



Enhanced 3-epi-25-hydroxyvitamin D₃ signal leads to overestimation of its concentration and amplifies interference in 25-hydroxyvitamin D LC-MS/MS assays

N Flynn, F Lam and A Dawney

Abstract

Background: 3-epi-25-hydroxyvitamin D₃ (3-epi-25OHD₃) interferes in most liquid chromatography-tandem mass spectrometry (LC-MS/MS) assays for 25-hydroxyvitamin D (25OHD). The clinical significance of this is unclear, with concentrations from undetectable to 230 nmol/L reported. Many studies have quantified 3-epi-25OHD₃ based on 25OHD₃ calibrators or other indirect methods, and we speculated that this contributes to the observed variability in reported 3-epi-25OHD₃ concentrations.

Methods: We compared continuous MS/MS infusions of 3-epi-25OHD₃ and 25OHD₃ solutions, spiked both analytes into the same serum matrix and analysed patient samples to assess the effect of three different quantitation methods on 3-epi-25OHD₃ concentration. Experiments were performed on an LC-MS/MS system using a phenyl column which does not resolve 3-epi-25OHD₃, and a modified method utilizing a Zorbax SB-CN column that chromatographically resolves 3-epi-25OHD₃ from 25OHD₃.

Results: A greater 3-epi-25OHD₃ signal, compared with 25OHD₃, was observed during equimolar post-column continuous infusion of analyte solutions, and following analysis of a serum pool spiked with both analytes. 3-epi-25OHD₃ signal enhancement was dependent on mobile phase composition. Compared with 3-epi-25OHD₃ calibrators, indirect quantitation methods resulted in up to 10 times as many samples having 3-epi-25OHD₃ concentrations \geq 10 nmol/L, and an approximately fourfold increase in the maximum observed 3-epi-25OHD₃ concentration to 95 nmol/L.

Conclusions: Enhanced 3-epi-25OHD₃ signal leads to overestimation of its concentrations in the indirect quantitation methods used in many previous studies. The enhanced signal may contribute to greater interference in some 25OHD LC-MS/MS assays than others. We highlight that equimolar responses cannot be assumed in LC-MS/MS systems, even if two molecules are structurally similar.

Keywords

3-epi-25-hydroxyvitamin D₃, 25-hydroxyvitamin D, epimer, LC-MS/MS, ionization

Accepted: 21st June 2013

Introduction

There is interference from 3-epi-25-hydroxyvitamin D₃ (3-epi-25OHD₃) in most liquid chromatography-tandem mass spectrometry (LC-MS/MS) 25-hydroxyvitamin D (25OHD) assays. The clinical significance of this is unclear, with some studies reporting concentrations up to 230 nmol/L,¹ but others that

Department of Clinical Biochemistry, University College London Hospitals NHS Foundation Trust, London, UK

Corresponding author:

A Dawney, Department of Clinical Biochemistry, University College London Hospitals NHS Foundation Trust, 60 Whitfield Street, London, W1T 4EU, UK.

Email: anne.dawney@uclh.nhs.uk

3-epi-25OHD₃ forms a minor proportion of total 25OHD₃.^{2,3} This may reflect patient selection, since 3-epi-25OHD₃ has been reported to be higher in infants than adults.

Some studies assume that equimolar 3-epi-25OHD₃ and 25OHD₃ produce equal signals in LC-MS/MS assays. 3-epi-25OHD₃ quantitation has been based on 25OHD₃ calibrators¹ or the difference in 25OHD₃ results between an assay which chromatographically separates the epimers and one that does not.⁴ We noticed high 3-epi-25OHD₃ recovery in our routine 25OHD LC-MS/MS assay and speculated that 3-epi-25OHD₃ produces a greater signal than 25OHD₃, enhancing interference in LC-MS/MS assays and overestimating 3-epi-25OHD₃ in assays lacking dedicated calibrators.

To investigate this, we spiked 3-epi-25OHD₃ and 25OHD₃ into serum, compared analyte infusions and analysed patient samples to assess different 3-epi-25OHD₃ quantitation methods. We used two LC-MS/MS methods: our routine 'co-elution method' in which 25OHD₃ and 3-epi-25OHD₃ co-elute, and a 'resolving method' that chromatographically separates the epimers.

Materials and methods

Materials

25OHD₃ monohydrate, 3-epi-25OHD₃, methanol, ammonium acetate and zinc sulphate heptahydrate were from Sigma-Aldrich (St Louis, MO, USA). 26,26,26,27,27,27-d₆-25OHD₃ was from Synthetica (Oslo, Norway). Ethanol was from VWR International (Radnor, PA, USA). Isopropanol and formic acid were from Fisher Scientific (Waltham, MA, USA). All solvents were LC-MS grade. Deionized water was taken from an ELGA Purelab Option-Q15BP water deionizer (ELGA Process Water, Marlow, UK) with a resistivity reading of 18.2 MΩ-cm.

Master solutions of 25OHD₃ and 3-epi-25OHD₃ at a concentration of 2.5 mmol/L were prepared in ethanol. The master solution was diluted 1/250 in ethanol to produce stock solutions of approximately 10 μmol/L. Concentrations of stock 3-epi-25OHD₃ and 25OHD₃ solutions were determined from their absorbances at 264 nm using a molar extinction coefficient $\epsilon_0 = 18,200 \text{ L mol}^{-1} \text{ cm}^{-1}$,² a UVIKON spectrophotometer (NorthStar Scientific, Potton, UK) and quartz cuvettes. 25OHD-deficient human serum pools were prepared from surplus patient samples with total 25OHD < 10 nmol/L. Dedicated 3-epi-25OHD₃ calibrators were prepared in-house from 25OHD-deficient serum spiked with 3-epi-25OHD₃. ChromSystems 3PLUS1[®] Calibrator Set (Chromsystems, Munich, Germany) was used unmodified for 25OHD₃

calibration. ChromSystems MassCheck 25-hydroxyvitamin D₂/D₃ serum controls (Level I and II) were used as quality control (QC) material for 25OHD₂ and 25OHD₃. For 3-epi-25OHD₃, QC material was prepared by spiking 3-epi-25OHD₃ into a human serum pool.

Specimen processing

Samples underwent semi-automated solid phase extraction (SPE)⁵ and analysis on a Waters ACQUITY UPLC-TQD (Waters, Milford, MA, USA). Briefly, 150 μL sample was mixed with 20 μL 26,26,26,27,27,27-d₆-25OHD₃ internal standard and proteins precipitated using ZnSO₄ and methanol. Following centrifugation, 600 μL of supernatant was transferred to a pre-conditioned Oasis HLB (30 μm) μElution plate (Waters, Milford, MA, USA). Sample cleanup on the μElution plate was via the use of aqueous methanol solutions. Analytes were eluted by 5% isopropanol/95% methanol into 96-well polypropylene plates (Waters, Milford, MA, USA). The 96-well plate was heat-sealed, vortexed and centrifuged before analysis.

MS parameters

Analytes underwent positive electrospray ionization and detection by multiple reaction monitoring. The 25OHD₃ and 3-epi-25OHD₃ response was the 401.3 > 159.1 peak area (collision energy 28 eV) relative to the d₆-25OHD₃ internal standard 407.3 > 159.1 peak area (collision energy 28 eV). The 401.3 > 365.2 transition at a collision energy of 10 eV was used as a qualifier transition for both 25OHD₃ and 3-epi-25OHD₃. For infusion experiments, we also monitored the 401.3 > 383.5 transition at a collision energy of 10 eV. The cone voltage was set at 22 V and the capillary voltage at 2.5 kV for all 25OHD₃ and 3-epi-25OHD₃ transitions.

Chromatography

Mobile phase A was water and mobile phase B was methanol, each supplemented with 2 mmol/L ammonium acetate and 0.1% (v/v) formic acid.

The co-elution method used a Waters ACQUITY UPLC BEH Phenyl column (1.7 μm, 2.1 × 50 mm) (Waters, Milford, MA, USA) with mobile phase B increasing from 65% to 98% over 3.6 min at 0.45 ml/min followed by re-equilibration at 65% B. Analytes co-eluted at 2.7 min with a runtime of 5 min. The limit of quantitation was 7 nmol/L based on a signal-to-noise ratio of 10. Inter-batch % coefficient of variation (%CV) for 25OHD₃ was < 10% at 42 nmol/L and 97 nmol/L. Recoveries of 25OHD₃ spiked into serum were within 20% of target.

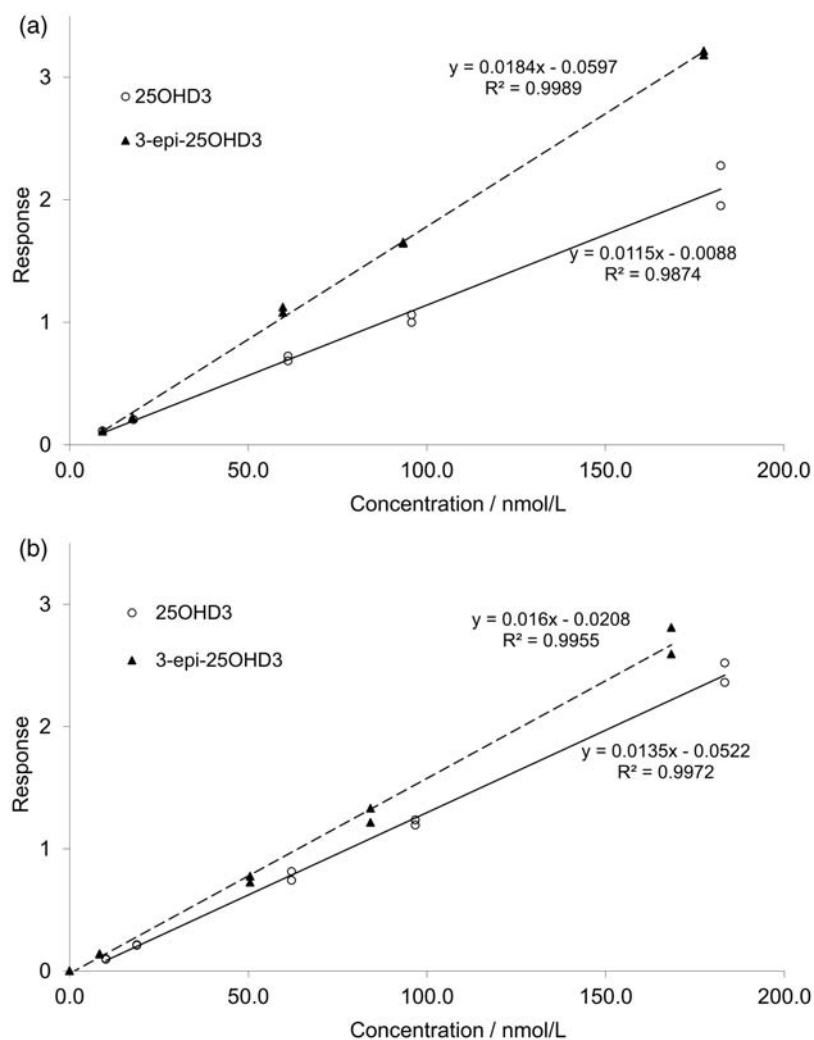


Figure 1. Measured response for 25OHD-deficient serum spiked with 25OHD₃ and 3-epi-25OHD₃ in (a) the co-elution method and (b) the resolving method.

The resolving method used an Agilent Zorbax SB-CN column (1.8 μ m, 2.1 \times 50 mm) (Agilent Technologies, Santa Clara, CA, USA) with mobile phase B held at 55% for 24 min at 0.4 mL/min to resolve 25OHD₃ (retention time 17.0 min) from 3-epi-25OHD₃ (retention time 19.6 min). The column was washed at 98% B and reequilibrated at 55% B. The limit of quantitation was 10 nmol/L based on a signal-to-noise ratio of 10. Interbatch %CVs were < 10% at 42 nmol/L and 91 nmol/L for 25OHD₃, and 7.8% at 15 nmol/L and 11.7% at 50 nmol/L for 3-epi-25OHD₃. Recoveries of 25OHD₃ and 3-epi-25OHD₃ spiked into serum were within 20% of target.

Spiking experiments

For spiking experiments, 25OHD-deficient serum was spiked with either 25OHD₃ or 3-epi-25OHD₃. In the

co-elution method, the response for both sets of samples was the combined (25OHD₃ + 3-epi-25OHD₃) peak area divided by the d₆-25OHD₃ internal standard peak area, as 25OHD₃ and 3-epi-25OHD₃ co-eluted. The concentration was calculated as the concentration measured in the base pool (9 nmol/L) plus the concentration of 25OHD₃ or 3-epi-25OHD₃ spiked in the sample.

In the resolving method, the response for 25OHD₃ spiked serum was the 25OHD₃ peak area divided by the d₆-25OHD₃ internal standard peak area, and the concentration was the 25OHD₃ concentration measured in the base pool (10 nmol/L), plus the concentration of 25OHD₃ spiked in the sample. The response for 3-epi-25OHD₃ was the 3-epi-25OHD₃ peak area divided by the d₆-25OHD₃ internal standard peak area, and the concentration was the 3-epi-25OHD₃ concentration spiked (3-epi-25OHD₃ was not detected in the base pool).

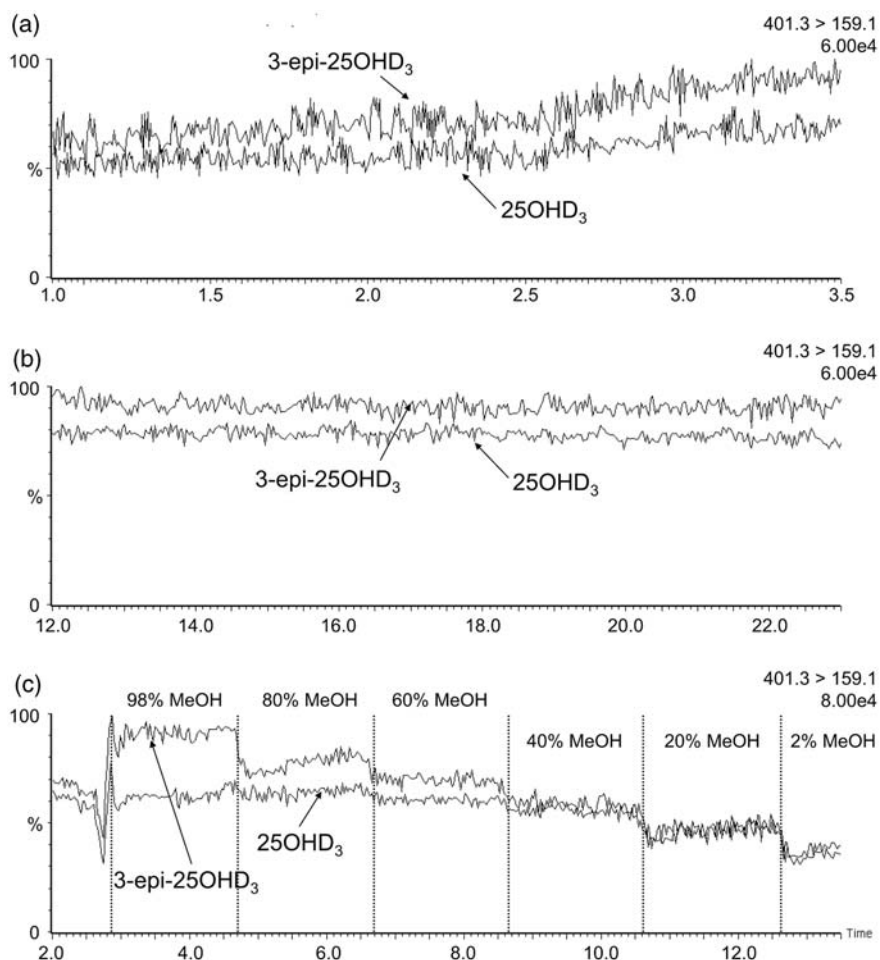


Figure 2. Chromatographs for the 401.3 > 159.1 transition during infusions of equal concentrations of 3-epi-25OHD₃ and 25OHD₃. (a) Infusion with the co-elution method chromatography conditions; (b) Infusion with the resolving method chromatography conditions; (c) Infusions where the mobile phase composition was changed in regular intervals. Signal intensity is given as a percentage of the ion count shown in the top right of each panel.

Infusion experiments

For infusion experiments, 1 $\mu\text{mol/L}$ 3-epi-25OHD₃ and 25OHD₃ solutions were prepared by dilution of stock solutions with 80:20 methanol:isopropanol and infused post-column with mobile phase.

Analysis of patients' samples

Samples were analysed from 341 patients, of which 211 were randomly selected and 130 were chosen from children (<18 years) or because they had 25OHD concentrations > 50 nmol/L. The study included 227 female and 113 male patients (gender unknown for one subject). Seven subjects were aged < 1 year (youngest 8 days), 87 aged 1–18 years, and 247 aged > 18 years (oldest 88 years). Quantification of 3-epi-25OHD₃ was performed:

1. using dedicated calibrators in the resolving method;

2. using 25OHD₃ calibrators in the resolving method;
3. as the difference between 25OHD₃ results in the co-elution and resolving methods.

Results

Equimolar 3-epi-25OHD₃ gave a greater response than 25OHD₃ when the same serum pool was spiked with either compound, being approximately 60% greater in the co-elution method, and 20% greater in the resolving method (Figure 1). This was also true when analytes were quantified using the 401.3 > 365.2 transition (data not shown).

When infusing equimolar solutions into the MS, the 3-epi-25OHD₃ signal was greater than 25OHD₃ for the 401.3 > 159.1 transition (Figure 2). The epimer signal enhancement during analyte elution (~2.7 min) in the co-elution method (Figure 2(a)) was double the signal

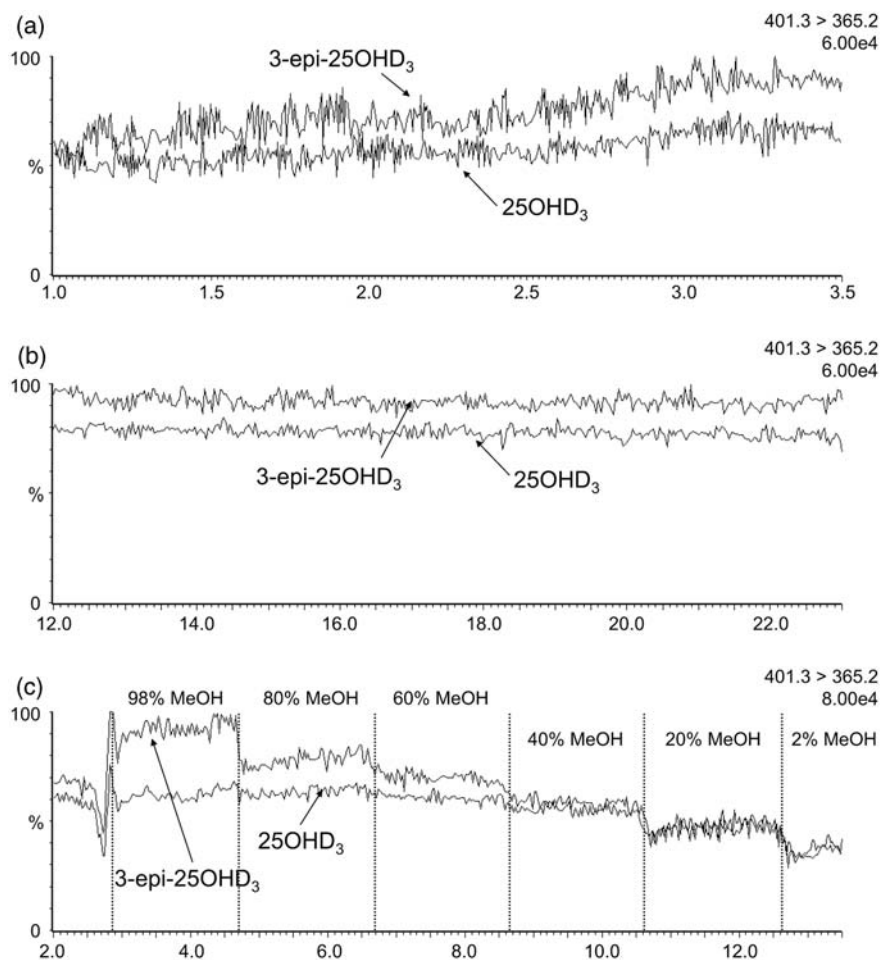


Figure 3. Chromatograms for the 401.3 > 365.2 transition during infusions of equal concentrations of 3-epi-25OHD₃ and 25OHD₃. (a) Infusion with the co-elution method chromatography conditions; (b) Infusion with the resolving method chromatography conditions; (c) Infusion where the mobile phase composition was changed in regular intervals. Signal intensity is given as a percentage of the ion count shown in the top right of each panel.

enhancement seen in the isocratic resolving method (Figure 2(b)). This was verified on a second independently prepared set of infusion solutions. Increasing methanol concentrations increased the difference between epimer signals (Figure 2(c)). The same pattern was observed when infusions were monitored with either 401.3 > 365.2 (Figure 3) or 401.3 > 383.5 (Figure 4) transitions.

When quantified using dedicated calibrators, 3-epi-25OHD₃ was present at between 10 and 22 nmol/L in five of 341 patient samples (Table 1). Using 25OHD₃ calibrators, the number of samples which had 3-epi-25OHD₃ concentrations \geq 10 nmol/L was increased two to threefold. Quantitation of 3-epi-25OHD₃ as the difference in 25OHD₃ between co-elution and resolving methods resulted in 10 times as many samples with 3-epi-25OHD₃ \geq 10 nmol/L, and an approximately four-fold increase in the maximum concentration to 95 nmol/L.

Discussion

We demonstrated enhanced 3-epi-25OHD₃ signal relative to equimolar 25OHD₃ during infusions and in spiked human serum. Enhanced signal caused overestimation of 3-epi-25OHD₃ concentrations when quantified using 25OHD₃ calibrators or as the difference in 25OHD₃ results between an assay that chromatographically separates the epimers and a method in which they co-elute.

It is unclear if enhanced 3-epi-25OHD₃ signal occurs in all LC-MS/MS systems. In a Vitamin D external quality assessment scheme (DEQAS), 3-epi-25OHD₃ interference studies (sample 405) showed an average cross-reactivity $>$ 100% in LC-MS/MS methods.⁶ The wide spread of LC-MS/MS results for this sample (%CV 23.5% vs. %CV 10.8% in the base sample) suggest variable 3-epi-25OHD₃ interference, possibly due to variable enhancement of 3-epi-25OHD₃ signal. Our analyte infusion studies showed signal enhancement for

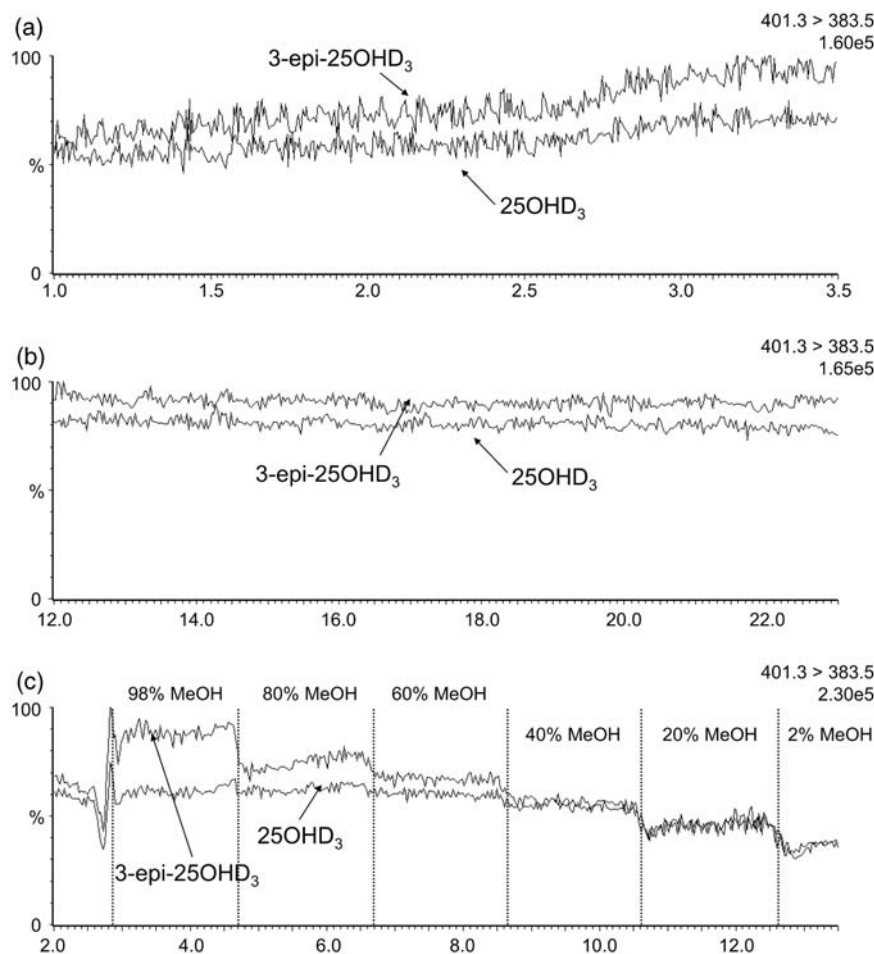


Figure 4. Chromatographs for the 401.3 > 383.5 transition during infusions of equal concentrations of 3-epi-25OHD₃ and 25OHD₃. (a) Infusion with the co-elution method chromatography conditions; (b) Infusion with the resolving method chromatography conditions; (c) Infusion where the mobile phase composition was changed in regular intervals. Signal intensity is given as a percentage of the ion count shown in the top right of each panel.

the 401.3 > 159.1, 401.3 > 365.2 and 401.3 > 383.5 transitions used by most LC-MS/MS 25OHD assays,⁷ suggesting that many LC-MS/MS systems might be affected by 3-epi-25OHD₃ signal enhancement.

In both infusion and spiking experiments, the 3-epi-25OHD₃ signal enhancement was two to three-fold greater in the co-elution than in the resolving method. Infusion experiments showed increasing signal enhancement at higher methanol concentrations; since 3-epi-25OHD₃ elutes at approximately 55% methanol in the resolving method and approximately 80% in the co-elution method, this may explain the greater signal enhancement in the latter. This dependence of signal enhancement on mobile phase composition may also contribute to the variable 3-epi-25OHD₃ interference between DEQAS participants in the interference studies noted above.

As the molar extinction coefficient for 3-epi-25OHD₃ is unknown, we used the same value as for 25OHD₃ to assign concentration, an approach used by others.²

The inversion of stereochemistry at C3 is chemically distant from the triene chromophore, but differences in UV absorption cannot be excluded. However, we would not expect the difference in UV absorption to exceed the < 10% difference between 25OHD₃ ($\epsilon_0 = 18,200 \text{ L mol}^{-1} \text{ cm}^{-1}$) and 25OHD₂ ($\epsilon_0 = 19,400 \text{ L mol}^{-1} \text{ cm}^{-1}$) absorbance. Any difference in 3-epi-25OHD₃ and 25OHD₃ concentrations due to inaccurate assignments of molar extinction coefficients cannot explain the different magnitudes of signal enhancement between the two methods, the variation of signal enhancement with methanol concentrations or the over-recovery of 3-epi-25OHD₃ by LC-MS/MS users in a DEQAS 3-epi-25OHD₃ interference experiment.⁶

Signal enhancement could occur due to extraction, ionization or fragmentation differences. As 3-epi-25OHD₃ enhancement was observed during infusion experiments, extraction differences alone cannot be responsible. Since 3-epi-25OHD₃ and 25OHD₃ fragment similarly⁸ and enhancement was observed for

Table 1. 3-epi-25OHD₃ measurement in 341 samples using three different modes of quantitation.

	3-epi-25OHD ₃ calibrators	25OHD ₃ calibrators	Difference between 25OHD ₃ results
Number of samples with [3-epi-25OHD ₃] ≥ 10 nmol/L (% of all samples)	5 (1.5%)	13 (4%)	50 (15%)
Maximum [3-epi-25OHD ₃] nmol/L	22	23	95
Maximum [3-epi-25OHD ₃] as % of 25OHD ₃ concentration	20	27	78

Using 3-epi-25OHD₃ calibrators in the resolving method, using 25OHD₃ calibrators in the resolving method, and as the difference between 25OHD₃ results in the co-elution and resolving methods.

401.3 > 159.1, 401.3 > 365.2 and 401.3 > 383.5 transitions during infusion experiments, signal enhancement is likely due to greater 3-epi-25OHD₃ ionization.

Ion suppressing substances could cause ionization differences in the resolving method if co-eluting with either analyte. However, no ion suppression was observed when analytes were co-infused with extracted serum during validation of the resolving method (data not shown), and infusion experiments show a consistently greater signal for 3-epi-25OHD₃. Ion suppression is an unsatisfactory explanation in the co-elution method, where analytes would be subjected to similar ion suppression. We suggest that intrinsic differences between 3-epi-25OHD₃ and 25OHD₃ ionization exist, modulated by factors such as mobile phase composition.

In some cases, quantitation of 3-epi-25OHD₃ as the difference between 25OHD₃ concentrations in the co-elution and resolving methods caused concentrations to increase four to fivefold compared with using dedicated calibrators. This cannot be explained solely by 3-epi-25OHD₃ signal enhancement and could be due to other unidentified 25OHD₃ isobars.

Studies using dedicated 3-epi-25OHD₃ calibrators reported low concentrations of this analyte, forming a minor fraction of total 25OHD₃,^{2,3} whereas studies using indirect quantitation have reported higher results, with 3-epi-25OHD₃ in excess of 25OHD₃ in some individuals.^{1,4} Our results suggest that previously unrecognized differences in epimer ionization contribute to these discrepancies.

Accurate 3-epi-25OHD₃ measurement could also be affected by the lack of an isotope labeled 3-epi-25OHD₃ internal standard, which was unavailable during this study. In the resolving method, we used d₆-25OHD₃ as internal standard for 3-epi-25OHD₃ and ensured that chromatography was sufficient to resolve ion suppressing substances.

The resolving method was developed to investigate 3-epi-25OHD₃ interference in 25OHD analysis. The limit of detection of the method is higher than has been reported for other 3-epi-25OHD₃ assays.^{2,3} However, our intention was not to develop a reference method for 3-epi-25OHD₃ but rather to demonstrate the effect

that different quantitation models can have on 3-epi-25OHD₃ quantitation. Similarly, the resolving method's extended runtime precludes routine use. However, 3-epi-25OHD₃ can be partially separated within 6.5 min.⁹ We advise that LC-MS/MS users quantitatively assess 3-epi-25OHD₃ interference in their 25OHD assays and consider modifications to minimize interference. We also advise using 3-epi-25OHD₃ calibrators if direct quantitation of this isomer is required.

Non-stoichiometric cross-reactivity is a familiar concept in immunoassays; we highlight that equimolar responses cannot be assumed in LC-MS/MS systems, even if two molecules are structurally similar. Furthermore, as cross-reactivity can differ between immunoassays due to differing antibody specificity and/or assay configuration, variable ionization is a possible mechanism for differing degrees of interference between LC-MS/MS assays.

Acknowledgements

None

Declaration of conflicting interests

None declared

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Ethical approval

Not required

Guarantor

AD

Contributorship

All authors contributed to study design. NF and FL performed experiments. NF analysed data and wrote the first draft of the manuscript. All authors reviewed and edited the manuscript and approved the final version.

References

1. Singh RJ, Taylor RL, Reddy GS, et al. C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. *J Clin Endocrinol Metab* 2006; 91: 3055–3061.
2. Schleicher RL, Encisco SE, Chaudhary-Webb M, et al. Isotope dilution ultra performance liquid chromatograph-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D₂, 25-hydroxyvitamin D₃ and 3-epi-25-hydroxyvitamin D₃ in human serum. *Clin Chim Acta* 2011; 412: 1594–1599.
3. Lensmeyer G, Poquette M, Wiebe D, et al. The C-3 epimer of 25-hydroxyvitamin D₃ is present in adult serum. *J Clin Endocrinol Metab* 2012; 97: 163–168.
4. Strathmann FG, Sadilkova K, Laha TJ, et al. 3-epi-25 hydroxyvitamin D concentrations are not correlated with age in a cohort of infants and adults. *Clin Chim Acta* 2012; 413: 203–206.
5. Molloy BJ, Calton LJ and Cooper DP. *A new semi-automated solid-phase extraction method for the high-throughput analysis of 25-hydroxyvitamin D in serum*. Waters Application Note APNT10122095, Waters: Milford, MA, USA, 2009.
6. *Vitamin D External Quality Assessment Scheme (DEQAS) 25(OH)D report*, October 2011 distribution. London: Charing Cross Hospital, 2011.
7. *Vitamin D External Quality Assessment Scheme (DEQAS) review*. London: Charing Cross Hospital, 2012.
8. Stepman HCM, Vanderroost A, Stöckl D, et al. Full-scan mass spectral evidence for 3-epi-25-hydroxyvitamin D₃ in serum of infants and adults. *Clin Chem Lab Med* 2011; 49: 253–256.
9. Van den Ouweland JMW, Beijers AM and van Daal H. Fast separation of 25-hydroxyvitamin D₃ from 3-epi-25-hydroxyvitamin D₃ in human serum by liquid chromatography-tandem mass spectrometry: variable prevalence of 3-epi-25-hydroxyvitamin D₃ in infants, children and adults. *Clin Chem* 2011; 57: 1618–1619.