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ORIGINAL RESEARCH

Validation of a high-performance liquid chromatographic method for the simultaneous assessment of vitamin D isoforms

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Background: Asthma is one of the common conditions clinicians encounter in children. Various studies report the association of poor vitamin D (Vit D) status of mothers during pregnancy, with an increased risk of asthma in their children, especially in HIV-positive mothers. Simultaneous measurement of Vit D metabolites, rather than measuring total Vit D, would improve assessment of Vit D metabolism in these children. This study aimed to validate a high-performance liquid chromatographic (HPLC) method using ultraviolet detection for the measurement of five Vit D isoforms (25 OHD2 and D3; 1,25 $[OH]_2$D2 and D3 and 24,25 $[OH]_2$D) simultaneously.

Methodology: Serum samples from 80 paediatric patients with asthma were subjected to solid phase extraction and 4-phenyl-1,2,4-triazole-3,5-dione-derivatisation. Chromatographic separation was achieved with a pentafluoro-phenylpropyl (PFPP) analytical column. Total 25 OH Vit D was measured on an automated immunochemistry analyser for comparison purposes.

Results and discussion: Good linearity was achieved for 25 OHD2, 25 OHD3 and 24,25 (OH)2D with $r^2 \geq 0.99$, with limits of detection ≤ 5.0 ng/ml and lower limits of quantitation ≤ 10.0 ng/m. The 1,25 $(OH)_2$D metabolites, present in the blood at pg/ml levels, could not be quantified within the required ranges. Comparing total 25 OH Vit D immunoassay and HPLC results showed a poor correlation ($r^2 = 0.073$), possibly due to cross-reactivity of the antibodies in the immunoassay with other Vit D isoforms.

Conclusion: This method provides a precise and accurate, with a potential for high throughput, determination of Vit D isoforms in the routine laboratory.

Keywords: vitamin D isoforms, PFPP column, HPLC

Introduction

Vitamin D (Vit D) is an essential component in the control of calcium and phosphorus levels.[1] In addition to its role in maintaining bone health, it plays a role in preventing cancerous cells from spreading, and by protecting against certain immune-mediated disorders.[2] Normally, Vit D obtained from exposure to the sun, food and supplements has to undergo two hydroxylation steps in the body for activation. Vit D is transported to the liver, bound to protein, where it goes through a hydroxylation process; firstly, to form dietary 25 hydroxy Vit D (25 OHD), which is a storage form of Vit D (inactive form). A second hydroxylation process takes place in the kidneys where 25 OHD is again hydroxylated by the enzyme 1α-hydroxylase, to form active 1,25 dihydroxy Vit D (1,25 OH_2D).[3,4] Notably, in the kidneys, Vit D is inactivated to 24,25-dihydroxy Vit D (24,25 OH_2D) by 25-hydroxyvitamin D3-24-hydroxylase.[5]

Literature reports that asthma is one of the most common conditions in children.[6,7] Some researchers have suggested that the increased prevalence of asthma is a result of a rise in Vit D deficiency among paediatric populations.[8] Poor maternal Vit D status during pregnancy may contribute to the child's risk of having asthmatic symptoms.[9] Viard et al. reported that a deficiency of Vit D is often observed in patients with HIV, concomitantly associated with a higher risk of the disease's progression.[10]

Until recently, concentrations of the storage form of Vit D (25 OHD) have been most commonly used in the determination of Vit D status.[11-13] Since Vit D metabolism is a dynamic process balancing the level of active and inactive metabolites in tissue, recent developments have focused on the simultaneous evaluation of both storage form (25 OHD2 and D3) and metabolites (1,25 OH2D2 and D3; 24,25 OH2D) in the assessment of their status. Studies[14,15] have been done in an attempt to separate these five Vit D isoforms; however, two high-performance liquid chromatography (HPLC) columns (reverse phase and normal phase) are required, resulting in high cost and extended analytical time.

The present study attempted to employ a single (mid-polarity) column with a HPLC-UV method in the simultaneous identification and assessment of 25 OHD2 and D3; 1,25 OH_2D2 and D3; 24,25 OH_2D, utilising one sample injection onto the column.

Materials and methods

Study population

Initially, 80 paediatric patients aged between two and 11 years, clinically presenting with asthma, were recruited for the study at Dr George Mukhari Academic Hospital (DGMAH). Forty of these patients were HIV positive, and 40 HIV negative. The Sefako Makgatho Health Sciences University Research Ethics Committee granted Ethics Approval (Ref: SMUREC/M47/2017). The legal guardians of the participants were briefed about the study and written consent was obtained. Serum samples were collected when patients attended the outpatient clinic. Samples were appropriately transported to the laboratory for Vit D analysis. The HIV status of the participants was established by a review of clinical information from each participant's hospital file.

Materials, apparatus and instrumentation

Acetonitrile and formic acid were manufactured by Merck (Darmstadt, Germany). Methanol was supplied by Acros (New Jersey, USA). Strata C18 solid phase extraction (SPE) cartridges were supplied by Phenomenex (Torrance CA, USA). 4-phenyl-1, 2, 4-triazoline-3, 5 dione (PTAD) was supplied by Sigma-Aldrich (St Louis MO, USA). Five analytical standards, including 25 OHD2 and D3; 1,25 OH_2D2 and D3; 24,25 OH_2D were purchased from Sigma Aldrich (St Louis MO, USA). 25 OHD2/D3 serum controls (CE-IVD certified; ISO13485; a low and a high level) were supplied by Recipe products (Dessauerstraβe, Germany); lyophilised serum 24,25 OH_2D and 1,25 OH_2D2 and D3 controls (a low and a high level) were supplied by Sigma-Aldrich (St Louis MO, USA). The total 25 OHD immunoassay kit was manufactured by Abbott Diagnostics (Lake Forest IL, USA). The Brownlee Choice pentafluoro phenylpropyl (PFPP) column (150 mm × 4.6 mm, 5µm) was manufactured by Perkin Elmer (Waltham MA, USA). The Alliance 2695 Separations Module: high-performance liquid chromatograph (2998), Lenovo data system and Empower 3.0 chromatographic software was manufactured by Waters (Milford MA, USA). Horizon technology XcelVap® automated evaporation system was supplied by Biotage (Lake Forest CA, USA). The Architect i8200 automated biochemistry analyser was manufactured by Abbott Laboratories (Abbott Park IL, USA).

Sample preparation procedure and laboratory analysis

Serum samples (500 µl aliquots stored in microcentrifuge tubes at -20 °C for a month), 25 OHD2 and D3; 24,25 OH_2D, 1,25 OH_2D2 and D3 controls and analytical standards (stored at -70 °C) were thawed and equilibrated at room temperature for 20 minutes. Acetonitrile (100%) was added to the samples (to remove the Vit D binding protein) and the mixtures were vortexed for five seconds. The treated samples were left overnight at 4 °C, followed by centrifugation at 3500 *Xg* for five minutes. The supernatants were extracted using SPE: for each sample, a C18 cartridge, methanol, water and gravity flow (assisted by vacuum) was used. Extracted samples, controls and standards were dried under nitrogen gas at 37 °C, followed by a derivatising procedure; PTAD (200 µl) was added to the dried samples, controls and standards, which were incubated for three hours in the dark at room temperature. After derivatisation, the colour of the samples had changed from a dark pink to yellow. A 200 µl volume of the derivatised patient samples, Vit D controls and standards were transferred to autosampler vials for analysis on the HPLC system.

Chromatographic procedure: high-performance liquid chromatographic-ultraviolet detection (HPLC-UV)

The Vit D isoforms were separated on a mid-polar PFPP analytical column (150 mm × 4.6 mm, 5 µm). The composition of the mobile phases was 0.01% formic acid in 100% acetonitrile (A) and 0.01% formic acid (B). Volumes of 20 µl of the patient samples, Vit D controls and standards were injected onto the HPLC column. A gradient was run from an initial 95% A and 5% B, progressing to 35% A and 65% B in four minutes, changing to 40% A and 60% B in the next 10 minutes and returning to 95% A for the equilibration phase, resulting in a total run time of 15 minutes. The temperature of the column was fixed at 35 ˚C and a flow rate of 1.0 ml/min was maintained. A blank was injected after every five samples to prevent carryover. Detection was carried out from 265 to 330 nm, which covers the wavelength range of the Vit D isoforms.

Validation of the method

For the preparation of standard curves and linearity range, a stock standard solution of the Vit D analytes was diluted in 100% acetonitrile to prepare ascending concentrations for the calibration curve, covering a concentration range of 2.5–75 ng/ml for 25 OHD2 and D3 ,and 24,25 OH_2D; 15–100 pg/ml for 1,25 OH_2D2 and D3. Serial injections of the dilutions were performed. The peak areas were calculated for the analytes in each dilution and plotted against the different concentrations. Precision and accuracy were determined based on the repeatability (within and between run) of triplicate injections of two different levels (low and high) of a commercially quality control sample, at different time intervals on the same day, for 10 days. The experiments were conducted in triplicate over 10 days. The coefficients of variation (CVs)[16] were calculated for within and between run analyses. The LOD and LLQ were determined by the lowest concentration at which the analyte in a standard solution is found through serial dilution, with the chromatographic peak having a minimum signal to noise ratio of 3:1 (LOD) and with the peak having a signal to noise ratio of 10:1 (LLQ). The 1,25 OH_2D2 and D3 were also prepared and analysed in 12.5–75 ng/ml concentrations to assess the sensitivity of the UV detector and to confirm the retention time for the active metabolites.

Determination of vitamin D status of the paediatric patients with HPLC-UV

Serum from 80 asthmatic children (40 HIV-positive children and 40 HIV-negative children), were assessed using the proposed method to determine their Vit D status.

Comparative method to HPLC-UV

Determination of total 25 OHD using chemiluminescent micro-particle immunoassay (CMIA)

An analysis of total 25 OHD for 80 patients was performed by using the Abbott Architect i8200 automated immunochemistry analyser. Three control levels (low, medium and high) were assayed in a minimum of two replicates at two different times per day for five days.

Statistical analysis

For statistical analysis, patients' demographic and clinical profiles were entered into the Statistical Package for Social Sciences (SPSS) version 24 software and Microsoft Excel, 2016. Linear range with regression coefficient (r), limit of detection, limit of quantitation and retention time were recorded for each Vit D isoform. For comparison of immunoassay and HPLC results, a Spearman's correlation graph was used.

Table I: Calibration statistics for vitamin D isoforms on high-performance liquid chromatographic-ultraviolet detection

Analyte (ng/ml)	Linearity (Range) (ng/ml)	r^2	LOD (ng/ml)	LLQ (ng/ml)	Retention time (~min)
25 OHD2	2.5–75	0.997	2.50	5.0	8
25 OHD3	2.5–75	0.998	1.25	2.50	6
24,25 $(OH)_2D$	5.0–75	0.994	5.0	10.0	10

Results

The current study was conducted to validate a mid-polarity PFPP column using HPLC-UV analysis. Linearity for three Vit D isoforms (ng/ml), LOD and LLQ (ng/ml) and retention time (~min) are shown in Table I. The mean, standard deviation (SD)[16] and CV were calculated and the results are shown in Table II. The peaks for Vit D isoforms are shown in Figure 1.

Table II: Mean, SD and %CV results (within and between run) for vitamin D isoforms at different concentrations on high-performance liquid chromatographic-ultraviolet detection

	Within run			Between run		
	Levels (ng/ml)	SD	CV (%)	Levels (ng/ml)	SD	CV (%)
25 OHD2	10	0.48	4.85	10	0.37	3.74
	60	1.84	3.05	60	1.57	2.60
25 OHD3	10	0.31	3.12	10	0.25	2.48
	60	1.58	2.61	60	1.54	2.55
24,25 $(OH)_2D$	12	0.50	4.24	12	0.47	3.90
	60	1.95	3.21	60	1.90	3.12

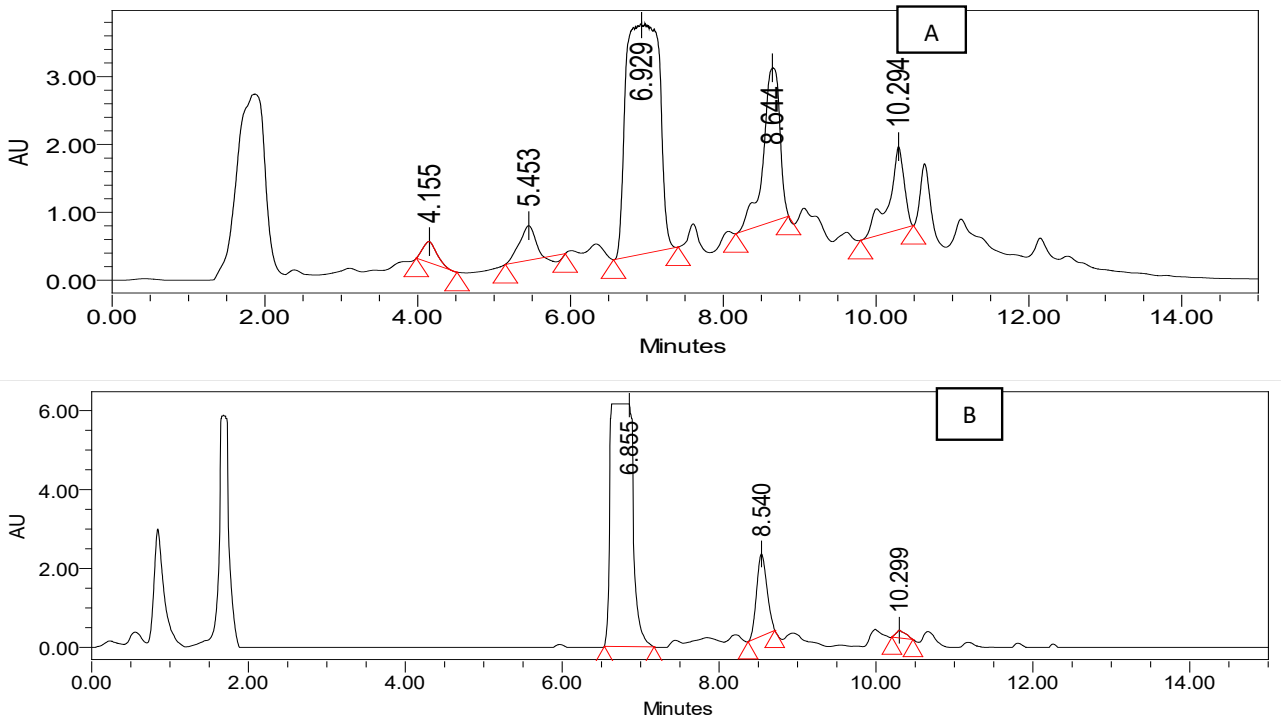

Figure 1: Chromatograms illustrating five peaks for vitamin D isoforms (25 OHD2 and D3; 24,25 $(OH)_2D$; 1,25 $(OH)_2D2$ and D3) at highest concentration in ng/ml. A). Three peaks of Vit D isoforms (25 OHD2 and D3; 24,25 $(OH)_2D$) in ng/ml and 1,25 $(OH)_2D2$ and D3 isoforms in pg/ml. B). Retention times for 1,25 $(OH)_2D2$ (~ 4 min); 1,25 $(OH)_2D3$ (~ 5 min); 25 OHD3 (~ 6 min); 25 OHD2 (~ 8 min); 24,25 $(OH)_2D$ (~ 10 min)

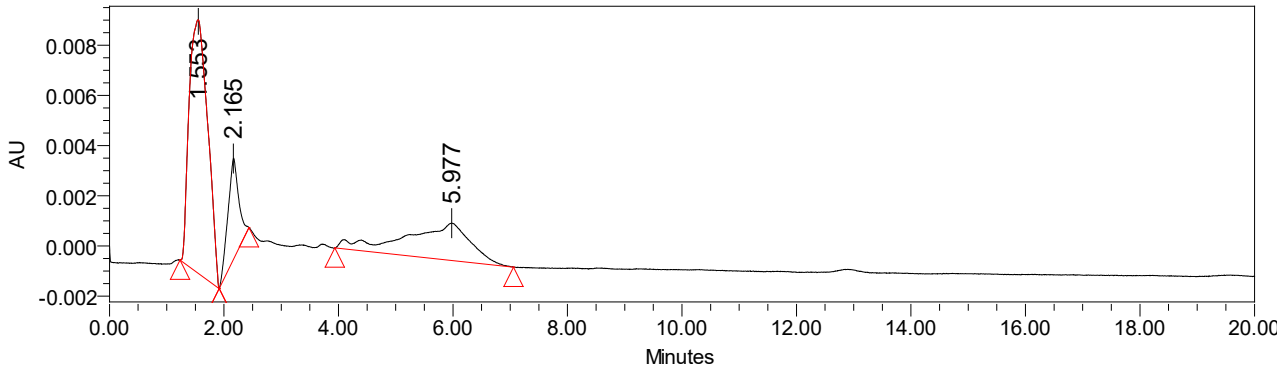

Figure 2: Poor chromatographic separation and co-elution of the analytes for a mixture of underivatised vitamin D isoforms (ng/ml) using HPLC-UV: 25 OHD3; (~ 1.5 min); 25 OHD2 (~ 2.1 min); 24,25 $(OH)_2D$ (~ 5.9 min)

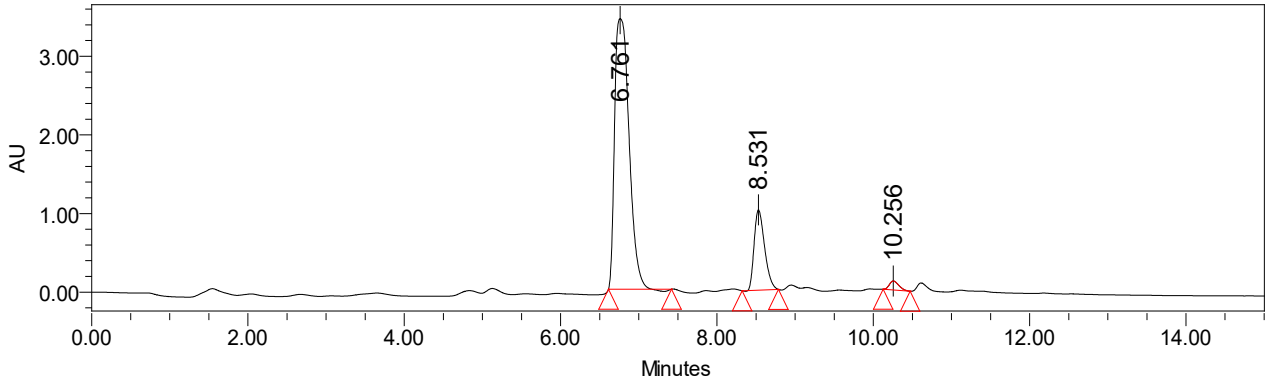

Figure 3: Good chromatographic separation and no co-elution of the analytes for a mixture of PTAD-derivatised vitamin D isoforms sample using HPLC-UV: 25 OHD3 (~ 6.7 min); 25 OHD2 (~ 8.5 min); 24,25$(OH)_2D3$ (~ 10.2 min)

4-phenyl-1,2,4-triazole-3,5 dione derivatisation of standard materials

A chromatogram (Figure 2) shows poor separation of un-derivatised Vit D isoforms (ng/ml concentrations), which may be due to similarities in the properties of the analytes. Improved separation was achieved when these properties (polar groups such as O-H, COOH added to the Vit D compound) were changed by derivatising with PTAD, as illustrated in Figure 3.

Method comparison of total 25 OHD (storage form) between high-performance liquid chromatographic-ultraviolet detection and immunoassay

Table III: Mean values obtained from analyses by immunoassay and the proposed high-performance liquid chromatographic-ultraviolet detection method for a total 25 OHD

	Mean: Immunoassay (*n* = 80)	**Mean: HPLC (*n* = 80)**
Total 25 OHD = 25 OHD2 + 25 OHD3 (ng/ml)	38.9	32.2

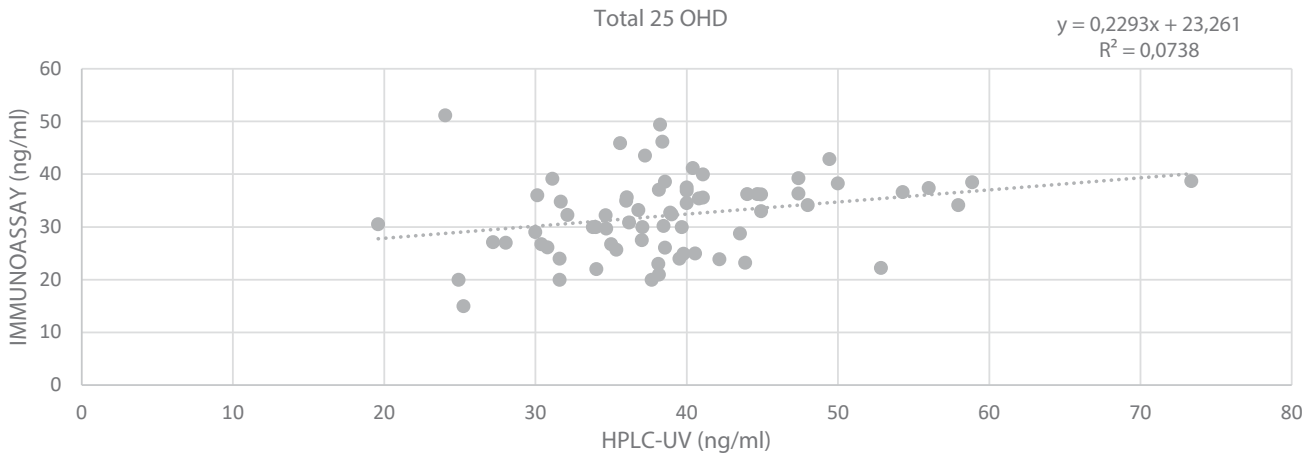

Figure 4: Correlation between HPLC-UV and immunoassay for total 25 OH D ($r^2 = 0.073$)

Vitamin D status of children recruited for the study according to high-performance liquid chromatographic–ultraviolet analysis

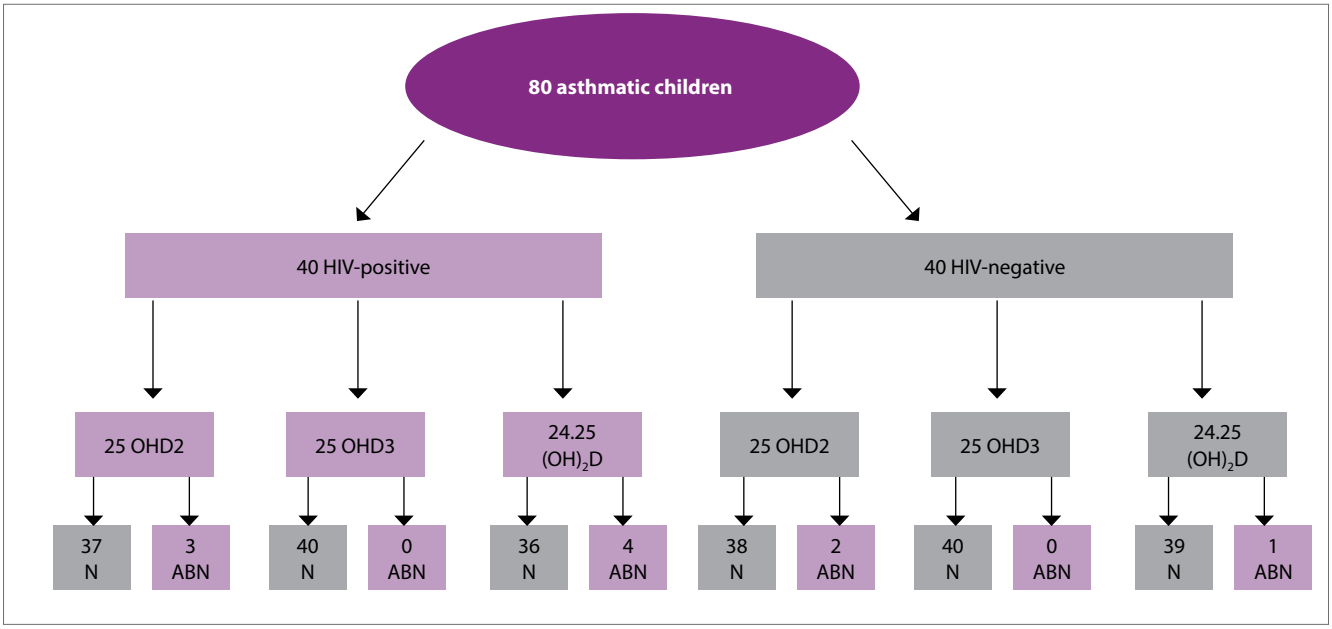

Figure 5: Flow diagram of 80 asthmatic children divided into one group of 40 HIV-positive and one group of 40 HIV-negative participants, and assessment of their vitamin D status using the HPLC results. Participants with levels ≤ 10 ng/ml were considered to have abnormal values (ABN); those with a range of (10–75 ng/ml) were considered to have normal values (N)

Discussion

In this study, optimisation of the chromatographic separation of Vit D storage forms 2 and 3, and inactive form 24,25 was achieved by using a mid-polarity PFPP column. The active form 1,25 was only detectable in ng/ml concentration and not in the pg/ml range. Unfortunately, typically, clinical cut-off ranges are at pg/ml.

Additionally, these results show an excellent relationship between the isoforms concentrations (5.0–75 ng/ml) as determined by the peak area which produced good correlations; $r^2 \geq 0.99$ (Table I). Studies conducted by Turpienen et al.[3] and Kumar et al.[17] also reported on good linearity and correlation; $r^2 \geq 0.99$ for Vit D3 concentration.

The LOD values obtained were as follows: 2.50 ng/ml for 25 OH D2, 1.25 ng/ml for 25 OHD3 and 5.0 ng/ml for 24,25 $(OH)_2D$. The LLQ for 25 OH D2 was 5.0 ng/ml, for 25 OHD3 was 2.50 ng/ml, and for 24,25 $(OH)_2D$ was 5.0 ng/ml. Kumar et al.,[17] Lensmeyer et al.[18] and Al Qadi et al.[19] used different methods to those of the current study and had different findings. In the present study, HPLC-UV results showed good sensitivity for five peaks in the ng/ml range, but only three peaks (25 OHD2, 25 OHD3, 24,25 $(OH)_2D$) could be quantified within the required concentration ranges (ng/ml). The remaining two active forms (1,25 $(OH)_2D2$ and 1,25 $(OH)_2D3$) could not be detected or quantitated in the patient samples (as well as the standards) because of the low concentrations (pg/ml) in which they are present in blood samples. Analysis of commercial controls containing 25 OHD 2 and 3, 24,25 $(OH)_2D$, on the mid-polar HPLC column also showed good precision and repeatability with CVs ≤ 5%. An acceptable CV for HPLC method is ≤ 15%.[20]

In this project, the use of the derivatising reagent PTAD, which reacted with the Vit D analytes, resulted in a 100-fold increase in analytical response. In terms of selectivity, the derivatising agent improved chromatographic separation of the analytes, especially those analytes that are closely related in structure. The chromatography results showed no interferences close to the retention times of the Vit D isoforms. These results confirmed that employing PTAD as a derivatisation reagent, co-elution of the sample analytes was prevented, resulting in good chromatographic separation (Figure 3). Ding et al.[15] and Aronov et al.[21] had similar findings to this; the derivatising agent improved the ionisation efficiency, chromatographic separation of the vitamin D isoforms on liquid chromatography mass spectrometry (LC/MS).

The patient results of this study revealed a poor correlation between the immunoassay and HPLC methods ($r^2 = 0.0738$). The immunoassay intra- and inter-assay precision was good with the CV ranging between 1.8% and 3.5%. The accepted CV for this CMIA method is less than 10%. When comparing the mean of the total 25 OHD values of the two methods (Table III), a higher mean value was obtained from the immunoassay. One factor that may have contributed to these higher levels is the cross-reactivity of the antibodies in the immunoassay with other Vit D isoforms (such as the inactive and active forms). However, this effect was not seen with the proposed HPLC method, possibly due to the high selectivity provided by the extraction of each isoform, and resolution by the column. Turpeinen et al.[3] and Kimball and Vieth[22] reported on the poor correlation between the Liaison assay (immunoassay) and an HPLC method; although the HPLC method was different to the one that was used in the present

study. The implication of these variabilities shows that a patient's sample should only be tested using one method for monitoring of Vit D levels and not using two methods interchangeably.

The analytical range of 10–75 ng/ml of the current results covers the Vit D status for deficiency, sufficiency and insufficiency, which has been described in studies by the American Academy of Pediatrics (AAP) and the Institute of Medicine (IOM)[23-25] In the present study, three of the participants from the HIV-positive group had 25 OHD2 concentrations ≤ 10 ng/ml, and four participants had 24,25 $(OH)_2D$ concentrations ≤ 12 ng/ml. Two of the participants from the HIV-negative group had 25 OHD2 concentrations ≤ 10 ng/ml, and one participant had a 24,25 $(OH)_2D$ concentration of < 12 ng/ml when samples were analysed using the proposed HPLC-UV method (Figure 5). Even though the total 25OHD of 38.9 ng/ml appeared to be within the normal range using the immunoassay method, the HPLC-UV results showed a deficiency of 25 OHD2 in both groups in these patients. These results confirm the importance of analysing Vit D isoforms with HPLC because of the selectivity that is achieved during separation. In addition, identifying Vit D deficiency at an early stage may assist paediatricians and clinicians in prescribing Vit D supplementation for Vit D deficient children. This may benefit patients from a slowing down of the progression of HIV to AIDS and might also reduce the risk of the recurrence of asthma attacks in these children. It could also assist in monitoring of Vit D levels, particularly in patients infected with HIV or affected by other factors (such as treatment) that are connected to the virus itself.[26,27]

A limitation of this study was that the active Vit D isoforms, ([1,25 (OH)2D2 and 1,25 (OH)2D3]), could not be detected in the required biological concentration range (pg/ml) with HPLC when using UV detection. Unfortunately, without extremely expensive high-end equipment fitted with ultra-sensitive detection systems at our facility, we were unable to achieve the desired LLQ for the two active Vit D isoforms.

Conclusion

In the present study, the five Vit D isoforms of interest were separated with a mid-polarity PFPP column in the ng/ml concentration range. Three of the isoforms ([25 OHD2, 25 OHD3, and 24,25 $(OH)_2D3$]) could be quantified and this was helpful, especially with the inactive form (24,25), providing better information of Vit D metabolism in patients than the total storage form alone.

The HPLC-UV method using a mid-polarity PFPP column can be regarded as simple, precise, accurate and has the potential of high throughput of samples for the determination of total storage Vit D and inactive Vit D in the routine laboratory setting. This is potentially beneficial for patients who may have abnormal Vit D metabolism where the inactivation of Vit D exceeds activation at the tissue level.

The extraction and separation method illustrated in the study can also be transferred to instruments such as the liquid chromatography-tandem mass spectrometry (LC-MS/MS); equipped with more sensitive detection capabilities or high-resolution accurate mass spectrometers such as time-of-flight or orbitrap-based analysers, in particular when performing full scan mass spectrometric procedures.

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Conflict of interest

The author declares that there is no conflict of interest in this research project.

Ethical approval

Ethics approval was obtained from the Sefako Makgatho University Research Ethics Committee, SMUREC/M47/2017

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