**Regular Article**

**Cost-Effective HPLC-UV Method for Quantification of Vitamin D₂ and D₃ in Dried Blood Spot: A Potential Adjunct to Newborn Screening for Prophylaxis of Intractable Paediatric Seizures**

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Introduction

25-Hydroxyvitamin D (25-(OH)D) deficiency is recently been described as one of the multiple factors responsible for pediatric seizures. 25-Hydroxyvitamin D3 and 25-Hydroxyvitamin D2 are the well-known markers to determine Vitamin D status. In this work we report the development of a sensitive and cost-effective HPLC technique for the quantification of the vitamin D metabolites from dried blood spot samples (DBS). The metabolites were extracted using acetonitrile–methanol–0.1% formic acid (60:20:20 (v/v/v)) and analyzed on an Acclaim C₁₈ column (150 × 4.6 mm i.d., 3 µm) at a flow rate of 1 mL/min. The method was linear in the range of 10–80 ng/mL. Limit of detection and limit of quantification (LOQ) of the method were 5 and 10 ng/mL respectively. Extensive stability studies demonstrated the analytes to be stable in stock and matrix with a percent change within the acceptable range of ±15%. Comparison of the newly developed HPLC-DBS method with the reported LC-MS-DBS and electrochemiluminescence immunoassay (ECLIA) methods followed by Bland–Altman analysis demonstrated a bias of 0.08 and −0.14, respectively proving the methods are comparable. Application of the developed method to a pediatric seizure cohort depicted 46.6% of cases as deficient and 26.6% as insufficient for 25-(OH)D. Among deficient cases 8 samples were below 10 ng/mL and exact amount was not calculated since these were below the LOQ levels. The mean ± standard deviation (S.D.) in the remaining 6 deficient cases was 13.22 ± 2.80 ng/mL. The levels in healthy infants were 33.9 ± 6.11 ng/mL. The method can be used routinely for assessing 25-(OH)D deficiency in newborn.

**Key words** vitamin D estimation; LC; dried blood spot; stability study

**Abstract**

25-Hydroxyvitamin D (25-(OH)D) deficiency is recently been described as one of the multiple factors responsible for pediatric seizures. 25-Hydroxyvitamin D3 and 25-Hydroxyvitamin D2 are the well-known markers to determine Vitamin D status. In this work we report the development of a sensitive and cost-effective HPLC technique for the quantification of the vitamin D metabolites from dried blood spot samples (DBS). The metabolites were extracted using acetonitrile–methanol–0.1% formic acid (60:20:20 (v/v/v)) and analyzed on an Acclaim C₁₈ column (150 × 4.6 mm i.d., 3 µm) at a flow rate of 1 mL/min. The method was linear in the range of 10–80 ng/mL. Limit of detection and limit of quantification (LOQ) of the method were 5 and 10 ng/mL respectively. Extensive stability studies demonstrated the analytes to be stable in stock and matrix with a percent change within the acceptable range of ±15%. Comparison of the newly developed HPLC-DBS method with the reported LC-MS-DBS and electrochemiluminescence immunoassay (ECLIA) methods followed by Bland–Altman analysis demonstrated a bias of 0.08 and −0.14, respectively proving the methods are comparable. Application of the developed method to a pediatric seizure cohort depicted 46.6% of cases as deficient and 26.6% as insufficient for 25-(OH)D. Among deficient cases 8 samples were below 10 ng/mL and exact amount was not calculated since these were below the LOQ levels. The mean ± standard deviation (S.D.) in the remaining 6 deficient cases was 13.22 ± 2.80 ng/mL. The levels in healthy infants were 33.9 ± 6.11 ng/mL. The method can be used routinely for assessing 25-(OH)D deficiency in newborn.
Experimental

Materials and Reagents 25-Hydroxyvitamin D2 (99.8%) and 25-hydroxyvitamin D3 (99.8%) were purchased from Sigma-Aldrich (St. Louis, U.S.A.). Montelukast Sodium (98.5%) was purchased from Optimus Drugs Pvt. Ltd. (Hyderabad, India). Acetonitrile (ACN) (HPLC grade) and formic acid 85% (AR grade) were purchased from Merck (Kenilworth, U.S.A.). Methanol (AR grade) was purchased from Finar Limited (Gujarat, India). In house Milli Q water (Siemens Ultra Clear) was used. Acclaim C18 column (150 × 4.6 mm i.d., 3 µm) was purchased from Thermo Scientific (Massachusetts, U.S.A.).

Instrumentation The LC method development and validation were carried out on a Shimadzu LC-20 AD prominence which is equipped with a Shimadzu LC-20 AD prominence pump, Shimadzu SPD-20AV UV-Visible detector, Shimadzu SIL-20AC HT auto sampler and a Shimadzu CTO-10AS column compartment. The data were collected on a PC equipped with LC solutions (version 1.25).

Sample Collection For preparing the control and calibration DBS samples, we collected anonymized leftover human blood samples in ethylene diamine tetra acetic acid tubes from the department of Biochemistry, Kasturba Hospital, Manipal. For the method comparison studies, we collected at random 50 anonymized left-over patient blood samples send to the department of Biochemistry, Kasturba Hospital, Manipal for assessing 25-(OH)D levels. To demonstrate method application, we applied it to a pediatric seizure cohort comprising of 30 neonates. For this work we obtained ethical clearance from Manipal academy of higher education Ethics Committee at Manipal (MUEC/010/2017 dated 08.05.2017). DBS samples were collected for a period of six months from July 2017 to December 2017 from 30 infants presenting with refractory epilepsy. Age and sex matched controls were also collected during the same period from another 30 healthy infants.

Calibrators and Quality Controls Calibrators and quality controls were prepared in left over blood samples. Since the analytes of interest are endogenous in nature the blank subtraction technique was followed for the preparation of the calibrators and quality controls. The basal level of all analytes in the matrix was determined by the standard addition approach. This endogenous analyte to internal standard (IS) ratio was subtracted from the spiked DBS analyte to IS ratio for all calibrators and quality controls. 25-(OH)D2 and 25-(OH)D3 were purchased as 100 µg/mL solution in ethanol. Suitable dilutions were performed to obtain a primary stock solution of 10 µg/mL in acetonitrile and a secondary stock of 2000 ng/mL in blood. Suitable aliquots of the secondary stock were pipetted into 1 mL of blood to prepare working stocks of 250–7000 ng/mL. Forty microliters of this working stock was pipetted onto a Whatman 903 filter paper to prepare DBS calibration and quality control as per Table 1. The spots were left to dry at room temperature for three hours and then stored in envelopes with dessicants at −80°C until analysis. The DBS calibrants and quality controls were extracted similar to a clinical sample. Montelukast sodium at a concentration of 10 µg/mL was used as the IS. The blank DBS (blank matrix without IS and analyte) and Zero DBS (blank matrix with IS) were prepared using fresh un-sampled DBS filter paper cards similar to Fallah and Peighambardoust. A 11.1 mm disk was punched from the un-sampled DBS filter paper card and subjected to extraction with a mixture of ACN–methanol–0.1% formic acid (60:20:20 (v/v)) to prepare the blank DBS. The Zero DBS was prepared by subjecting the un-sampled DBS filter paper cards (11.1 mm punch) to extraction with an internal standard prepared in a mixture of ACN–methanol–0.1% formic acid (60:20:20 (v/v)).

HPLC-UV Method Development The method development was undertaken to achieve separation between 25-(OH)D2 and 25-(OH)D3. The hurdle was to obtain enough selectivity and resolution among the metabolites and blood components within a reasonable run time. For selecting the wavelength, the UV absorption spectra of 25-(OH)D2 and 25-(OH)D3 solutions were studied and an absorption maximum was observed at 266 nm. For choosing the column, literature was scanned and the C18 column was chosen as the stationary phase. 25-(OH)D2 and 25-(OH)D3 are lipophilic in nature with a logP of 7.5 and 7.13 respectively making them excellent candidates for reverse phase chromatography. Acclaim C18 column (150 × 4.6 mm i.d., 3 µm) was seen suitable for the retention of both 25-(OH)D2 and 25-(OH)D3 within 10 min. Trials were conducted at various pH of 3 to 7 for a fixed mobile phase (90:10% (v/v) (acetonitrile: buffer), flow rate of 1 mL/min and ambient temperature conditions. Increasing the pH was not seen to influence the retention time but an influence on the peak areas were observed with a higher response at lower pH. Hence pH 3 was seen suitable for further trials. Further the mobile phase composition was altered with a percent change in the organic from 95–70% (v/v) to improve

Table 1. Method Performance Specifications for 25-(OH)D2 and 25-(OH)D3

| Analyte   | Calibration range (ng/mL) | LOQ (ng/mL) | LOD (ng/mL) | QC Levels (ng/mL) | Accuracy (%) | Precision (%) | Percent recovery (%) |
|-----------|---------------------------|-------------|------------|-------------------|--------------|---------------|---------------------|
|           | Inter                     | Intra       | Inter      | Intra             |              |               |                     |
| 25-(OH)D2 | 10–80                     | 10          | 5          | LLOQ (10)         | 107.24       | 103.62        | 4.63                | 0.074              |
|           |                           |             |            | LQC (20)          | 101.40       | 99.97         | 1.43                | 0.020              |
|           |                           |             |            | MQC (50)          | 102.57       | 102.08        | 1.66                | 0.062              |
|           |                           |             |            | HQC (80)          | 101.78       | 100.59        | 1.27                | 0.150              |
| 25-(OH)D3 | 10–80                     | 10          | 5          | LLOQ (10)         | 103.61       | 107.21        | 2.25                | 4.023              |
|           |                           |             |            | LQC (20)          | 99.67        | 101.48        | 1.66                | 0.44               |
|           |                           |             |            | MQC (50)          | 102.08       | 102.52        | 3.38                | 1.86               |
|           |                           |             |            | HQC (80)          | 100.59       | 101.73        | 2.34                | 1.18               |

25-(OH)D3: 25-hydroxyvitamin D3; 25-(OH)D2: 25-hydroxyvitamin D2; LLOQ: lower limit of quantification, LQC: low quality control, MQC: medium quality control, HQC: high quality control.
the resolution between 25-(OH)D2 and 25-(OH)D3. 95% of organic eluted the metabolites from the column and led to a decrease in the resolution (Rs = 1.1) between the compounds. Decreasing the organic composition to 90, 80 and 70% led to resolutions of 4.1, 5.5 and 6.5 between the metabolites. Additionally, with increased resolution an increase in run time was also observed with a maximum of 30 min observed at 70% organic phase. Hence 90 parts organic and 10 parts buffer was chosen as this resulted in a total run time of 15 min. Column temperature (25–40°C) was further studied for its influence on resolution and run time. Increasing the temperature to 40°C was seen to result in poor resolution (Rs = 1.1) and optimum resolution (Rs = 3.1) was seen to be achieved at 35°C.

**Extraction Optimization from DBS**

Extraction optimization trials were undertaken to achieve maximum recovery with negligible interference. 11.1 mm disk was punched from the DBS and transferred to Eppendorf tube. This punch was then subjected to various extraction optimization trials. The extraction procedure involved treating the disc with a suitable solvent and shaking at different speeds and different temperatures. Acetonitrile, methanol, ethanol, ethyl ether, dichloromethane and acetone were attempted. One hundred percent acetonitrile resulted in 40.12 and 38.15% recovery for 25-(OH)D2 and 25-(OH)D3, respectively. For further improvement in recovery, acetonitrile and methanol in various parts were attempted (80:20, 70:30, 60:40, 50:50 (v/v)). Sixty parts of acetonitrile and 40 parts of methanol was seen to produce 55.12 and 58.18% recovery for 25-(OH)D2 and 25-(OH)D3. For further improvement in recovery 20 parts of 0.1% formic acid was incorporated into the above extraction solvent. This attempt increased the recovery to 65.13 and 68.98% respectively. Further studies were undertaken for influence of rotation speed (RPM 400, 800, 1350) and rotation time (0.5, 1, 2, 3, 4, 5, 6 and 7 h). Increased recovery was observed with increased rotation time. A maximum recovery of 75.14% was observed for both metabolites at 6 h. No further increase in percent recovery was observed after 6 h. The rotation speed was not seen to influence the recovery. Further attempts to increase recovery was undertaken by incorporating an incubation step and undertaking trials at various incubation time (15, 30 and 60 min) and incubation temperature (35, 40, 45 and 50°C). 94.61 and 97.13% recovery were observed for 25-(OH)D2 and 25-(OH)D3 at 15 min and at 50°C.

**Method Validation**

Method validation studies were performed based on recommendations of Food and Drug Administration (FDA). A single batch of six DBS samples prepared from anonymized blood was processed with internal standard to assess the specificity of the method. Limit of detection (LOD) and limit of quantification (LOQ) were determined using standard solutions serially diluted until a signal to noise (S/N) 3 and 10 were obtained respectively. The LOD was determined to be 0.1 ng/mL and LOQ 0.4 ng/mL. The precision and accuracy experiments were executed using four independent batches comprising of six replicates of lower limit of quantification control (LLOQC), lower quality control (LQC), medium quality control (MQC) and higher quality control (HQC) samples (after subtraction of endogenous level as mentioned earlier). The responses obtained were compared with the nominal concentration value at each level. The extraction recoveries of analytes were determined by comparing the responses (after subtraction of endogenous level as mentioned above) of six replicates at LQC, MQC and HQC levels with neat standard solutions of same concentrations prepared in the mobile phase. To assess the carry over, blank samples were injected after the upper limit of quantification (ULOQ) samples. Inter and intra-day precision studies were performed by analyzing two independent batches consisting of six replicates at each quality control (QC) levels (after subtraction of endogenous level as mentioned above) over four separate days.

Hematocrit effect is an additional parameter that needs to be performed for DBS sampling technique to study the influence of hematocrit on the quantification of the analyte. Five DBS samples were prepared by spiking MQC at each hematocrit level of 20, 30, 40, 50 and 60% and the concentrations were back calculated using a linear regression equation. If there is an influence, it needs to be considered during the quantification of the analytes. ANOVA was used to statistically analyze the results and a p value less than 0.05 was considered statistically significant.

**Stability Studies**

Stock solution stability was performed using five replicates of MQC at room temperature for 8.0 h and at 2–8°C for 30 d. Stability of analytes in matrix were evaluated at bench top (1 to 7 h, room temperature), freeze thaw (3 cycles, −80°C) and stability in autosampler (4°C, 6 to 48 h). Long-term stability was assessed at −80°C for 7 to 180 d. Six replicates of LQC and HQC were subjected under each condition for the requisite time. On the day of the analysis all the stability samples were thawed unassisted at room temperature and compared with initial results at each QC level and the percent change was calculated.

**Method Comparison Studies**

Method comparison studies were performed using 50 anonymized patient samples (age group: 18–60 years) from the department of Biochemistry, Kasturba Hospital, Manipal. These samples were directed to the laboratory for 25-(OH)D estimation. On receipt of the left-over sample, the vacutainers were gently shaken on a shaker at 400 RPM and 40 μL of blood was spotted onto a 903-filter paper card. The cards were left to dry at room temperature for 3 h. These DBS samples were analyzed by the procedure described in our method and by the LC-MS/MS method reported by Eyles et al. Each batch consisted of blank (mobile phase), zero standard (with internal standard), a set of ten calibrators and QC samples (bracketed and placed at regular intervals among the unknown samples).

From the DBS values corresponding serum values were calculated based on the equation described by Heath et al., after correction of hematocrit values to 0.45. Serum samples were separated and analyzed by the Electrochemical Luminescence Immunoassay (ECLIA) method to compare the above results with true value in serum. The results obtained by the HPLC-UV method were compared against the LC-MS/MS method and the ECLIA method. The comparability of the method was assessed statistically by the Bland–Altman test using SPSS 16.0.

**Application of the Developed Method to the Pediatric Seizure Cohort**

For this study we collected dried blood spots of 30 infants presenting with intractable (require more than dual therapy to control seizures) epilepsy. The patient data collected from the seizure cohort include demographic data, seizure type, seizure frequency and age of onset of sei-
We also collected the dried blood spots from healthy age and sex matched controls (n = 30). The inclusion criteria for the study were as follows: Term neonates with no identifiable cause of seizures (infections, hypoglycaemia, hypocalcaemia, electrolytic abnormalities, asphyxia and structural abnormalities). For healthy neonates we set the inclusion criteria as: Healthy term neonates on breast feeding, born between 37–42 weeks of gestation, weight more than 2500 g, both male and female babies, no antibiotic or vitamin supplementation, with or without physiological jaundice, caesarean or vaginal delivery. The samples were dried at room temperature for three hours followed by which the samples were transferred to envelopes with desiccants. The envelopes were then transferred to zip lock plastic bags and were stored at −80°C. The samples were analyzed by the developed HPLC method and the total 25-(OH) D levels were estimated. The total 25-(OH) D status is determined by the sum of the levels of 25-(OH) D2 and 25-(OH) D3 in the body. A nine-point calibration curve was constructed based on the peak area ratios of the analyte to the internal standard. The sum of the peak area ratios of 25-(OH) D2 and 25-(OH) D3 was plotted on the X axis and concentration on the Y axis. These values were converted to corresponding serum values after correction of 0.49 hematocrit value for infants.

Results and Discussion

A circulating level of <20 ng/mL 25-(OH) D reflect vitamin D deficiency and a level of 21–29 ng/mL is considered insufficiency. Levels greater than 30 ng/mL is considered sufficient.\(^\text{23}^\) 25-(OH) D2 and 25-(OH) D3 are lipophilic in nature with a log P of 7.5 and 7.13 respectively making them excellent candidates for reverse phase chromatography. Montelukast was chosen as the internal standard as it demonstrated sufficient separation from the metabolites and other endogenous compounds. Isocratic reverse phase employing an Acclaim C18 column (150 × 4.6 mm i.d., 3 µm) was seen suitable for the retention of both 25-(OH) D2 and 25-(OH) D3 within 10 min. The mobile phase consisted of acetonitrile–formic acid buffer (pH 3) (90 : 10% (v/v)) at a flow rate of 1 mL/min with an injection volume of 20 µL. The column oven and auto-sampler temperature was set at 35 and 4°C, respectively. The readings were recorded at 266 nm using a UV-Visible detector. These chromatographic conditions were seen to provide excellent peak symmetry (Tailing factor: 1.10), height equivalent to a theoretical plate (>10000) and good area response for both the analytes. The retention times of 25-(OH) D2, 25-(OH) D3 and montelukast sodium were 7.2, 6.3 and 4.2 min, respectively. The blank chromatogram and a sample chromatogram obtained at this optimized condition is presented in Fig. 1.

Following procedure was optimized for extraction of analyte from DBS. Using a leather puncher, disks of size 11.1 mm were prepared from the DBS. The disks were then transferred to an Eppendorf tube and ACN–methanol–0.1% formic acid buffer with a ratio 60:20:20 (v/v/v) was added. The sample was shaken at 600 RPM for 6 h followed by incubation for 15 min at 50°C. The Eppendorf was then centrifuged at 10000 RPM for 10 min and the supernatant was evaporated under a gentle stream of nitrogen for 15 min. The residue was reconstituted in 100 µL mobile phase and 20 µL was injected into the HPLC system for analysis.
The study of method performance parameters demonstrated a LOD of 5 ng/mL and a LOQ of 10 ng/mL. The method was seen to be specific as no significant endogenous interference was observed at the retention times of the metabolites and the internal standard (Montelukast). Compared to earlier reported methods, our HPLC-UV method demonstrated linearity over a wide range from 10–80 ng/mL with \( r^2 \) of 0.998 for both 25-(OH) D2 and 25-(OH) D3 (Fig. 2). Inter and intra-day precision % CV at various quality control levels for the metabolites was less than 4.63%. These results are within the acceptance criteria and the % CV attained is similar to earlier reported DBS methods. The results are presented in Table 1.

The results of the study of influence of hematocrit on 25-(OH)D2 and 25-(OH)D3 are shown in Table 2. No influence of hematocrit on the levels of 25-(OH) D2 and 25-(OH) D3 was observed in our experiment. The no influence of hematocrit observed in our experiment can be attributed to the use of a whole DBS spot for the analysis. Jensen et al. report a +16 to −22% bias in the tested hematocrit range of 0.30 to 0.60 when using a 3.2 mm punch. The work by Kavaskoff et al. demonstrates the quantification of 25-(OH)D to be influenced by spot volume, punch size and chromatographic effect when a 3.2 mm punch is taken. These parameters were not seen necessary to be performed as we were using a 11.1 mm punch i.e., an entire DBS sector for the analysis.

| Time point | Percent change of 25-(OH)D3 | Percent change of 25-(OH)D2 |
|------------|-----------------------------|-----------------------------|
|            | LQC                         | HQC                         | LQC                         | HQC                         |
| 0 cycle    | 0.00                        | 0.00                        | 0.00                        | 0.00                        |
| 3 cycles   | 0.59                        | −0.33                       | −0.69                       | 0.74                        |

Table 2. Results of Influence of Haematocrit on 25-(OH)D2 and 25-(OH)D3

The results of the study of influence of hematocrit at MQC level demonstrated a minimal standard diviation (S.D.) and yielded a \( p \) value of 0.56 and 0.13 for 25-(OH)D3 and 25-(OH)D2 respectively (Table 2). Hence there is no influence of hematocrit on the levels of 25-(OH)D3 and 25-(OH)D2 respectively. The no influence of hematocrit observed in our experiment can be attributed to the use of a whole DBS spot for the analysis. Jensen et al. report a +16 to −22% bias in the tested hematocrit range of 0.30 to 0.60 when using a 3.2 mm punch. The work by Kavaskoff et al. demonstrates the quantification of 25-(OH)D to be influenced by spot volume, punch size and chromatographic effect when a 3.2 mm punch is taken. These parameters were not seen necessary to be performed as we were using a 11.1 mm punch i.e., an entire DBS sector for the analysis.

Figure 3. Graphical Representation of the Results of Stock Solution Stability Studies and Bench Top Stability Studies of Low Quality Controls (LQC) and High-Quality Controls (HQC) of 25-Hydroxy Vitamin D2 and 25-Hydroxy Vitamin D3.

The X axis represents the percent change from the initial value and the Y axis represents the time in hours for which the study was conducted. The dotted lines represent the acceptance limit as recommended by U.S. FDA.
It is important to understand the influence of temperature on analyte integrity to maintain optimum conditions during storage, transportation and analysis. Stability studies reported in literature demonstrated steady analyte integrity in long term conditions (7 months at room temperature), autosampler stability (4°C, 18 h), freeze thaw cycles (10 cycles), and in the presence of light at room temperature. For stock solution stability we observed a change of less than 3% for the metabolites stored at 2–8°C (30 d) and at room temperature (8 h). This is well within the acceptance limit of ±10%. The bench top stability studies undertaken to understand the influence of laboratory conditions witnessed negligible percent change (less than 1%) in the first one hour. At 8 h, bench top, for LQC levels we observed a −0.81 and −0.34% change from initial value for 25-(OH)D3 and 25-(OH)D2. Further at HQC levels a percent change of −1.17 and −0.14% from baseline was observed. The processed sample stability in the autosampler by 48 h demonstrated a percent change of −0.85 and −0.23% for 25-(OH)D3 and 25-(OH)D2 at LQC levels. Additionally, at HQC levels we observed a change of −1.23 and −3.31% for 25-(OH)D3 and 25-(OH)D2 respectively. The metabolites demonstrated stability after three freeze thaw cycles and the percent change was less than 0.74% at LQC and HQC levels. Long term stability study for a period of six months demonstrated a percent change of −1.15 and −3.27% for 25-(OH)D3 and 25-(OH)D2, respectively at LQC levels. At HQC levels these changes were −1.62 and −2.26%, respectively. In all stability conditions the percent change was within the recom-
Table 4. Patient Characteristics

| Sample size | n = 30 |
|-------------|-------|
| Demographic characteristics (number, percentage) | |
| Female | 17 (56.71) |
| Male | 13 (43.33) |
| Mean gestational age (weeks) | 37 ± 5.00 |
| Mean age (d) | 32 ± 10.00 |
| Seizure type (number, percentage) | |
| Focal tonic | 8 (26.61%) |
| Focal clonic | 10 (33.33%) |
| Generalised tonic | 8 (26.61%) |
| Myoclonic | 2 (6.66%) |
| Subtle | 1 (3.33%) |
| Motor automation | 1 (3.33%) |
| Seizure frequency (number, percentage) | |
| Once daily | 15 (50.00%) |
| Twice daily | 10 (33.31%) |
| More than twice | 5 (16.63%) |
| Mean age of onset of seizure (d) | 59 ± 12 |
| 25-(OH)D status (number, percentage) | |
| Deficiency (<20ng/mL) | 14 (46.61%) |
| Insufficiency (21–29ng/mL) | 8 (26.65%) |
| Normal (<30ng/mL) | 8 (26.65%) |

were below the LOQ levels. The mean ± S.D. in the remaining 6 deficient cases was 13.22 ± 2.80ng/mL. The 25-(OH)D values in healthy infants were 33.9 ± 6.11ng/mL. Patient characteristics are presented in Table 4. Our study results are consistent with the earlier studies which report 4-78% 25-(OH)D deficiency in seizing children.\(^5\)\(^,\)\(^6\)\(^,\)\(^7\) 25-(OH)D is known to protect against excitotoxicity in the brain by its ability to increase levels of glutathione and reduce levels of reactive oxygen species in the brain.\(^3\)\(^7\)

### Conclusion

A simple, sensitive and cost-effective method has been developed and validated for dried blood spot samples on HPLC-UV. The method involves no derivatization. The method involves the use of HPLC which is a less expensive instrumentation compared to LC-MS/MS thereby reducing the overall cost of analysis per sample. The stability studies demonstrated analyte integrity in all tested conditions. Our method was comparable to the reported and established methods. Application of the developed method has been demonstrated in seizure cohort. This method can apply for assessing 25-(OH)D deficiency in newborn. A prophylactic treatment with Vitamin D can save the child from the multitude of complications arising due to 25-(OH)D deficiency especially the intractable paediatric seizure due to 25-(OH)D deficiency. The method can be used as an adjunct to newborn screening. The cost effectiveness of the method makes it affordable to the low and middle income countries.

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### Conflict of Interest

The authors declare no conflict of interest.

### References

1. Caprio M., Infante M., Calanchini M., Mammi C., Fabbri A., *Bulim. Obes.*, 22, 27–41 (2017).
2. Eyles D., Burne T., McGrath J., “Vitamin D,” Elsevier, 2011, pp. 565–582.
3. Sebaaly A., Bachour F., Bayoud W., Adib G., Bedran F., Daher C., Maalouf G., *J. Med. Liban.*, 63, 87–93 (2015).
4. El-Fakhri N., McDevitt H., Shaikh M. G., Halsey C., Ahmed S. F., *Harm. Res. Paediatr.*, 81, 363–378 (2014).
5. Hatun S., Ozkan B., Orbak Z., Doterey H., Cizmecioglu F., Toprak D., *Calkoglu A. S.*, *J. Nutr.*, 135, 279–282 (2005).
6. Christiansen C., Rodbro P., *Sjo O.*, *BMJ.*, 2, 258–259 (1974).
7. Holló A., Clemens Z., Kamondi A., Lakatos P., Szűcs A., *Epilepsy Behav.*, 24, 131–133 (2012).
8. Holló A., Clemens Z., Lakatos P., *Int. J. Neurosci.*, 124, 378–393 (2014).
9. He C.-S., Gleeson M., Fraser W. D., *ISRN Nutr.*, 2013, 723139 (2013).
10. Valcour A., Zierold C., Podgorski A. L., Olson G. T., Wall J. V.
11) Coldwell R. D., Porteous C. E., Trafford D. J. H., Makin H. L. J., Steroids., 49, 155–196 (1987).
12) Lensmeyer G. L., Wiebe D. A., Binkley N., Dreznner M. K., Clin. Chem., 52, 1120–1126 (2006).
13) Diwesh Kumar S., Chawla D., Tripathi A. K., Pharm. Anal. Acta, 6, 410 (2015).
14) Snellman G., Melhus H., Gedeborg R., Byberg L., Berglund L., Wernroth L., Michaëlsson K., Clin. Chim. Acta, 403, 145–151 (2009).
15) Heath A. K., Williamson E. J., Ebeling P. R., Kvaskoff D., Eyles D. W., English D. R., J. Clin. Endocrinol. Metab., 99, 3319–3324 (2014).
16) Holick M. F., Chen T. C., Am. J. Clin. Nutr., 87, 1080S–1086S (2008).
17) Hedman C. J., Wiebe D. A., Dey S., Plath J., Kemnitz J. W., J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 953–954, 62–67 (2014).
18) Jones G., Kaufmann M., J. Steroid Biochem. Mol. Biol., 164, 110–114 (2016).
19) Wang Z., Senn T., Kalhorn T., Zheng E., Zheng S., Davis C. L., Hebert M. F., Lin Y. S., Thummel K. E., Anal. Biochem., 418, 126–133 (2013).
20) Volmer D. A., Mendes L. R. B. C., Stokes C. S., Mass Spectrom. Rev., 34, 2–23 (2015).
21) Thakare R., Chhonker Y. S., Gautam N., Alamoudi J. A., Alnouti Y., J. Pharm. Biomed. Anal., 128, 426–437 (2016).
22) Fallah E., Peighambarzost S. H., Heal. Promot. Perspect., 2, 180–189 (2012).
23) Food and Drug Administration. “Guidance for Industry: Bioanalytical Method Validation”: https://www.fda.gov/downloads/drugs/guidances/ucm070107.pdf.
24) Gachet M. S., Rhy P., Bosch O. G., Quednow B. B., Gertsch J., J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 976–977, 6–18 (2015).
25) Eyles D., Anderson C., Ko P., Jones A., Thomas A., Burne T., Mortensen P. B., Nørgaard-Pedersen B., Hougaard D. M., McGrath J., Clin. Chim. Acta, 403, 145–151 (2009).
26) Heath A. K., Williamson E. J., Ebeling P. R., Kvaskoff D., Eyles D. W., English D. R., J. Clin. Endocrinol. Metab., 99, 3319–3324 (2014).
27) Holick M. F., Chen T. C., Am. J. Clin. Nutr., 87, 1080S–1086S (2008).
28) Kvaskoff D., Heath A. K., Simila H. A., Ko P., English D. R., Eyles D. W., Clin. Chem., 62, 639–646 (2016).
29) Jensen B. P., Saraf R., Ma J., Berry S., Grant C. C., Camargo C. A., Jr., Sies C. W., Clin. Chim. Acta, 481, 61–68 (2018).
30) Higashi T., Suzuki M., Hanai J., Inagaki S., Min J. Z., Shimada K., J. Pharm. Biomed. Anal., 403, 725–732 (2011).
31) Kvaskoff D., Ko P., Simila H. A., Eyles D. W., J. Chromatogr. B Analyt. Technol. Biomed. Life Sci., 901, 47–52 (2012).
32) Bergqvist A. G. C., Schall J. I., Stallings V. A., Epilepsia, 48, 66–71 (2007).
33) Karaoğlu P., Polat İ., Ayanoğlu M., Yiş U., Hız S., Araştırmaya K., Derg. İzmir Dr. Behçet Uz Çocuk Hast. Derg., 4, (2014).
34) Harms L. R., Burne T. H. J., Eyles D. W., McGrath J. J., Best Pract. Res. Clin. Endocrinol. Metab., 25, 657–669 (2011).