JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

# Quality and stability of extemporaneous pyridoxal phosphate preparations used in the treatment of paediatric epilepsy

Abeer H. A. Mohamed-Ahmed[a,*], Matthew P. Wilson[b,*], Maedelyn Albuera[a], Ting Chen[a], Philippa B. Mills[b], Emma J. Footitt[c], Peter T. Clayton[b] and Catherine Tuleu[a]

[a]UCL School of Pharmacy, University College London, London, UK, [b]Institute of Child Health, University College London, London, UK and [c]Metabolic Medicine Unit, Great Ormond Street Hospital for Children NHS Foundation Trust, London, UK

**Keywords**
Dietary supplements; epilepsy; pyridox(am)ine 5′-phosphate oxidase deficiency; pyridoxal-5-phosphate; stability

**Correspondence**
Abeer H. A. Mohamed-Ahmed, UCL School of Pharmacy, University College London, 29-39 Brunswick Square, London WC1N 1AX, UK.
E-mail: abeer.ahm0065d@ucl.ac.uk

Received June 17, 2016
Accepted January 12, 2017

doi: 10.1111/jphp.12701

*These authors contributed equally to the manuscript.

## Abstract

**Objectives** To assess the pyridoxal 5′-phosphate (PLP) content and stability of extemporaneous PLP liquids prepared from dietary supplements used for the treatment of vitamin $B_6$-dependent epilepsy.

**Methods** Pyridoxal 5′-phosphate liquids were prepared in accordance with the guidelines given to patients from marketed 50 mg PLP dietary capsules and tablets. The PLP content and its stability were evaluated under conditions resembling the clinical setting using reverse phase HPLC and mass spectrometry.

**Key findings** Pyridoxal 5′-phosphate content in most of the extemporaneously prepared liquids from dietary supplements was found to be different from the expected amount (~16–60 mg). Most of these PLP extemporaneous liquids were stable at room temperature (protected from light) after 24 h but unstable after 4 h when exposed to light. A key photodegradation product of PLP in water was confirmed as 4-pyridoxic acid 5′-phosphate (PAP).

**Conclusion** Pyridoxal 5′-phosphate tablets from Solgar® were found to be the most reliable product for the preparation of extemporaneous PLP liquids. This work highlighted the difference between the marketed PLP dietary supplements quality and the importance of proper storage of aqueous PLP. There is a need to develop pharmaceutical forms of PLP that ensure dose accuracy and avoid potentially unsafe impurities with the aim of enhancing safety and compliance.

## Introduction

Pyridoxal-5-phosphate (PLP) is the biologically active form of vitamin $B_6$ (Figure 1) and it is involved in the catalysis of more than 140 enzymatic reactions in the body including those involved in the synthesis and degradation of neurotransmitters. There are multiple disorders relating to a deficiency of intracellular PLP. Several of these results in neurological dysfunction and epilepsy, an unsurprising outcome given the integral role PLP plays in neurotransmitter metabolism.[1] Pyridox(am)ine 5′-phosphate oxidase (PNPO) is a flavin mononucleotide (FMN)-dependent enzyme required for the synthesis of PLP from dietary pyridoxine (PN), pyridoxamine (PM) and their phosphates (PNP and PMP), as well as the glucoside of pyridoxine. It is also essential for the recycling of PLP from degraded enzymes in a 'salvage pathway'.[2]

PNPO deficiency is an autosomal recessive inborn error of metabolism that leads to neonatal epileptic encephalopathy. This disorder is characterised by drug-resistant seizures that can be fatal. Classically, seizures are responsive exclusively to PLP but patients show a good neurodevelopmental outcome when treated promptly.[3–5] Recently, it has become apparent that in some cases of PNPO deficiency, pyridoxine is an effective treatment.[6–9] However, in more than half of the patients, PLP is still the only effective form of treatment. PLP has also been shown to aid seizure control in 11.7% of idiopathic intractable epilepsies and is seen to produce a better outcome than pyridoxine.[10]

 

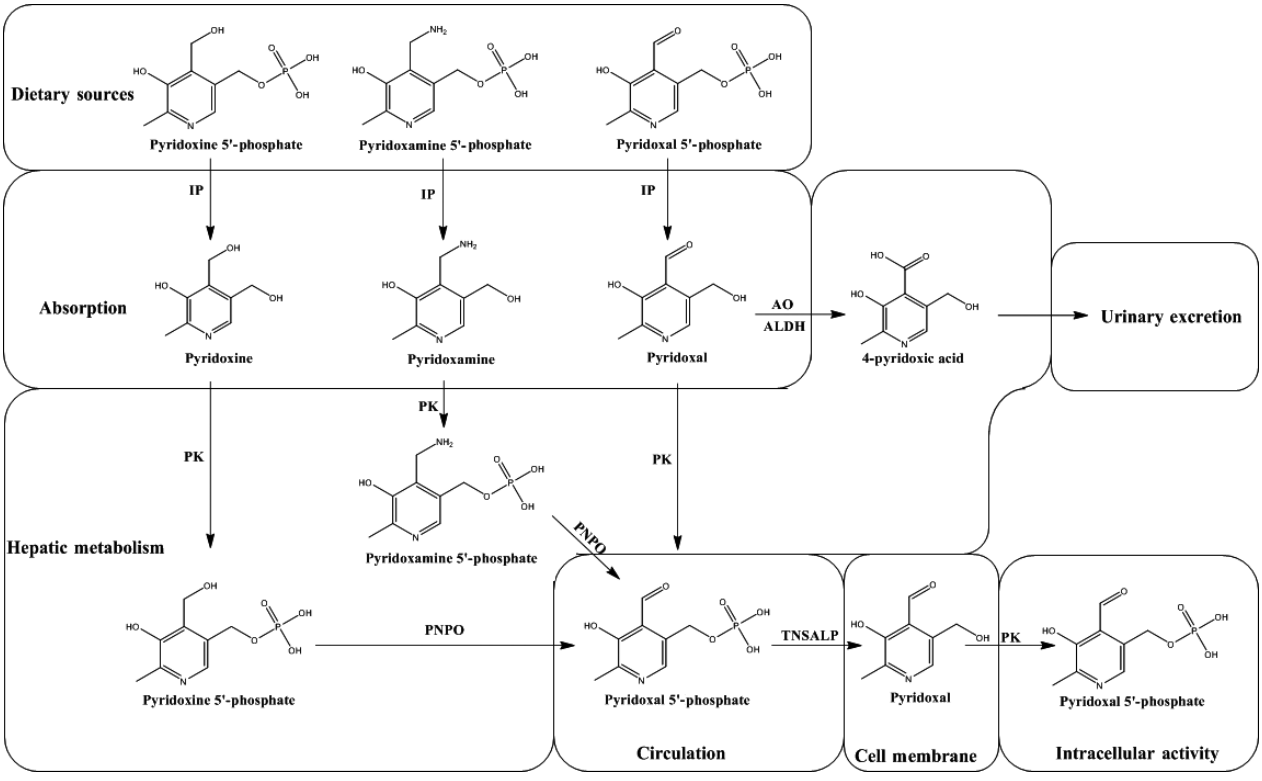


**Figure 1** The human vitamin B6 pathway from dietary to intracellular pyridoxal 5′-phosphate showing the activity of intestinal phosphatases, pyridoxal kinase, pyridox(am)ine 5-phosphate oxidase, tissue non-specific alkaline phosphatase, aldehyde oxidase and aldehyde dehydrogenases (adapted from Clayton 2006).[1]

There is currently no pharmaceutically licensed form of PLP; it is only available in the form of dietary supplements. There are several PLP dietary supplements in the form of capsules, tablets and liquid (Appendix 1). Unlike medicines, dietary supplements are not strictly regulated and their manufacture is not standardised. Therefore, the content of PLP in these dietary supplements should be determined to ensure dose accuracy. Patients with PNPO deficiency are treated by dissolving a crushed tablet or the content of capsule (50 mg PLP) in 5–10 ml water before oral administration or via a nasogastric tube. Because of the difficulties experienced in the preparation, this aqueous PLP liquid is often prepared in advance and given to the patient some hours later. Often, PLP tablets are enteric coated and hence difficult to crush them. Additionally, large volumes of liquid are required because of the poor solubility, and high amounts of PLP are needed for seizure control. Along with poor palatability, these large volumes have been known to cause vomiting in patients. Overall, the current acceptability of PLP when used as an anti-seizure medication is a major concern, and it increases the burden of disease on both patient and carer.

It has been reported that some patients receiving this aqueous form of PLP develop deranged liver function tests (LFTs) and, with time, hepatic cirrhosis.[11,12] Transient LFT increases were shown to occur in 14/28 patients treated with PLP for infantile spasms, these were resolved on cessation of PLP supplementation.[13] In addition, one reported homocystinuria patient treated with 1000 mg/day of PLP developed hepatitis and deranged LFTs within 4 days of his dose being raised.[14]The cause of this liver toxicity is not clear but is hypothesised that it may occur as a result of either the high doses of PLP itself (30–100 mg/kg/day in 4–6 doses) or ingestion of compounds arising as a result of degradation of PLP when in solution. Importantly, hepatic disease is not seen in PNPO patients supplemented with high dose of pyridoxine or in other $B_6$ responsive disorders treated similarly.

Pyridoxal 5′-phosphate is known to be unstable in aqueous form and undergoes photodegradation. Several photolysis products of PLP were first postulated nearly sixty years ago.[15] This photochemical reaction is irreversible and the degradation compounds formed are oxygen concentration-dependent and PLP concentration-dependent.[16] One of the degradation products has been identified as 4-pyridoxic acid 5′- phosphate (PAP), formed by the oxidation of the aldehyde at the 4′ position of PLP (Figure 2). In the presence of $O_2$, PAP is readily formed when PLP is present at

  

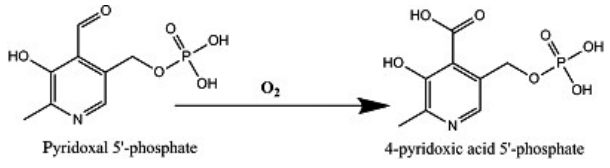


**Figure 2** The photooxidation of pyridoxal 5′-phosphate to PAP.

low concentrations, however, at high concentrations other yet unidentified species, with a peak absorbance of 288 nm are also formed, these are postulated to be dimers of PLP.[16] These higher concentrations are likely more representative of those seen on the use of PLP as an anti-seizure medication. It is possible that one or more currently unidentified degradation products are hepatotoxic and responsible for the liver dysfunction recently identified in patients on high-dose PLP supplementation.

The aims of our work are to understand what patients receive when marketed supplement products are manipulated to prepare a liquid dosage form to be administered to children for the treatment of PLP-responsive seizures by: (1) Assessing the available marketed PLP dietary supplements when prepared in aqueous solution, (2) Investigating the stability of the liquid PLP derived from different dietary supplements depending upon the storage conditions (temperature and light), (3) Characterising the photostability of PLP in aqueous solution. It is evident that this work is novel because; (i) this is the first study in which the quality of the marketed PLP products has been assessed and, (ii) the knowledge of the quality of PLP products (PLP content and stability) will help understand their clinical efficacy and safety.

## Materials and Methods

### Materials

Pyridoxal phosphate hydrate (>98%) was purchased from Sigma Aldrich. The marketed PLP dietary products include: Solgar 50 mg tablets (Batch No. 746087-03), Country Life tablets (Lot No. 13L622A), Thorne capsules (LB11606), Food Science capsules (21412000 1115), Biocare capsules (BN 32711) and Metabolics PLP liquid (800 μg/drop) were purchased from the Amazon website. PLP Bonusan tablets (Batch No. 0822) were purchased from Bonusan company. PLP Vitacost tablets (Batch No. 151027) were purchased from Vitacost company, Their composition is described in Appendix 1. HPLC grade methanol and trifluoroacetic acid (TFA) were purchased from Fisher Scientific. A custom synthesised PAP standard was purchased from Dr Herman ten Brink, Vrije Universiteit Medisch Centrum, Amsterdam, The Netherlands. $D_3$-PLP was purchased from Buchem BV, Apeldoorn, The Netherlands. HPLC grade methanol was purchased from VWR, both LC-MS grade heptafluorobutyric acid (HFBA) and acetic acid were purchased from Sigma.

### Weight uniformity of dosage form unit [British Pharmacopoeia (BP) 2014]

Pharmaceutical tablets and capsules must comply with the tests for content uniformity or the test for uniformity of mass. The tablets were weighed directly on a precision balance whilst the content of the capsules was evaluated by taking the difference between the mass of each of the twenty filled capsules and the average mass of 20 empty capsule shells. Data were expressed as an average of content mass ± standard deviation (mg). The uniformity of content was measured using high performance liquid chromatography (HPLC).

### Quantification of pyridoxal 5′-phosphate in dietary supplements using HPLC

Pyridoxal 5′-phosphate was quantified using reverse phase HPLC. Quantification and stability evaluations were performed using an Agilent Technologies 1200 Series HPLC system (Agilent Technologies, Palo Alto USA), equipped with an autosampler (injection volume of 10 μl) and a UV-VIS detector, set to perform measurements at a fixed absorption maximum wavelength of 285 nm at 25°C. A Synergi Polar (RP) HPLC column from Phenomenex (250 × 4.60 mm, 4 μm), was used with a gradient elution method at a flow rate of 1.0 ml/min. The mobile phase was initially 95% water (containing 0.2% trifluoroacetic acid) and 5% methanol. The percentage of methanol increased slowly to 40% over 20 min then gradually reduced to 5% over 5 min. The run time was 25 min. The retention time of PLP under the HPLC conditions was 5.5 min. Area under the peak was used to quantify PLP using a calibration curve (Appendix 2).

Seven marketed PLP dietary products were quantified. The crushed PLP tablets (using a mortar and pestle) and capsule contents were dissolved in two volumes of deionised water (10 and 50 ml volumetric flasks) resulting in a yellow cloudy solution at theoretical concentration of 50 mg/10 ml (5 mg/ml) or 50 mg/50 ml (1 mg/ml). Pure PLP dissolved in the same volume of water was used as control. These liquids were protected from exposure to light with foil and left to stir for 40 min at room temperature. The whole suspensions were filtered using a micro-syringe filter (Millex PES membrane 0.22 μm) before being analysed by HPLC.

The maximum solubility of PLP in distilled water was investigated under the same conditions as the quantification test. A supersaturated solution of PLP in water (10 mg/ml) was prepared, protected from light exposure by foil, and left to stir for 40 min at room temperature. This suspension was filtered and diluted tenfold before analysis

by HPLC. All experiments were repeated thrice. Data were expressed as average concentration (mg/ml) or PLP amount (mg) ± standard deviation.

### Stability testing of marketed pyridoxal 5′-phosphate products using HPLC-UV/VIS

The content of PLP capsules or crushed tablets were suspended in deionised water (10 ml volumetric flasks) to achieve a 5 mg/ml strength. For liquid PLP (Metabolics), 4.5 ml (11.5 mg/ml calculated concentration) of the liquid was withdrawn and diluted using water to a PLP concentration of 5 mg/ml. Pure PLP powder (50 mg) was dissolved in distilled water (10 ml) and used as a control. These solutions were protected from light exposure by foil and left to stir for 40 min. The stability studies were conducted at room temperature (25°C) over a 24 h period.

For light stability studies, the liquids were exposed to light with 2 Philips TL 8W/35 fluorescent lamps enclosed in a wooden box fitted with a cooling fan 240 V AC running at a speed of 50–60 Hz with a 23 W power to keep the atmosphere at ambient temperature. The PLP liquids were exposed for 4 h along with a solution of pure PLP as a control. PLP liquids were filtered and then analysed by HPLC. All the stability tests were repeated thrice. Results from the stability tests were expressed as the mean of the amount of PLP remaining (%) ± standard deviation.

### Assessment of pyridoxal 5′-phosphate Photodegradation by LC-MS/MS

The rate of PLP photodegradation was measured by the incubation of freshly prepared solutions at a theoretical concentration of 5 mg/ml, according to the stated PLP content in each product. The marketed formulations or analytical grade PLP were incubated for 1, 4 and 24 h under the same conditions described in the above photostability studies ($n = 3$). Solutions were analysed using a modified version of the LC-MS/MS method published by Footitt *et al.*[17] Samples were diluted to 100 nM and an equal volume of 0.3 N TCA containing 50 nmol/l $D_3$-PLP internal standard was added.

LC-MS/MS analysis was performed using a Waters H-Class FTN LC linked to a Waters Xevo TQ-S mass spectrometer in Multiple Reaction Monitoring (MRM) mode. An HSS T3 column was used with an HSS T3 guard and the mobile phases employed in the analysis consisted of A: 3.7% acetic acid, 0.01% HFBA and B: 100% methanol at a flow rate of 0.4 ml/min. The mobile phase composition graduated linearly from 97.5% A, 2.5% B at 0 min to 0.01% A, 99.9% B at 4.5 min before returning to initial conditions for a re-equilibration step of 2 min. The injected volume of the sample was 8 µl.

Pyridoxal 5′-phosphate and PAP were quantified by measuring the ratio of the peak area to that of $D_3$-PLP. Calibration curves for PLP and PAP were constructed in MQ-$H_20$ using a $D_3$-PLP internal standard concentration of 25 nmol/l. These calibration curves were shown to be linear between 1–200 nmol/l (PLP: $R^2 = 0.9982$; PAP: $R^2 = 0.9990$). (Figure S4) This allowed the accurate quantification of PLP and PAP, which were distinguishable by both retention time and m/z ratio (Table S1). Data acquisition and analysis were performed using Masslynx software. Statistical analysis was performed using Graphpad Prism version 5.00 for Windows.

### Investigation of pyridoxal 5′-phosphate photodegradants using LC-MS/MS

Preliminary mass spectra were obtained upon the photodegradation of 1 mM PLP solutions in deionised water exposed to sunlight. Investigative MS1, MS2, daughter ion and neutral loss scans (Figure S5) were performed by either direct or combined infusion mode using the Xevo TQ-S instrument described above. For specific identification of photoproducts previously separated by HPLC, the peaks of interest were collected and analysed in an identical fashion by either direct or combined infusion.

### Statistical analysis

Statistical analysis for the comparison of different PLP solutions prepared from the marketed products and pure PLP after stability testing was conducted using Kruskal-Wallis (using statistic programme Origin®). Conover-Iman test with P adjustment (bonferroni) was conducted using R (version 3.0.1) to compare between individual products.

## Results

### Weight uniformity and pyridoxal 5′-phosphate content

Weight variation is associated with non-uniformity of the amount of drug in solid formulations and poses a risk of dose variation; therefore, the weight uniformity of PLP dietary supplements was examined. The BP requirements for tablets weighing more than 250 mg is that no more than 2/20 tablets can deviate from the mean weight by more than 5% and none should deviate by more than 10%. For capsules weighing less than 300 mg the BP guidelines state that no more than 2/20 tablets can deviate from the mean by more than 10% and none should deviate by more than 20%.

Among the seven marketed PLP dietary products analysed, Food Science, Biocare and Bonusan did not comply with the BP requirements with weight variability being most marked for the Biocare® capsules (Table 1). PLP

  

tablets (Solgar, Country Life and Vitacost) and Thorne capsules met the BP requirements for weight uniformity (Table 1).

The content of PLP in the marketed products was assessed after dissolving the crushed tablets/contents of the capsules in 10 ml of deionised water as in the clinic or 50 ml to ensure complete dissolution of PLP (solubility of PLP is 5.7 mg/ml in water http://www.drugbank.ca/drugs/DB00114). The maximum experimentally calculated solubility of PLP in water at room temperature was 8.37 ± 0.94 mg/ml. According to the manufacturers, all the dietary products contained 50 mg PLP. Products from Solgar, Bonusan and Thorne contained the stated amount ~ 50 mg when dissolved in 10 ml water (Table 2) whilst Country Life, Food Science and Biocare products contained less than 50 mg (~2–30 mg) (Table 2). When the volume of water used for dissolution was increased to 50 ml, the same PLP content was obtained for all the products except for Country Life and Thorne (Table 2). The content of PLP in both Vitacost and Thorne was higher than 50 mg. The liquid form of PLP (Metabolics®) contained 11.01 ± 5.78 mg/ml PLP as well as significant amounts of degradation products. There is therefore a high risk of inaccurate dosing if the PLP dietary supplements from Biocare, Food Science, Vitacost, Thorne or Metabolics are used for preparation of PLP extemporaneous liquids.

**Table 1** Weight uniformity of pyridoxal 5′-phosphate dietary products. Twenty tablets or capsules content were weighed from each product. Data are expressed as mean ± standard deviation

| | BP criteria | | | |
|---|---|---|---|---|
| Product | Dosage form | Weight (mg) | No more than 2/20 masses deviate by 5% | None deviate by more than 10% |
| Solgar | Tablet | 350.7 ± 5.4 | √ | √ |
| Country Life | Tablet | 330.4 ± 4.9 | √ | √ |
| Vitacost | Tablet | 337.8 ± 4.1 | √ | √ |
| Bonusan | Tablet | 250.2 ± 10.7 | √ | **x** |
| Food Science | Capsule | 393.8 ± 10.0 | √ | **x** |
| Biocare | Capsule | 288.5 ± 21.2 | x | **x** |
| Thorne | Capsule | 197.2 ± 6.3 | √ | √ |

BP, British Pharmacopoeia.
**X** did not meet B.P requirements for weight uniformity test.

**Table 2** Pyridoxal 5′-phosphate content of dietary products dissolved in 10 and 50 ml deionised water. Data are expressed as mean ± standard deviation ($n$ = 3)

| | Amount of dissolved drug (mg) | | |
|---|---|---|---|
| Products | Dosage form | 10 ml water | 50 ml water |
| Country Life | Tablet | 15.9 ± 5.8 | 46.2 ± 4.4 |
| Solgar | Tablet | 51.3 ± 2.0 | 53.4 ± 2.2 |
| Bonusan | Tablet | 53.1 ± 1.0 | 51.4 ± 0.3 |
| Vitacost | Tablet | 62.7 ± 1.5 | 62.3 ± 1.7 |
| Food Science | Capsule | 30.9 ± 6.0 | 36.6 ± 4.4 |
| Biocare | Capsule | 2.1 ± 0.6 | 2.41 ± 0.1 |
| Thorne | Capsule | 54.7 ± 4.0 | 64.4 ± 2.8 |
| Pure PLP | Powder | 49.3 ± 1.1 | 51.2 ± 0.1 |

PLP, pyridoxal 5′-phosphate.

## Stability Studies

### Photostability of pyridoxal 5′-phosphate and its marketed products

#### *Quantification of 4-pyridoxic acid 5′-phosphate as a major degradation product of pyridoxal 5′-phosphate*

Analysis of a PLP solution by HPLC-UV/VIS after exposure to light revealed the presence of degradation products (Figure S2). In particular, a major product was seen to elute at 3.5 min. There were also two more minor photoproducts found to elute after PLP between 6.5 and 10 min HPLC-UV/VIS analysis (Figure S2).

To identify these compounds and study their rate of formation, mass spectral analysis was performed, followed by multiple reactions monitoring (MRM)-based LC-MS/MS for accurate quantification. Upon direct infusion of a photodegraded analytical grade PLP solution, several potential products were detected. Some of these were shown to contain phosphoric acid, signified by a loss of m/z 98 upon fragmentation with significant products seen at m/z 397.2, 381.1, 264.0, 262.9 and 219.9 (Figure S5). Because of its fragmentation pattern and abundancy, the product at m/z 264.0 was hypothesised to be 4-pyridoxic acid 5′-phosphate (PAP), the only previously confirmed PLP photodegradant.[16] Based on daughter scans of m/z 264, m/z 166.0 was identified as the most abundant product ion and a theoretical transition for PAP pertaining to a loss of phosphoric acid (m/z 264.0 > 166.0) was included in all subsequent LC-MS/MS analyses. An ion corresponding to this mass eluting at 0.78 min was evident after injection of the degraded PLP solution onto the LC-MS/MS with pyridoxal phosphate eluting at 0.94 min (Table S1).

The fragmentation and elution pattern of PAP was confirmed using the synthesised standard. Light irradiation of pure PLP over 24 h resulted in a dramatic decrease in PLP concentration. This was concomitant with the appearance of PAP, the concentration of which increased with time (Figure 3). Interestingly, at the concentrations of PLP used, the proportion of PAP formed after photolysis was found to only constitute 22.7 (±4.6%), 20.2 (±1.8%) and 27.5 (±4.6%) of the 'missing fraction' of PLP after 1, 4 and 24 h respectively. This confirms earlier work by Reiber (1972) that showed a large quantity of PLP is photolysed into compounds other than PAP.

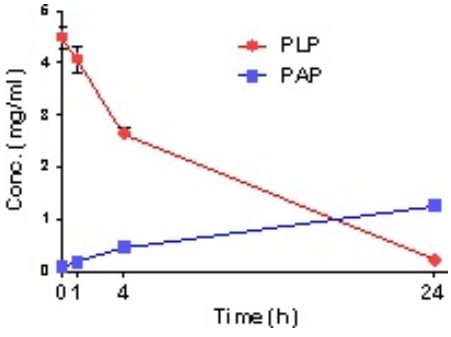


**Figure 3** LC-MS/MS analysis of the stability of analytical grade aqueous pyridoxal 5′-phosphate and the formation of 4-pyridoxic acid 5′-phosphate under light irradiation. Data are expressed as mean ± standard deviation ($n$ = 3).

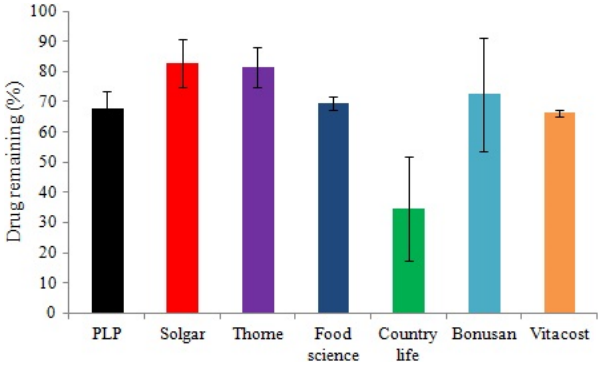


**Figure 4** The stability of marketed pyridoxal 5′-phosphate products dissolved in 10 ml water after 4 h of exposure to light. Data are expressed as mean ± standard deviation ($n$ = 3).

Subsequent analysis of the pure PAP standard on the HPLC/UV-VIS system revealed that PAP elutes at 7.2 min and does not correspond to the major photodegradation peak seen at 3.5 min (Figure S3). Upon collection of this peak and direct infusion into a Xevo TQ-S mass spectrometer, abundant ions were seen at m/z 492.9, 395.0, 297.1 and 247.2. These are hypothesised to correspond to $[M+H]^+$, $[M+H\text{-}H_3PO_4]^+$, $[M+H\text{-}2(H_3PO_4)]^+$ and $[M+2H]^{2+}$ of a diketone dimer of PLP, respectively, as predicted by Morrison and Long in 1958[15] Spectrophotometric analysis of this peak showed strong absorbance at 288 nm and is therefore considered highly likely to be one of the aforementioned '288 nm absorbing species' identified by Reiber in 1972.[16]

### *Photostability of marketed pyridoxal 5′-phosphate dietary supplements in solution*

Extemporaneously prepared aqueous liquids of marketed PLP dietary products were found to be unstable after 4 h exposure to light (Figure 4). Solgar and Thorne were the most stable (82.9 ± 7.9 and 81.7 ± 6.6% drug remaining respectively) followed by Bonusan (72.5 ± 18.9%), Food Science (69.7 ± 2.2%) and Vitacost (66.3 ± 1.2%); Country Life was the least stable (34.6 ± 7.2%). Pure PLP was unstable and displayed 68.0 ± 5.6% drug remaining. There was significant difference between all marketed PLP and pure PLP ($P$ = 0.025) (Figure 4). Country life was significantly unstable compared with Solgar and Thorne ($P$ = 0.003 and 0.011) (Figure 4).

The stability of the marketed liquid form of PLP (Metabolics®) was also assessed and revealed that only 47.0 ± 3.2% drug remained after 4 h exposure to light. This product was impure to begin with and contained degradation products irrespective of whether it had been stored in the fridge or at room temperature.

Several of the PLP dietary supplements identified as being the most reliable by HPLC-UV/VIS were further studied by LC-MS/MS and a similar loss of PLP was seen over time. In addition, in the marketed products, PAP was shown to form by photodegradation at a similar rate to that seen in pure PLP (Table 3).

### Stability of light protected marketed pyridoxal 5′-phosphate dietary supplements in solution at room temperature

At room temperature whilst protected from light, the stability of PLP was improved: with ~ 90% of drug remaining after 24 h (Figure 5) for all the products except for aqueous liquids made from Food Science (82.7 ± 1.8 drug) and Country Life (40.2 ± 6.9%) products**.** There was significant difference between all marketed PLP and pure PLP ($P$ = 0.012). Country life was significantly unstable compared with the pure PLP ($P$ = 0.029), Solgar ($P$ = 0.006), Bonusan ($P$ = 0.0002) and Vitacost ($P$ = 0.008) (Figure 5). Pure PLP solution was stable at room temperature (93.1 ± 4.8% drug remaining) but PLP Metabolics aqueous liquid (56.0 ± 0.8% drug remaining) was not.

## Discussion

Pyridoxal 5′-phosphate is used for the treatment of epilepsy caused by a deficiency of PNPO which, if untreated, can be fatal. Currently, there is no pharmaceutical form of PLP for paediatric use, so liquid forms of PLP are prepared from dietary products using crushed tablets or capsule contents mixed in a specific volume of tap water. There are major concerns about these extemporaneous PLP liquids regarding dose accuracy, stability and safety.

There are several issues with the feasibility of preparation of these extemporaneous liquids. At 25°C analytical grade

**Table 3** PLP remaining, taken as a percentage of initial pyridoxal 5′-phosphate (PLP), because of light degradation over 24 h, as measured by LC-MS/MS. Quantity of 4-pyridoxic acid 5′-phosphate formed over 24 h because ofphotodegradation of PLP, calculated as a percentage of PLP lost over the same period. Initial concentrations were nominally 5 mg/ml. Data are expressed as mean ± standard deviation ($n$ = 3)

| Products | Initial PLP (mg/ml) | PLP remaining (% of initial PLP) | PAP Formed (% of PLP lost) |
|---|---|---|---|
| Thorne | 5.7 ± 0.7 | 21.7 ± 9.3 | 26.6 ± 11.5 |
| Solgar | 4.1 ± 0.4 | 18.8 ± 3.8 | 23.1 ± 5.3 |
| Vitacost | 4.3 ± 0.1 | 16.0 ± 11.1 | 18.7 ± 10.7 |
| Pure PLP | 4.6 ± 0.7 | 16. 5 ± 2.7 | 27.5 ± 0.7 |

PAP, 4-pyridoxic acid 5′-phosphate.

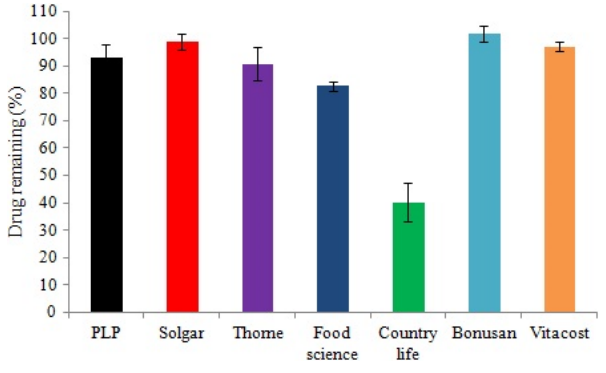


**Figure 5** The stability of marketed pyridoxal 5′-phosphate products in water (5 mg/ml PLP) after 24 h at room temperature (25°C). Data are expressed as mean ± standard deviation ($n$ = 3).

PLP requires continuous stirring for 40 min to completely dissolve at a concentration of 5 mg/ml, therefore, it is likely that PLP prepared from dietary products by manual shaking within a clinical setting or at home might not completely dissolve to give 5 mg/ml. This was evidenced by our findings; PLP contents in liquids that were prepared from dietary products according to common clinical usage were not accurate. Some of these products contained less than the 50 mg stated on the label (Country Life, Food Science, Biocare and Metabolics) and others more (Vitacost and Thorne). This variation in PLP content can result in dose inaccuracy leading to either inefficacy or toxicity.[18–20] The low amounts of PLP solubilised in some products are particularly dangerous, given that non-response to these supplements can be taken to be diagnostically indicative of a seizure disorder that does not respond to PLP.

Several dietary PLP products (Biocare, Bonusan and Food Science) did not comply with the B.P guidelines with regard to weight uniformity. This increases the risk of dose inaccuracy. Furthermore, some of the tablets (Solgar and Vitacost) are enteric coated making them difficult to crush and cause the generation of residues that can impair administration. When used clinically as a liquid formulation, carers usually only take the supernatant after solubilising PLP and leave any insoluble excipients behind, not knowing what is left, but in this context pragmatism prevails. This study has replicated the clinical scenario by only measuring PLP in the supernatant of the solutions prepared for analysis and has not measured any PLP that may be sequestered in the insoluble fraction. It is possible (indeed likely given its reactivity) that additional PLP is bound to other insoluble excipients (Appendix 1). If these products are taken as a dietary supplement it is likely the acidic conditions found in the stomach will release all PLP found therein. Moreover, it is also thought that these experiments being performed in the laboratory possibly improved the preparation methods of these PLP liquids and are not strictly what an inexperienced parent would do.

All of the extemporaneous liquids prepared from the tested marketed PLP products showed degradation when exposed to light even for short periods of time (4 h). This was consistent with the studies of Morrison and Long[15] and Ubbink,[21] which showed that pure PLP solutions were unstable after 4 h incubation in simulated daylight (66.5 ± 4.3% drug remaining). Morrison and Long[15] observed that pure PLP dissolved in air-free water completely photolysed after one hour in bright summer sunlight. Our advice is that PLP should always be made up immediately before administration. However, if this is impossible, it is imperative to protect PLP aqueous liquids from light when prepared in advance to avoid risk of dose inaccuracy and potential toxicity from any degradation products that may form. Unfortunately, this might be difficult to implement in the patient's home.

Most of the extemporaneous liquids prepared from the tested marketed PLP products and pure PLP were stable at room temperature when protected from light (~90% drug remaining). This was not consistent with the study of Shephard and Labadarios,[22] who have reported that 95% of a solution of pure PLP was degraded (mostly by hydrolysis to PL) after storage at room temperature in the dark for 24 h. The concentration of PLP is known to affect its degradation pattern.[14] This might explain the different findings in Shephard and Labadario's studies in which PLP was assessed at low concentrations (1 μg/ml), while in our studies a higher concentration relevant to the clinical setting was investigated (5 mg/ml).

The photodegradation products of PLP were further characterised using LC-MS/MS and showed that after 24 h exposure to light 27.4% of degraded PLP had been converted to PAP with a further 72.6% degraded to other products including a diketone dimer of PLP. This is crucial as these degradation products could cause toxic side effects. PAP, naturally not present in humans, has been shown to inhibit PLP-dependent enzymes and thus theoretically could be a cause of the hepatotoxicity seen in PNPO

patients on high-dose PLP supplementation.[23] However, *in vivo* it is likely that PAP is hydrolysed and subsequently excreted as pyridoxic acid in urine.[24,25] It is known that the B6 vitamers are mostly absorbed in their non-phosphorylated form and do not cross the gastro-intestinal wall in appreciable amounts before hydrolysis.[26] In addition, upon measurement of the B6 vitamer blood levels of patients on PLP supplementation, PAP is not detected in significant amounts (Unpublished data Laboratory of Mills P, 2016).

While it seems unlikely that PAP is a major toxic degradation product, other structural analogues of the B6 vitamers are already known to have toxic effects. Gingko toxin (4′-O-methylpyridoxine), a naturally occurring plant extract, causes seizures thought to be because of inhibition of the pyridoxal kinase enzyme, responsible for phosphorylation of the B6 vitamers: pyridoxal, pyridoxamine and pyridoxine.[27,28] If other structural analogues of PLP are also capable of inhibition of B6-metabolising or PLP-dependent enzymes, the effects could be wide ranging and could indeed result in hepatic dysfunction.

Coman *et al.* postulate that the hepatic cirrhosis seen on high-dose PLP supplementation results because of the aberrant purinoceptor activation of hepatic stellate cells (HSC).[11] P2 receptors of HSC are linked with a fibrogenic response through increased collagen production. PLP itself acts on P2 receptors in an antagonist role but other PLP analogues show both agonistic and antagonist effects. It is possible that degradation products or metabolites of PLP may cause the hepatic fibrosis and deranged LFTs seen in PNPO deficient patients on high doses of PLP by purinoceptor activation.

The identification of other photodegradants of PLP goes beyond the scope of this study, but would be an important focus for any future work investigating the hepatotoxicity seen in certain patients on large doses of PLP.

## Conclusions

Pyridoxal 5′-phosphate from Solgar, Thorne, Bonusan and Vitacost are the most stable products at room temperature, protected from light. Bonusan tablets are of non-uniform weight. Throne and Vitacost contain higher than stated amounts of PLP [~ 60 mg] which increases the risk of dose inaccuracy and toxicity. Although Solgar was of the best quality, none of the currently marketed products are truly suitable for long-term use at high doses because of possible liver toxicity. There is a need for a more stable pharmaceutically licensed treatment that is easily prepared and administered to PLP-responsive patients.

The quantity of PAP formed upon the photodegradation of PLP in aqueous solution was accurately quantified. This accounted for 27% of the PLP lost/24 h. The implications of this are significant for patients with seizure disorders on high doses of PLP as are the presence of other degradation products (73% of total degraded PLP) which could be responsible for hepatic cirrhosis in these patients.

We recommend that (1) high-dose PLP supplements be prepared immediately before administration and protected from light to avoid degradation in solution, (2) PLP dietary supplements that do not meet the standards required for pharmaceutical products should be avoided and (3) the LFTs of all patients on this treatment should be closely monitored.

## Declarations

### Acknowledgement

We would like to thank Dr. Yucheng Sheng for his help with the statistical analysis and Dr. Elin Haf Davies for her support and mentoring.

## References

1. Clayton PT. B6-responsive disorders: a model of vitamin dependency. *J Inherit Metab Dis* 2006; 29: 317–326.
2. Musayev FN *et al.* Structure and properties of recombinant human pyridoxine 5′-phosphate oxidase. *Protein Sci* 2003; 12: 1455–1463.
3. Mills PB *et al.* Neonatal epileptic encephalopathy caused by mutations in the PNPO gene encoding pyridox (am)ine 5′-phosphate oxidase. *Hum Mol Genet* 2005; 14: 1077–1086.
4. Hatch J *et al.* Normal Neurodevelopmental Outcomes in PNPO Deficiency: a Case Series and Literature Review. *JIMD Rep* 2015; 16: 1392–1394.
5. Clayton PT *et al.* Neonatal epileptic encephalopathy. *Lancet* 2003; 361: 1614.
6. Mills PB *et al.* Epilepsy due to PNPO mutations: genotype, environment and treatment affect presentation and outcome. *Brain* 2014; 137: 1350–1360.
7. Plecko B *et al.* Pyridoxine responsiveness in novel mutations of the PNPO gene. *Neurology* 2014; 82: 1425–1433.
8. Pearl PL *et al.* Partial Pyridoxine Responsiveness in PNPO Deficiency. *JIMD Rep* 2013; 9: 139–142.
9. Pearl PL, Gospe SM Jr. Pyridoxine or pyridoxal-5′-phosphate for neonatal epilepsy: the distinction just got murkier. *Neurology* 2014; 82: 1392–1394.
10. Wang HS *et al.* Pyridoxal phosphate is better than pyridoxine for controlling idiopathic intractable epilepsy. *Arch Dis Child* 2005; 90: 512–515.
11. Coman D *et al.* PNPO Deficiency and Cirrhosis: expanding the Clinical

Phenotype? *JIMD Rep* 2015; 25: 71–75.
12. Sudarsanam A *et al.* Cirrhosis associated with pyridoxal 5′-phosphate treatment of pyridoxamine 5′-phosphate oxidase deficiency. *JIMD Rep* 2014; 17: 67–70.
13. Takuma Y, Seki T. Combination therapy of infantile spasms with high-dose pyridoxal phosphate and low-dose corticotropin. *J Child Neurol* 1996; 11: 35–40.
14. Yoshida I *et al.* Pyridoxal phosphate-induced liver injury in a patient with homocystinuria. *J Inherit Metab Dis* 1985; 8: 91.
15. Morrison AL, Long RF. The photolysis of pyridoxal phosphate. *Journal of the Chemical Society (Resumed)* 1958; 211–215.
16. Reiber H. Photochemical reactions of vitamin B 6 compounds, isolation and properties of products. *Biochim Biophys Acta* 1972; 279: 310–315.
17. Footitt EJ *et al.* Measurement of plasma B6 vitamer profiles in children with inborn errors of vitamin B6 metabolism using an LC-MS/MS method. *J Inherit Metab Dis* 2013; 36: 139–145.
18. Hartmann H *et al.* Status epilepticus in a neonate treated with pyridoxine because of a familial recurrence risk for antiquitin deficiency: pyridoxine toxicity? *Dev Med Child Neurol* 2011; 53: 1150–1153.
19. Hammen A *et al.* A paradoxical rise of neonatal seizures after treatment with vitamin B6. *Eur J Paediatr Neurol* 1998; 2: 319–322.
20. Salazar P, Tapia R. Seizures induced by intracerebral administration of pyridoxal-5′-phosphate: effect of GABAergic drugs and glutamate receptor antagonists. *Neuropharmacology* 2001; 41: 546–553.
21. Ubbink JB *et al.* Stability of pyridoxal-5-phosphate semicarbazone: applications in plasma vitamin B6 analysis and population surveys of vitamin B6 nutritional status. *J Chromatogr* 1985; 342: 277–284.
22. Shephard GS, Labadarios D. Degradation of vitamin B6 standard solutions. *Clin Chim Acta* 1986; 160: 307–311.
23. Churchich JE. Fluorescence changes induced by binding of 4-pyridoxic acid 5 -phosphate to proteins. *J Biol Chem* 1972; 247: 6953–6959.
24. Fonda ML. Purification and characterization of vitamin B6-phosphate phosphatase from human erythrocytes. *J Biol Chem* 1992; 267: 15978–15983.
25. Gao GJ, Fonda ML. Kinetic analysis and chemical modification of vitamin B6 phosphatase from human erythrocytes. *J Biol Chem* 1994; 269: 7163–7168.
26. Albersen M *et al.* The intestine plays a substantial role in human vitamin B6 metabolism: a Caco-2 cell model. *PLoS One* 2013; 8: e54113.
27. Kastner U *et al.* The human pyridoxal kinase, a plausible target for ginkgotoxin from Ginkgo biloba. *FEBS J* 2007; 274: 1036–1045.
28. Gandhi AK *et al.* Crystal structures of human pyridoxal kinase in complex with the neurotoxins, ginkgotoxin and theophylline: insights into pyridoxal kinase inhibition. *PLoS One* 2012; 7: e40954.

## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix 1.** PLP dietary products on the market (none have marketing authorisation in the EU)

**Appendix 2.** Calibration curve

**Figure S1**. Standard Calibration Curve of PLP in water. Equation of the line is $y = 10643x$. Data is expressed as mean ± standard deviation ($n = 3$)

**Figure S2.** Chromatogram of Pure PLP after 4 h exposure to light. Intact PLP appears at retention time 5.8 min, while the main degradation product appears at 3.5 min

**Figure S3.** Chromatogram of Pure PAP appears at retention time 7.2 min

**Figure S4.** Calibration curves for PLP and PAP, $R^2$ values are 0.98824 and 0.9990 respectively. Response is calculated by normalising the peak areas of the MRMs for PLP and PAP against that of $D_3$-PLP

**Figure S5.** Neutral loss (98) mass spectra of a PLP solution before (a) and after (b) incubation for 5 h in sunlight. The major degradation products seen to lose a phosphoric acid residue (m/z 98) can be seen at m/z's 397.21, 381.15, 264.01(pyridoxic acid phosphate), 262.97 and 219.99. m/z's 270.12 and 269.81 are likely PLP $[M+Na]^+$

**Table S1.** The mass spectral specifications for detection of PLP, PAP and D3-PLP. All are detected in positive ion mode.