ARTICLE INFO

*Article history:*
Received 18 September 2009
Accepted 11 November 2009
Available online 18 November 2009

*Keywords:*
Vitamin D metabolite
Synthetic vitamin D analogue
Biological sample
Liquid chromatography–mass spectrometry
Diagnostic assessment
Derivatization

ABSTRACT

The measurements of the serum/plasma concentrations of vitamin D metabolites are widely used for the diagnostic assessment and follow-up of several diseases, such as chronic renal failure and osteoporosis. These metabolites have usually been measured by protein binding assays, such as radioimmunoassay and radioreceptor assay. Although these techniques will doubtless continue to be the methods of choice for routine use in the clinical field, their specificity and accuracy are sometimes poor due to interference from other metabolites and lipids. Among the alternative methods, liquid chromatography (LC) coupled with mass spectrometry (MS) has been used for the analysis of these metabolites and even synthetic vitamin D analogues (therapeutic agents) due to its sensitivity and selectivity. This article reviews recent advances in the determination of vitamin D metabolites and related compounds in biological samples using LC–MS.



**Contents**



## 1. Introduction

The so-called vitamin D (D) consists of six different compounds, $D_2$–$D_7$, which differ in their side chain structures at the 17β-position of the 9,10-seco-steroid. However, actually two compounds, $D_3$ and $D_2$, and their metabolites circulate in human biological fluids. Therefore, D means both $D_3$ and $D_2$ in this review. Both $D_3$ and $D_2$ are absorbed from the diet, but nutritionally adequate amounts of $D_3$ are biosynthesized from its precursor, 7-dehydrocholesterol, in the skin upon irradiation of ultraviolet (UV) light followed by thermal isomerization (Fig. 1). Both $D_3$ and $D_2$ are metabolized in the same way in the human body and show similar biological activities. $D_2$ is solely derived from plant sources; relevant serum/plasma concentrations of its metabolites are observed only after ingestion of a $D_2$ drug preparation [1].

D is hydroxylated in the liver to 25-hydroxyvitamin D [25(OH)D], which is further metabolized in the kidney to form the active metabolite, 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D] [2]. 25(OH)D is the major circulating metabolite and its 3-sulfate might be the storage form [3]. Alternatively, 25(OH)D is thought to be catabolized *via* conversion into (24*R*)-24,25-dihydroxyvitamin D [24,25$(OH)_2$D] and other oxidative metabolites at carbons 23 and 26 of the side chain followed by conjugation [4], although independent biological effects of 24,25$(OH)_2$D are also known [5–7]. Recently, the 3-epimers of the D metabolites were found in biological fluids [8–10], which are biologically inactive, but show the new metabolic pathway of D. 1,25$(OH)_2$D is the most potent metabolite for the stimulation of intestinal calcium transport, the

☆ This paper was presented at the 34th Annual Meeting of the Japanese Society for Biomedical Mass Spectrometry, Osaka, Japan, 10–11 September 2009.

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doi:10.1016/j.jchromb.2009.11.026

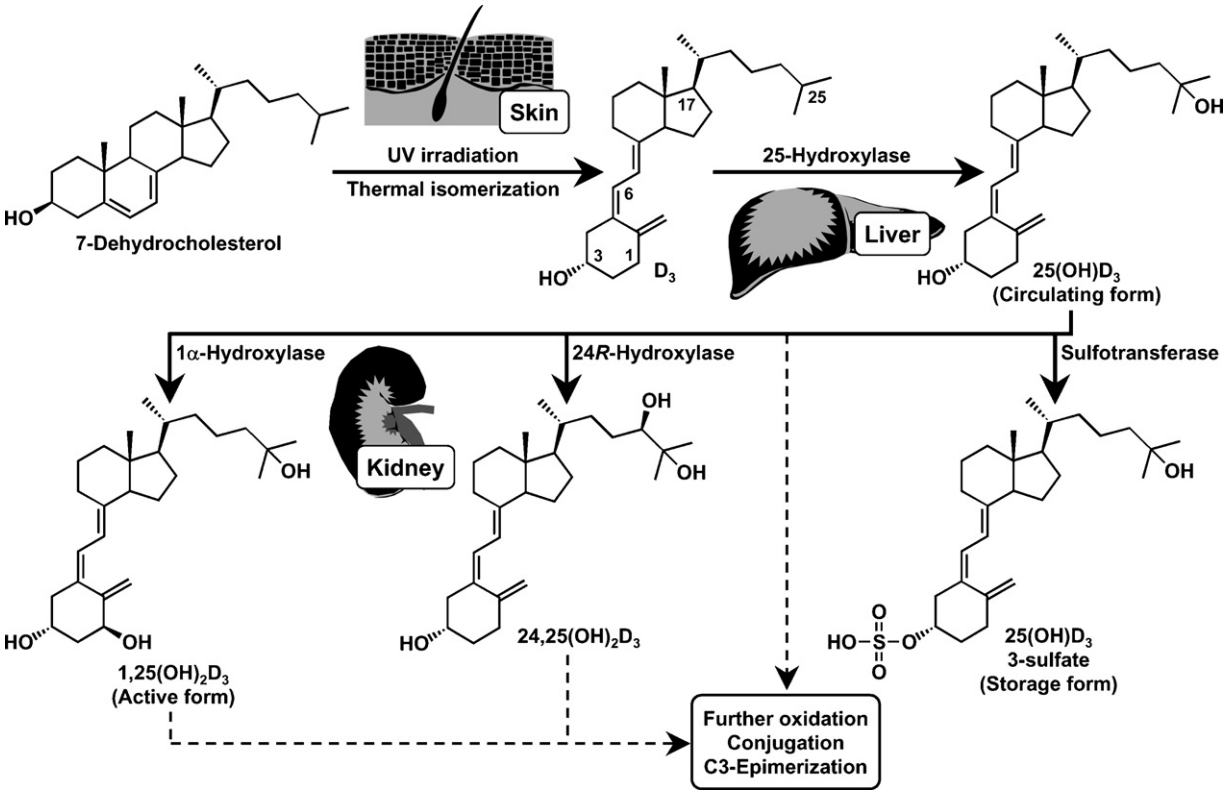


**Fig. 1.** Biosynthesis and metabolism of $D_3$ in man.

mobilization of calcium from bones and for the prevention of rickets [11].

$1,25(OH)_2D_3$ (Rocaltrol®) and its synthetic pro-drug, 1α-hydroxyvitamin $D_3$ [$1(OH)D_3$; Alfarol® and Onealfa®], have been clinically used for the treatment of rickets, hypovitaminosis, hypocalcemia, chronic renal failure and osteoporosis [12]. In 1981, Abe et al. first reported the new activity of $1,25(OH)_2D_3$, namely its ability to differentiate the myeloid leukemia cells into normal monocytes-macrophages [13]. However, the possibility of using $1,25(OH)_2D_3$ or $1(OH)D_3$ as an antileukemic drug was not feasible because of its potential calcemic effects. Based on these observations, numerous noncalcemic analogs with the potential to treat leukemia and other diseases have been developed [14]. For example, maxacalcitol (Oxarol®) and eldecalcitol (ED-71) have been developed for the treatment of secondary hyperparathyroidism and osteoporosis, respectively (Fig. 2). The former has already been used as a medicine and the latter will soon be approved as a medicine [15].

The measurements of the serum/plasma concentrations of D metabolites are widely used for the diagnostic assessment and the follow-up of several diseases (osteoporosis, renal osteodystrophy, parathyroid gland disorders and sarcoidosis) [16,17]. A pharmacokinetic study is required to develop D-related medicines, in which the sensitive and specific determination of the target compounds in biological samples is absolutely imperative. Furthermore, the structural elucidation of the formed product based on *in vitro* and *in vivo* experiments using the limited sample volume is also requisite to clarify the metabolism of D compounds.

The endogenous metabolites have been usually measured by protein binding assays, such as competitive protein binding assay (CPBA) using D-binding globulin (DBP), immunoassay or radioreceptor assay (RRA) using D receptor (VDR). Although these techniques will doubtless continue to be the methods of choice for routine use in clinical fields, their specificity and accuracy are sometimes poor due to interference from other metabolites and lipids. Recently, liquid chromatography (LC) coupled with electrospray ionization (ESI)– or atmospheric pressure chemical ionization (APCI)–mass spectrometry (MS) has been used for the analysis of D compounds (endogenous metabolites and synthetic therapeutic agents), due to its sensitivity, specificity and versatility [18]. Furthermore, LC–MS is suitable for profile analysis and a powerful tool for structural elucidation, which cannot be done by protein binding assays.

In this article, the authors present an overview of the LC–MS analyses of D metabolites and related compounds in biological samples, which were published from January 2004 to June 2009. However, in order to disclose the background, previous papers are arbitrarily cited. No attention has been paid to D compounds in food matrices.

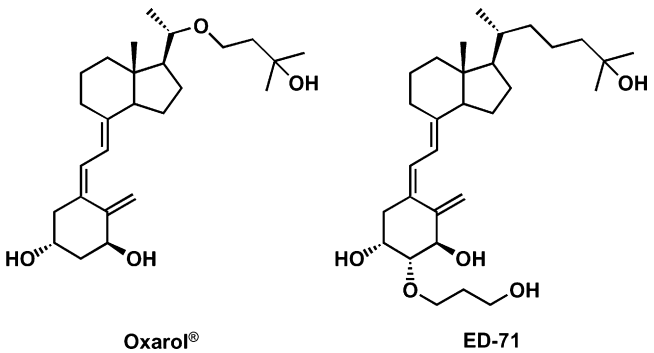


**Fig. 2.** Representative D derivatives that received clinical approval (Oxarol®) and will soon be approved as a medicine (ED-71).

**Table 1**
Representative methods for the determination of D metabolites and related compounds published from January 2004 to June 2009.

| Analyte | Sample treatment | LC conditions (analytical column, mobile phase and flow rate) | MS (instrument, ionization mode and detection) | Sensitivity | Ref. |
|---|---|---|---|---|---|
| 25(OH)$D_3$ IS: $^2H_3$,$^{13}C_1$-25(OH)$D_3$ | Human serum (0.2 mL) → deproteinization (acetonitrile) → on line RP-SPE → column switching technique | LiChrospher 100RP-18 (125 mm × 4.0 mm i.d., Merck), methanol–0.5 mM ammonium acetate and 0.85 mL/min | Quattro (Micromass), positive ESI and SRM ($[M+H]^+ \rightarrow m/z$ 159) | Not described | [34] |
| 25(OH)$D_2$ and 25(OH)$D_3$ IS: $^2H_6$-25(OH)$D_3$ | Human serum (0.1 mL) → deproteinization (methanol–propanol) → LLE (hexane) | Hypersil BDS $C_8$ (51 mm × 2.1 mm i.d., ThermoHypersil), gradient (methanol–water containing 0.05% formic acid) and 0.3 mL/min | API 3000 (Applied Biosystems), positive ESI and SRM ($[M+H]^+ \rightarrow m/z$ 395 [25(OH)$D_2$] and $m/z$ 383 [25(OH)$D_3$]) | Measurable range: 4.95–316 pmol/mL [25(OH)$D_2$] and 4.0–256 pmol/mL [25(OH)$D_3$] | [35] |
| 25(OH)$D_2$ and 25(OH)$D_3$ IS: $^2H_3$-$\Delta^9$-Tetrahydro-cannabinol | Human serum (0.2 mL) → LLE (heptane) | XTerra C18 (50 mm × 2.1 mm i.d., Waters), methanol–2 mM ammonium acetate containing 0.1% formic acid and 0.l mL/min | Quattro (Micromass), positive ESI and SRM ($[M+H]^+ \rightarrow m/z$ 355 [25(OH)$D_2$] and $m/z$ 365 [25(OH)$D_3$]) | LOD: 90 pg/mL [25(OH)$D_2$] and 60 pg/mL [25(OH)$D_3$] | [36] |
| 25(OH)$D_2$ and 25(OH)$D_3$ IS: $^2H_6$-25(OH)$D_3$ | Human serum/plasma (0.2 mL) → deproteinization (methanol) → automated RP-SPE | ACQUITY UPLC BEH C8 (50 mm × 2.1 mm i.d., Waters), gradient (methanol–2 mM ammonium acetate containing 0.1% formic acid) and 0.35 mL/min | ACQUITY TQD (Waters), positive ESI and SRM ($[M+H]^+ \rightarrow m/z$ 83 [25(OH)$D_2$] and $m/z$ 159 [25(OH)$D_3$]) | LLOQ: 7.5 nmol/L [25(OH)$D_2$] and 4.0 nmol/L [25(OH)$D_3$] | [37] |
| 25(OH)$D_2$ and 25(OH)$D_3$ IS: $^2H_6$-25(OH)$D_3$ | Human plasma (0.1 mL) → deproteinization (methanol) → RP-SPE | CAPCELL PAK C18 UG120 (250 mm × 4.6 mm i.d., Shiseido), methanol–water and 0.5 mL/min | API 3000 (Applied Biosystems), positive APCI and SRM ($[M+H]^+ \rightarrow m/z$ 355 [25(OH)$D_2$] and $m/z$ 257 [25(OH)$D_3$]) | LOD (signal/noise = 3): 2.5 ng/mL Measurable range: 5–100 ng/mL | [38] |
| 25(OH)$D_2$, 25(OH)$D_3$, 3-epi-25(OH)$D_2$ and 3-epi-25(OH)$D_3$ IS: $^2H_6$-25(OH)$D_3$ | Human serum (0.2 mL) → deproteinization (acetonitrile) → on-line RP-SPE | Chirex (R)-PGLY and DNB (250 mm × 4.6 mm i.d., Phenomenex), gradient (methanol–water containing 0.005% formic acid) and 0.9 mL/min | API 4000 (Applied Biosystems), positive APCI and SRM ($[M+H]^+ \rightarrow m/z$ 395 [25(OH)$D_2$ and 3-epi-25(OH)$D_2$] and $m/z$ 383 [25(OH)$D_3$ and 3-epi-25(OH)$D_3$]) | Not described | [39] |
| 25(OH)$D_2$ and 25(OH)$D_3$ IS:1(OH)$D_3$ | Human serum (0.5 mL) → deproteinization (methanol–acetonitrile) | ZivakDs (100 mm × 4.6 mm i.d., Zivak), methanol–5% formic acid and 0.5 mL/min | Thermo Finnigan LCQ Deca (Thermo Electron), positive APCI and SRM ($[M+H]^+ \rightarrow m/z$ 395 [25(OH)$D_2$] and $m/z$ 383 [25(OH)$D_3$]) | Measurable range: 10–500 ng/mL | [40] |
| 25(OH)$D_2$ and 25(OH)$D_3$ IS: $^2H_6$-25(OH)$D_3$ | Human serum (0.2 mL) → deproteinization (acetonitrile) → on-line RP-SPE | SUPELCOSIL LC-18-DB (30 mm × 3.0 mm i.d., Supelco), ethanol–water and 0.5 mL/min | Quattro (Micromass), positive APCI and SRM ($[M+H]^+ \rightarrow m/z$ 395 [25(OH)$D_2$] and $m/z$ 383 [25(OH)$D_3$]) | LOD: 1.86 ng/mL [25(OH)$D_2$] and 0.49 ng/mL [25(OH)$D_3$] | [41] |
| 25(OH)$D_3$ IS: 25(OH)$D_4$ | Human saliva (1.0 mL) → deproteinization (acetonitrile) → RPSPE → derivatization (PTAD) | YMC-Pack Pro C18 RS (150 mm × 2.0 mm i.d., YMC), methanol–10 mM ammonium formate containing 5 mM methylamine and 0.2 mL/min | API 2000 (Applied Biosystems), positive ESI and SRM ($[M+CH_3NH_3]^+ \rightarrow m/z$ 298) | LLOQ: 2.0 pg/mL | [51] |
| 25(OH)$D_2$ and 25(OH)$D_3$ IS: $^2H_3$-25(OH)$D_3$ | 3.2-mm in diameter dried blood spot (equivalent to 3.3 μL human whole blood) → extraction (water and then acetonitrile) → derivatization (PTAD) | ZORBAX SB-C18 (50 mm × 2.1 mm i.d., Waters), acetonitrile–water containing 0.1% formic acid and 0.35 mL/min | API 4000 QTRAP (Applied Biosystems), positive ESI and SRM ($[M+H-H_2O]^+ \rightarrow m/z$ 298 [25(OH)$D_2$ and 25(OH)$D_3$]) | LLOQ: 10.7 nmol/L [25(OH)$D_2$] and 7.7 nmol/L [25(OH)$D_3$] | [54] |

Table 1 (*Continued*)

| Analyte | Sample treatment | LC conditions (analytical column, mobile phase and flow rate) | MS (instrument, ionization mode and detection) | Sensitivity | Ref. |
|---|---|---|---|---|---|
| 25(OH)$D_2$, 25(OH)$D_3$, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ IS: $^2H_6$-25(OH)$D_3$ and $^2H_6$-1,25$(OH)_2D_3$ | Human serum (0.5 mL) → deproteinization (acetonitrile) → LLE (methyl *t*-butyl ether) or RPSPE → derivatization (PTAD) | ACQUITY UPLC BEH C18 (100 mm × 2.1 mm i.d., Waters), gradient (acetonitrile–water containing 0.1% formic acid → methanol) and 0.4 mL/min | Quattro Premier T (Micromass), positive ESI and SRM ($[M+H-H_2O]^+ \rightarrow m/z$ 298 [25(OH)$D_2$, 25(OH)$D_3$ and 24,25$(OH)_2D_3$] and $m/z$ 314 [1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$]) | LLOQ: 25 pg/mL | [58] |
| 1(OH)$D_3$ IS: $^2H_4$-1(OH)$D_3$ | Rat plasma (0.1 mL) → deproteinization (acetonitrile) → RPSPE → derivatization (FMTAD) | CAPCELL PAK C18 UG-120 (150 mm × 2.0 mm i.d., Shiseido), acetonitrile–10 mM ammonium acetate and 0.2 mL/min | Quattro LC (Micromass), positive ESI and SRM ($[M]^+ \rightarrow m/z$ 199) | LLOQ: 50 pg/mL | [60] |
| ED-71 and preED-71 IS: $^2H_6$-ED-71 | Human serum (1 mL) → RPSPE (×2) | Symmetry C18 (150 mm × 2.1 mm i.d., Waters), methanol–2 mM ammonium acetate and 0.2 mL/min | Quattro LC (Micromass), positive ESI and SRM ($[M+NH_4]^+ \rightarrow m/z$ 397) | LLOQ: 25 pg/mL | [61] |
| $D_3$ IS: $^2H_3$-$D_3$ | Human serum (1 mL) → deproteinization (ethanol) → LLE (hexane) → RPSPE Fat tissue (0.2–0.25 g) → homogenization (ethanol–water) → saponification (KOH) → LLE (hexane) → RPSPE | ProntoSIL C30 (250 mm × 4.6 mm i.d., MAC-MOD), gradient (methanol, methylene chloride) and 1.0 mL/min | Agilent 1100 (Agilent), positive APCI and SIM ($[M+H]^+$) | LOD (fat tissue): 0.081 pmol/sample | [63] |

## 2. Determination of 25-hydroxyvitamin D

Quantification of 25(OH)D in serum/plasma is the best-established indicator of the D status [19]. The routine quantification of circulating 25(OH)D has been done by protein binding assays using either DBP or anti-25(OH)D antibodies for analyte recognition for almost four decades [20–22]. Currently, CPBA has been generally replaced by immunoassays in the clinical field. The DiaSorin Corporation introduced the fully automated chemiluminescence-based immunoassay, LIAISON®, in 2004. The assay is co-specific for 25(OH)$D_3$ and 25(OH)$D_2$, so it reports a total 25(OH)D concentration [23]. This is the most presently used 25(OH)D assay system in the world for clinical diagnosis. The most recent addition to the automated electrochemiluminescence immunoassay for 25(OH)$D_3$, the Elecsys 25(OH)$D_3$ assay, is from Roche Diagnostics in 2008 [24]. This method follows a competitive assay principle in which the DBP is inactivated during incubation, and can only detect 25(OH)$D_3$ using a specific antibody.

The major difficulty in measuring 25(OH)D using the protein binding assays is attributable to the molecule itself. Its lipophilic nature renders it especially vulnerable to the matrix effects of all protein binding assays. In light of these analytical problems, a reference method for the quantification of 25(OH)D is desired to permit validation of routine protein binding assays, especially in comparison to the LC–MS or LC–tandem MS (MS/MS) method [17,25–27]. The analytical performance of the above-mentioned Elecsys 25(OH)$D_3$ assay was also compared to that of LC–MS/MS [24]. The obtained concentrations of 25(OH)$D_3$ were in good overall agreement with those determined by LC–MS/MS. However, a significant between-method variation was observed in individual patient samples. The use of serum rather than plasma is preferred for the Elecsys 25(OH)$D_3$ assay due to the higher results observed with plasma samples.

Chromatographic methods can separately and simultaneously quantify 25(OH)$D_3$ and 25(OH)$D_2$. Gas chromatography–MS methods were developed years ago, but they are extremely complex and did not gain popularity for quality-control programs or routine assay validation [28–30]. Several HPLC methods with UV detection have been described as well [31,32], but their routine use is limited due to the complex sample preparation requirements. A recently reported HPLC method is equipped with an automated clean-up system [33].

When properly performed, LC–MS is a very accurate testing method. Although the ionization efficiencies of the D metabolites are very low in either ESI or APCI, the serum/plasma 25(OH)$D_3$ can be measured using LC–positive ESI- [34–37] or positive APCI-MS [38–41] without derivatization when a relatively large volume of sample (0.1–0.5 mL) is used, because of its relatively high serum/plasma level (normal range, 10–40 ng/mL). Most of these methods [35–41] can simultaneously determine the 25(OH)$D_2$ levels, and the details are summarized in Table 1. Deuterium-labeled 25(OH)$D_3$ was often used as an internal standard (IS). All the methods listed in Table 1 employed selected reaction monitoring (SRM) for the quantification of 25(OH)D, and the transition from respective $[M+H]^+$ to the dehydrated ions ($[M+H-H_2O]^+$ or $[M+H-2H_2O]^+$) was generally used. As a methodology, LC–MS can favorably compare to the RIA technique [34–38,41] and is becoming increasingly popular for measuring 25(OH)D in biological samples, although LC–MS is inferior to RIA regarding the analysis throughput [35]. Carter and Jones examined the inter-laboratory precision in the 25(OH)D values determined using LC–MS [42]. The use of a common standard improved agreement among laboratories, which suggests that problems with assay standardization contribute to the poor inter-laboratory precision. This may be related to the nature of the matrix used for working standards or errors in the calibration stock standard solutions of 25(OH)D.

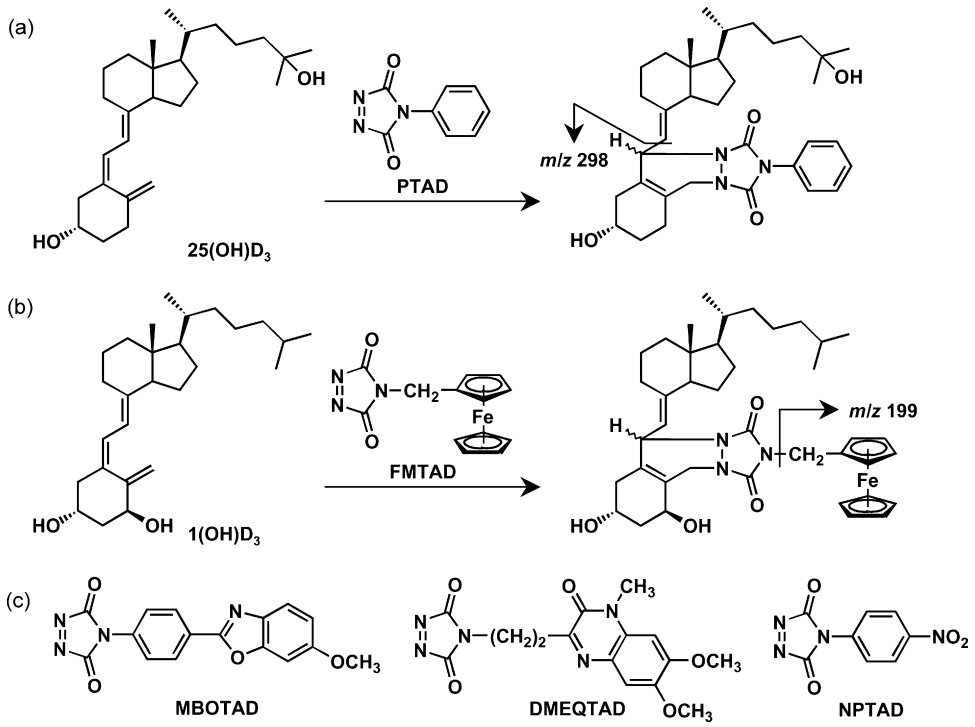


**Fig. 3.** Representative Cookson-type reagents: (a) derivatization of 25(OH)$D_3$ with PTAD and major product ion (*m/z* 298) in MS/MS, (b) derivatization of 1(OH)$D_3$ with FMTAD and major product ion (*m/z* 199) in MS/MS and (c) chemical structures of MBOTAD, DMEQTAD and NPTAD.

One particular problem with MS is its relative inability to discriminate between 25(OH)$D_3$ and its inactive 3-epimer. Taylor et al. have introduced LC–positive APCI-MS/MS for 25(OH)$D_2$ and 25(OH)$D_3$ testing [43], which cannot discriminate the inactive metabolites, and identified significantly elevated results in newborn infants. They modified the method to allow the separate detection of C-3 epimers [39]. They re-tested specimens from four patient groups (group 1; children less than 1-year-old, group 2; children 1–18-year-old, group 3; adults aged 20–87-year-old with liver disease, and group 4; adults aged 19–91-year-old without liver disease) with the new method. In 172 children from group 1 with detectable 25(OH)$D_2$ or 25(OH)$D_3$, they identified the C-3 epimers in 39 subjects (23%). The epimers contributed 9–61% of the total 25(OH)D. There was an inverse relationship between patient age in days and the epimer percentage. No C-3 epimers were detected in any of the other groups. They concluded that the measurement of 25(OH)D in children less than 1 year should therefore be performed with an assay that allows the accurate detection of 25(OH)D in the presence of its 3-epimers.

Saliva has recently been attracting attention as a new tool in clinical examinations and therapeutic drug monitoring due to the easy and noninvasive nature of its collection [44,45]. It is expected that saliva-based assessment of the D status will be highly beneficial to subjects. However, the major disadvantage of using saliva is the low analyte concentration, and therefore, the detection response of 25(OH)D in ESI- or APCI-MS should be significantly increased in order to use saliva for the above purpose. Derivatization has often been employed to enhance the detection response of a poorly ionizable compound in ESI- or APCI-MS [46]. For example, the application of Cookson-type reagents (4-substituted 1,2,4-triazoline-3,5-dione), which rapidly and quantitatively react with the *s-cis*-diene structure of D compounds to give the Diels–Alder adduct, has been examined in order to enhance the sensitivity of D compounds in various modes of ionization (Fig. 3). The Diels–Alder adduct consists of 6*R* and 6*S* isomers that give two peaks in its chromatographic behavior [47] and the formed ratio depends upon the structure of the D compounds. The formation of isomers has a drawback and advantage in sensitivity and selectivity, respectively. Among these reagents, 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione (MBOTAD) in positive APCI-MS/MS [47], 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD) in positive APCI-MS/MS [48] and 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione (NPTAD) in electron capture APCI-MS/MS [49] have been reported to be very effective in enhancing the sensitivity of D compounds (Fig. 3c). However, none of them are currently commercially available, which produces a practical problem. On the contrary, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) [50], a representative Cookson-type reagent, can be purchased from several chemical companies.

Higashi et al. developed a sensitive LC–positive ESI-MS/MS method for the determination of 25(OH)$D_3$ in human saliva [51] (Table 1). The saliva (1 mL) was deproteinized with acetonitrile, purified using a reversed-phase solid phase extraction (RPSPE) cartridge, derivatized with PTAD, and subjected to LC–MS/MS. The PTAD derivative was much more easily ionized in positive ESI-MS and efficiently produced a characteristic product ion during MS/MS (Figs. 3a and 4a), compared to the intact 25(OH)$D_3$, which enabled the sensitive detection using the SRM mode which resulted in a 100 times enhancement of the sensitivity. Methylamine was also used as the mobile phase additive to enhance the assay sensitivity (2 times); an intense adduct ion $[M+CH_3NH_3]^+$ was formed by the addition of methylamine (Fig. 4a). The lower limit of quantification (LLOQ) was 2.0 pg/mL (Fig. 4b) and the method was used for clinical studies. There was a positive linear relationship between the serum and salivary 25(OH)$D_3$ levels.

Low levels of 25(OH)D during early development is associated with a range of adverse health outcomes that emerge later in childhood or during adulthood; developmental D deficiency is associated with an increased risk of schizophrenia [52] and diabetes [53]. Most recently, Eyles et al. reported a sensitive LC–positive ESI-MS/MS assay of 25(OH)D in a neonatal dried blood spot, which is

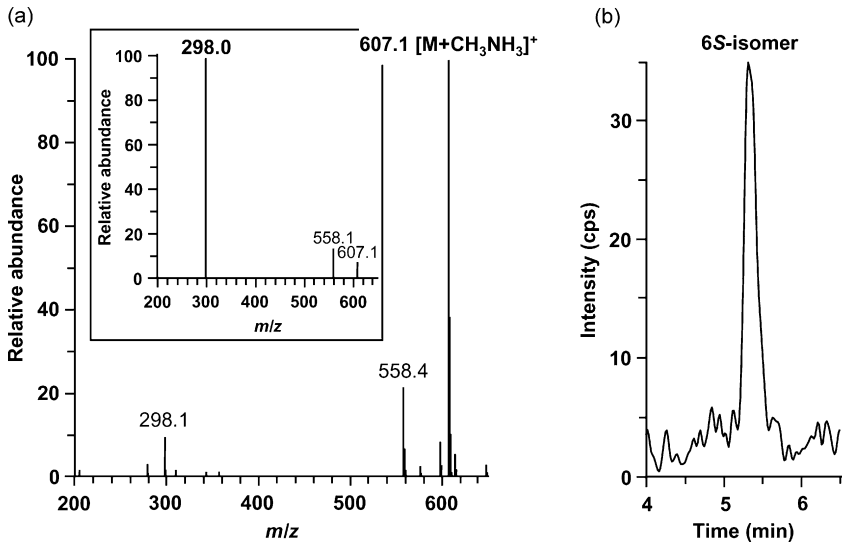


**Fig. 4.** (a) ESI-MS and -MS/MS (inset) spectra of PTAD derivative of 25(OH)$D_3$ and (b) chromatogram of salivary 25(OH)$D_3$ (2.0 pg/mL, LLOQ) as its PTAD derivative. The LC–MS/MS conditions were described in Ref. [51].

routinely used for a broad range of neonatal screening protocols [54] (Table 1). The method also employed the PTAD derivatization to enhance the assay sensitivity and its LLOQs were 7.7 nmol/L for 25(OH)$D_3$ and 10.7 nmol/L for 25(OH)$D_2$ when a 3.2-mm diameter disk (equivalent to 3.3 μL of whole blood) was used. Concerning the storage conditions, there was no significant effect of the different storage conditions (light exposure, room temperature storage for 2 weeks and 10 freeze–thaw cycles) on the measured values. The measurement of the neonatal dried blood spot clearly showed seasonal variations in the blood 25(OH)D level.

## 3. Determination of 1α,25-dihydroxyvitamin D

Quantification of 1,25$(OH)_2D_3$ in serum/plasma is widely used for the diagnostic assessment of chronic renal failure, hypoparathyroidism, osteomalacia and rickets [16,17]. Of all the steroid hormones, 1,25$(OH)_2D_3$ in serum/plasma represented the most difficult challenge to the analytical biochemist with respect to quantification. The metabolite circulated at extremely low concentrations (normal range, 15–60 pg/mL) has been conventionally measured by RRA using VDR or $^{125}$I-based RIA in the clinical field. These methods, however, require a complicated pretreatment of human serum/plasma samples to remove interfering substances, especially the cross-reactive D metabolites [55]. A convenient and reliable method for serum/plasma 1,25$(OH)_2D_3$ using LC–MS is required for a clinical evaluation.

The first LC–MS/MS assay of 1,25$(OH)_2D_3$ in biological fluids was reported in 2001 [56]. The LLOQ of the method was 20 pg/mL for a 1.0-mL serum aliquot. The assay was applied to the analysis of 1,25$(OH)_2D_3$ in rat and pig serums, but was not used for the human serum/plasma analysis. Recently, LC–coordination ion spray (CIS)-MS using $Li^+$ or $Ag^+$ as an additive has been reported for the assay of 1,25$(OH)_2D_3$, which showed an LLOQ of 25 pg/mL or 10 pg/mL, respectively [57]. The details of this technique have not been clarified, and the ESI source design is not perfectly suited for this type of ionization; long-term use of this method leads to a deposit of metal on the first quadrupole rods which might cause deterioration during use of the instrument.

Aronov et al. demonstrated that liquid–liquid extraction (LLE) or the RPSPE of D metabolites in combination with the PTAD derivatization followed by ultra-performance LC (UPLC)–positive ESI-MS/MS analysis provided a rapid and simultaneous quantification of 25$(OH)D_2$, 25$(OH)D_3$, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$ in 0.5 mL of human serum with LLOQs of 25 pg/mL [58] (Table 1). No significant interfering peak was found for any of the analytes except for 1,25$(OH)_2D_3$. The interfering peak was derived from a derivatized serum component and could not be suppressed by increasing the quadrupole resolution, chromatographic resolution or pretreatment. The method is still of less practical use for the determination of these D metabolites, except for 25$(OH)D_3$ and 24,25$(OH)_2D_3$, in the clinical field due to its sensitivity and selectivity. A promising LC–ESI-MS/MS assay for the plasma 1,25$(OH)_2D_3$ was reported at the 57th annual conference of the American Society for Mass Spectrometry (Philadelphia, PA, USA, May–June 2009) [59]. The pretreatment procedure consists of protein precipitation by acetonitrile and the PTAD-derivatization. The derivatized mixture was then loaded onto the selective SPE and sensitive capillary LC–MS/MS for analysis. The derivatization and use of capillary LC significantly enhanced the assay sensitivity; the limits of detection (LODs) of around 0.5 pg/mL were achieved for all target analytes [25$(OH)D_2$, 25$(OH)D_3$, 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$] using only 200 μL of plasma. Although the fact that it is not easy to measure the serum/plasma 1,25$(OH)_2D_3$ levels using LC–MS with a clinically available sample volume (less than 1 mL) is still unchanged, this method might be positioned as the breakthrough for the routine 1,25$(OH)_2D_3$ assay using LC–MS.

## 4. Determination of other vitamin D compounds

Although the significance of the determination of 24,25$(OH)_2D_3$ in serum/plasma (normal range, 1–4 ng/mL) in clinical field is not so high, a plasma 24,25$(OH)_2D_3$ assay method using LC–positive APCI-MS/MS combined with the MBOTAD derivatization was reported in 2001 [47]. A new determination method of 24,25$(OH)_2D_3$ in serum using PTAD as the derivatization reagent for the LC–positive ESI-MS/MS analysis has been reported as already described [58].

The determination of 1$(OH)D_3$ (Alfarol® and Onealfa®), the prodrug of 1,25$(OH)_2D_3$, in human plasma using LC–positive APCI-MS/MS combined with DMEQTAD derivatization followed by acetylation was reported in 2002 [48]. A new Cookson-type reagent, 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD), has been developed for the determination of 1$(OH)D_3$ in rat plasma using LC–positive ESI-MS/MS [60] (Fig. 3b and Table 1). FMTAD has the easily oxidizable ferrocene residue and converts the poorly ionizable 1$(OH)D_3$ into the highly ESI-active derivative. The analyte in rat plasma was extracted with acetonitrile and then purified

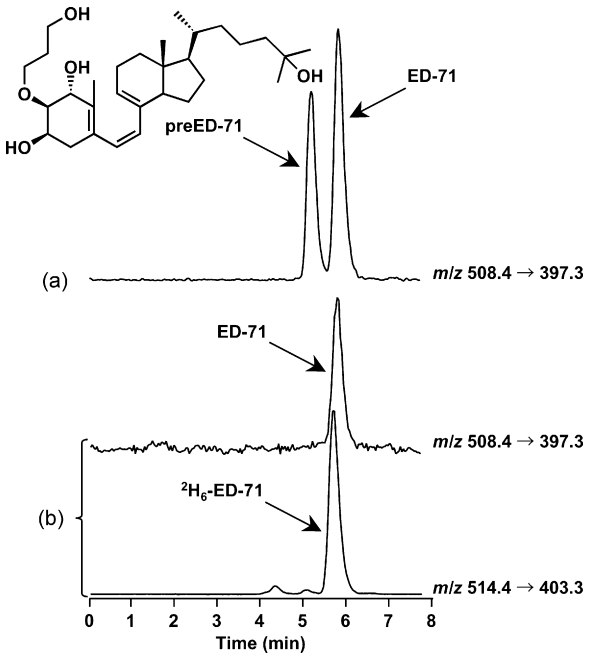


**Fig. 5.** SRM chromatograms of ED-71, preED-71 and $^2H_6$-ED-71 (IS): (a) authentic mixture of ED-71 and preED-71, (b) human serum obtained 5 h after a single oral administration of ED-71 (tablet, 1 μg). SRM transitions: *m/z* 508.4 → 397.3 for ED-71 and preED-71; *m/z* 514.4 → 403.3 for $^2H_6$-ED-71. Data were kindly provided by Dr. N. Murao (Chugai Pharmaceutical Co.).

using RPSPE 96-well plates. After the derivatization with FMTAD, the samples were subjected to LC–ESI-MS/MS employing a column switching system. The FMTAD derivatives provided $[M]^+$ in the positive ESI-MS. The major product ion at *m/z* 199 is attributable to the ferrocenylmethyl cation as shown in Fig. 3b. The method achieved an LLOQ of 5 pg from plasma (0.1 mL) and had a 200-fold higher sensitivity to that without derivatization. The method was fully validated with accuracy and reproducibility and was successfully applied to the pharmacokinetic study in rats.

D compounds are normally in thermal equilibrium with their pre-D isomers. The equilibrium of the reaction between D and pre-D significantly inclines toward D under normal conditions (neutral pH and room temperature) without biological fluids. In addition, it was reported that the pre-form of $1,25(OH)_2D_3$ lost most of its biological activity. However, the isomeric conversion into the pre-form after administration of a D compound has not been well understood. Furthermore, there is the possibility of causing overestimation in the determination of D compounds using LC–MS, because pre-forms have the same molecular weights as the D compounds. Much attention has not been focused on the separation of D compounds and their pre-forms, and recently, Murao et al. reported the analytical method for ED-71 and its pre-form in human serum using LC–ESI-MS/MS [61] (Table 1). ED-71 was singly orally administered to healthy volunteers (1 μg, tablet or capsule). The analyte in human serum (1 mL) was extracted using two RPSPE cartridges and the separation of ED-71 and its pre-form was done using a reversed-phase column (Fig. 5a). ESI-MS/MS analysis was operated using SRM in the positive ion mode. This method gave a satisfactory LLOQ (25 pg/mL) with good accuracy and precision. There was no peak of the pre-form in the SRM chromatogram obtained from a serum sample (Fig. 5b), which demonstrated that mostly the biologically active ED-71 was exposed to human blood stream. A good correlation was observed in the results obtained from the developed method and RRA combined with HPLC purification. The same group has already reported the determination of Oxarol® in human serum using LC–positive ESI-MS/MS in 1998, which was helpful for the pharmacokinetic study [62]. ED-71 and Oxarol® have ether bonds at the C-2 position and in the side chain, respectively (Fig. 2), and these bonds are easily cleaved during MS/MS, which enables the sensitive and selective detection using the SRM mode.

The literature describing the D content of fat tissue is extremely limited. Blum et al. measured the concentration of $D_3$ in the fat tissue and serum of obese adults using LC–MS [63] (Table 1). The fat tissue was treated with 30% KOH followed by RPSPE, the sample was analyzed by selected ion monitoring (SIM) of the LC–positive APCI-MS. Compatible with the long-standing concept that fat tissue is a storage site for D, the fat tissue and serum $D_3$ concentrations were positively correlated ($r = 0.68$, $P = 0.003$).

## 5. Analysis of metabolism of vitamin D compounds

The development of novel gene expression systems for cytochrome P450s (CYPs) together with that of analytical methods using LC–ESI-MS have opened the door to answering some questions about the D compound metabolism. CYP24 metabolized $25(OH)D_3$ and $1,25(OH)_2D_3$ at similar rates *in vitro*, but DBP present in the culture medium has a profound influence on the rate of $25(OH)D_3$ metabolism, but not the $1,25(OH)_2D_3$ metabolism [64]. The *in vitro* metabolites of $1,25(OH)_2D_2$ with human CYP24 were also isolated and identified using LC–APCI-MS [65]. Various transfected CYPs and hepatic cell lines combined with the LC–MS/MS analysis were used to investigate the metabolism of a spectrum of the clinically-relevant D analogs. In the case of $1(OH)D_3$, evidence of multiple sites of side-chain hydroxylation consistent with the generation of more than one active form was found [66].

LC–MS/MS also contributed to the finding of the C-3 epimerization pathway in the metabolism of D compounds [8,9]. Although little is known about the enzyme responsible for the C-3 epimerization, Higashi et al. demonstrated that hydroxysteroid dehydrogenase enzymes may catalyze the epimerization *in vitro* [67].

## 6. Conclusion

This article reviewed recent advances in the determination of D metabolites [25(OH)D, $1,25(OH)_2D$] and related compounds including synthetic D analogs in biological samples using LC–MS(/MS). As a routine assay, 25(OH)D is measured with commercial kits using immunoassay. However, LC–MS(/MS) might be the next most utilized procedure for the clinical assessment of circulating 25(OH)D due to its high sensitivity and specificity.

$1,25(OH)_2D_3$ circulates at extremely low concentrations and has been conventionally measured by RRA or $^{125}$I-based RIA in the clinical field. At present, LC–MS/MS is not a definitive means for the determination of this active metabolite because of lack of sensitivity and selectivity. However, a highly sensitive and selective method combined with derivatization and the capillary LC has been reported [59] and the authors believe that LC–MS/MS will perform a central role in the clinical testing of $1,25(OH)_2D_3$ in the near future.

LC–MS/MS has been successfully applied to the quantification of the synthetic analogs in biological fluids, which demonstrates that LC–MS/MS is a powerful tool for the development of D related medicines.

## Acknowledgements

Our thanks go to Drs. Noboru Kubodera, Naoaki Murao and Masaki Ishigai (Chugai Pharmaceutical Co., Tokyo, Japan) for their helpful suggestions.

## References


[1] P.B. Rapuri, J.C. Gallagher, G. Haynatzki, Calcif. Tissue Int. 74 (2004) 150.
[2] H.F. DeLuca, H.K. Schnoes, Annu. Rev. Biochem. 52 (1983) 411.
[3] T. Higashi, K. Mitamura, H. Ohmi, N. Yamada, K. Shimada, K. Tanaka, H. Honjo, Ann. Clin. Biochem. 36 (1999) 43.
[4] D.E. Prosser, G. Jones, Trends Biochem. Sci. 29 (2004) 664.
[5] B.D. Boyan, V.L. Sylvia, D.D. Dean, F. Del Toro, Z. Schwartz, Crit. Rev. Oral Biol. Med. 13 (2002) 143.
[6] Z. Schwartz, H. Ehland, V.L. Sylvia, D. Larsson, R.R. Hardin, V. Bingham, D. Lopez, D.D. Dean, B.D. Boyan, Endocrinology 143 (2002) 2775.
[7] B.D. Boyan, V.L. Sylvia, D.D. Dean, Z. Schwartz, Steroids 66 (2001) 363.
[8] T. Higashi, R. Kikuchi, K. Miura, K. Shimada, H. Hiyamizu, H. Ooi, Y. Iwabuchi, S. Hatakeyama, N. Kubodera, Biol. Pharm. Bull. 22 (1999) 767.
[9] H. Sekimoto, M.L. Siu-Caldera, A. Weiskopf, P. Vouros, K.R. Muralidharan, W.H. Okamura, M.R. Uskokovic, G.S. Reddy, FEBS Lett. 448 (1999) 278.
[10] M. Kamao, S. Tatematsu, S. Hatakeyama, T. Sakaki, N. Sawada, K. Inouye, K. Ozono, N. Kubodera, G.S. Reddy, T. Okano, J. Biol. Chem. 279 (2004) 15897.
[11] H.F. DeLuca, FASEB J. 2 (1988) 224.
[12] A. Shiraishi, S. Higashi, H. Ohkawa, N. Kubodera, T. Hirasawa, I. Ezawa, K. Ikeda, E. Ogata, Calcif. Tissue Int. 65 (1999) 311.
[13] E. Abe, C. Miyaura, C. Sakagami, H. Takeda, M. Konno, K. Yamazaki, T. Yoshiki, T. Suda, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 4990.
[14] A.J. Brown, E. Slatopolsky, Mol. Aspects Med. 29 (2008) 433.
[15] N. Kubodera, Curr. Bioact. Compd. 2 (2006) 301.
[16] N. Tsugawa, T. Okano, Clin. Calcium 16 (2006) 36.
[17] B.W. Hollis, R.L. Horst, J. Steroid Biochem. Mol. Biol. 103 (2007) 473.
[18] T. Higashi, T. Nishio, K. Shimada, Encyclo. Chromatogr., Taylor and Francis, Abingdon, U.K., 2007, doi:10.1081/E-ECHR-120043822.
[19] D.B. Endres, R.K. Rude, in: C.A. Burtis, E.R. Ashwood (Eds.), Tietz Textbook of Clinical Chemistry, 3rd ed., W.B. Saunders, Philadelphia, U.S.A., 1999, p. 1395.
[20] J.C. Haddad, K.J. Chyu, J. Clin. Endocrinol. 33 (1971) 992.
[21] B.W. Hollis, J.Q. Kamerud, S.R. Selvaag, J.D. Lorenz, J.L. Napoli, Clin. Chem. 39 (1993) 529.
[22] C. Lind, J. Chen, I. Byrjalsen, Clin. Chem. 43 (1997) 943.
[23] D.L. Ersfeld, D.S. Rao, J.-J. Body, J.L. Sackrison Jr., A.B. Miller, N. Parikh, T.L. Eskridge, A. Polinske, G.T. Olson, G.D. MacFarlane, Clin. Biochem. 37 (2004) 867.
[24] A. Leino, U. Turpeinen, P. Koskinen, Clin. Chem. 54 (2008) 2059.
[25] G.D. Carter, J.C. Jones, J.L. Berry, J. Steroid Biochem. Mol. Biol. 103 (2007) 480.
[26] H.J. Roth, H. Schmidt-Gayk, H. Weber, C. Niederau, Ann. Clin. Biochem. 45 (Pt. 2) (2008) 153.
[27] B.W. Hollis, Curr. Opin. Endocrinol. Diabetes Obes. 15 (2008) 489.
[28] A.P. De Leenheer, A.A. Cruyl, Anal. Biochem. 91 (1978) 293.
[29] I. Björkhem, I. Holmberg, Methods Enzymol. 67 (1980) 385.
[30] R.D. Coldwell, D.J. Trafford, M.J. Varley, D.N. Kirk, H.L. Makin, Clin. Chim. Acta 180 (1989) 157.
[31] K. Shimada, K. Mitamura, N. Kitama, M. Kawasaki, J. Chromatogr. B 689 (1997) 409.
[32] U. Turpeinen, U. Hohenthal, U.H. Stenman, Clin. Chem. 49 (2003) 1521.
[33] G.L. Lensmeyer, D.A. Wiebe, N. Binkley, M.K. Drezner, Clin. Chem. 52 (2006) 1120.
[34] M. Vogeser, A. Kyriantsoulis, E. Huber, U. Kobold, Clin. Chem. 50 (2004) 1415.
[35] Z. Maunsell, D.J. Wright, S.J. Rainbow, Clin. Chem. 51 (2005) 1683.
[36] A.K. Saenger, T.J. Laha, D.E. Bremner, S.M.H. Sadrzadeh, Am. J. Clin. Pathol. 125 (2006) 914.
[37] S. Knox, J. Harris, L. Calton, A.M. Wallace, Ann. Clin. Biochem. 46 (2009) 226.
[38] N. Tsugawa, Y. Suhara, M. Kamao, T. Okano, Anal. Chem. 77 (2005) 3001.
[39] R.J. Singh, R.L. Taylor, G.S. Reddy, S.K.G. Grebe, J. Clin. Endocrinol. Metab. 91 (2006) 3055.
[40] A.C. Gören, G. Bilsel, M. Bilsel, J. Chem. Metrl. 1 (2007) 1.
[41] H. Chen, L.F. McCoy, R.L. Schleicher, C.M. Pfeiffer, Clin. Chim. Acta 391 (2008) 6.
[42] G.D. Carter, J.C. Jones, Ann. Clin. Biochem. 46 (Pt. 1) (2009) 79.
[43] R.L. Taylor, S.K. Grebe, R.J. Singh, Clin. Chem. 51 (Suppl 6–05) (2005) A231.
[44] M. Gröschl, Clin. Chem. 54 (2008) 1759.
[45] R. Mullangi, S. Agrawal, N.R. Srinivas, Biomed. Chromatogr. 23 (2009) 3.
[46] T. Higashi, Chem. Pharm. Bull. 54 (2006) 1479.
[47] T. Higashi, D. Awada, K. Shimada, Biomed. Chromatogr. 15 (2001) 133.
[48] T. Higashi, D. Awada, K. Shimada, J. Chromatogr. B 772 (2002) 229.
[49] T. Higashi, A. Yamauchi, K. Shimada, Anal. Sci. 19 (2003) 941.
[50] R.C. Cookson, S.S. Gupte, I.D.R. Stevens, C.T. Watts, Org. Synth. 51 (1971) 121.
[51] T. Higashi, Y. Shibayama, M. Fuji, K. Shimada, Anal. Bioanal. Chem. 391 (2008) 229.
[52] J. McGrath, D. Eyles, B. Mowry, R. Yolken, S.L. Buka, Schizophr. Res. 63 (2003) 73.
[53] E. Hyppönen, E. Läärä, A. Reunanen, M.R. Järvelin, S.M. Virtanen, Lancet 358 (2001) 1500.
[54] D. Eyles, C. Anderson, P. Ko, A. Jones, A. Thomas, T. Burne, P.B. Mortensen, B. Nørgaard-Pedersen, D.M. Hougaard, J. McGrath, Clin. Chim. Acta 403 (2009) 145.
[55] N. Kobayashi, T. Imazu, J. Kitahori, H. Mano, K. Shimada, Anal. Biochem. 244 (1997) 374.
[56] A.M. Kissmeyer, K. Sonne, J. Chromatogr. A 935 (2001) 93.
[57] E. Fedorov, M. Coutu, J. Chapdelaine, L. Ciochina, J.-F. Larocque, M. Mancini, Proc. 56 ASMS Conf. 19 (2008) 97S.
[58] P.A. Aronov, L.M. Hall, K. Dettmer, C.B. Stephensen, B.D. Hammock, Anal. Bioanal. Chem. 391 (2008) 1917.
[59] X. Duan, E. Bang, H. Wang, B. Weinstock-Guttman, M. Ramanathan, J. Qu, Proc. 57th ASMS Conf. (2009) MOB pm 3:30.
[60] N. Murao, M. Ishigai, N. Sekiguchi, T. Takahashi, Y. Aso, Anal. Biochem. 346 (2005) 158.
[61] N. Murao, N. Ohishi, Y. Nabuchi, M. Ishigai, T. Kawanishi, Y. Aso, J. Chromatogr. B 823 (2005) 61.
[62] M. Ishigai, Y. Asoh, K. Kumaki, J. Chromatogr. B 706 (1998) 261.
[63] M. Blum, G. Dolnikowski, E. Seyoum, S.S. Harris, S.L. Booth, J. Peterson, E. Saltzman, B. Dawson-Hughes, Endocrine 33 (2008) 90.
[64] S. Masuda, M. Kaufmann, V. Byford, M. Gao, R. St-Arnaud, A. Arabian, H.L.J. Makin, J.C. Knutson, S. Strugnell, G. Jones, J. Steroid Biochem. Mol. Biol. 89–90 (2004) 149.
[65] N. Urushino, K. Yasuda, S. Ikushiro, M. Kamakura, M. Ohta, T. Sakaki, Biochem. Biophys. Res. Commun. 384 (2009) 144.
[66] G. Jones, V. Byford, S. West, S. Masuda, G. Ibrahim, M. Kaufmann, J.C. Knutson, S. Strugnell, R. Mehta, Anticancer Res. 26 (2006) 2589.
[67] T. Higashi, K. Sakajiri, K. Shimada, J. Pharm. Biomed. Anal. 36 (2004) 429.