REVIEW

Vitamin B₆: Deficiency diseases and methods of analysis

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Abstract: Vitamin B₆ (pyridoxine) is closely associated with the functions of the nervous, immune and endocrine systems. It also participates in the metabolic processes of proteins, lipids and carbohydrates. Pyridoxine deficiency may result in neurological disorders including convulsions and epileptic encephalopathy and may lead to infant abnormalities. The Intravenous administration of pyridoxine to patients results in a dramatic cessation of seizures. A number of analytical methods were developed for the determination of pyridoxine in different dosage forms, food materials and biological fluids. These include UV spectrometric, spectrofluorimetric, mass spectrometric, thin-layer and high-performance liquid chromatographic, electrophoretic, electrochemical and enzymatic methods. Most of these methods are capable of determining pyridoxine in the presence of other vitamins and complex systems in µg quantities. The development and applications of these methods in pharmaceutical and clinical analysis mostly during the last decade have been reviewed.

Keywords: Vitamin B₆, pyridoxine deficiency, spectrometric methods, chromatographic methods, electrochemical methods, enzymatic methods.

INTRODUCTION

Vitamin B₆ is a unique vitamin that is involved in the metabolism of proteins, lipids and carbohydrates. The metabolism of amino acids requires enzymes that use pyridoxal phosphate as the co-factor or prosthetic group. In the amino acid decarboxylase reaction that leads to the formation of monoamine neurotransmitters, vitamin B₆ is closely associated with the function of the nervous system. It also has an important role in immune and endocrine systems (Dakshinamurti et al., 2007; Sweetman, 2007). Thus, the biological role of pyridoxine in health and ailment is considered vital.

Vitamin B₆ was discovered by Paul Gyorgy (1934) as a factor distinct from riboflavin and the pellagra-preventive factor, niacin. It was chemically identified as 3-hydroxy-4,5-hydroxymethyl-2-methylpyridine and was synthesized by Harris and Folkers (1939). The active derivatives of pyridoxine are referred to as “vitamin B₆ vitamers” and include the group of naturally occurring derivatives: pyridoxine (pyridoxol), pyridoxal, and pyridoxamine and their phosphorylated derivatives having similar physiological actions. The term vitamin B₆ generically refers to all these chemically related compounds (Dakshinamurti et al., 2007). However, pyridoxine is the predominantly used form of vitamin B₆ in clinical treatment. A large number of biological reactions are catalyzed by pyridoxal-5'-phosphate-dependent enzymes (Christen and Mehta, 2001). Pyridoxine has a low toxicity and doses about 1000 mg/day for variable periods of time might be associated with neuropathy (Bendich and Choen, 1990).

Pyridoxine deficiency

In all species of pyridoxine-deficient animals impairment of somatic growth, a pellagra-like dermatitis, and ataxia have been observed (Gries and Scott, 1972). The most outstanding symptoms caused by pyridoxine deficiency are related to the nervous system and include hyperacuosis, hyperirritability, impaired alertness, abnormal health movements and convulsions in animals and humans (Dakshinamurti and Stephens, 1969). The corticosteroids and thyroid hormones may increase the requirement for pyridoxine and thus affect pyridoxal-5'-phosphate-dependent metabolic processes. There is an association between vitamin B₆ and anterior pituitary hormones that seem to involve the hypothalamus, 5-hydroxy-tryptamine and dopamine. The synthesis of the later two neurotransmitters by metabolic processes requires pyridoxal-5-phosphate (Ortiga et al., 2004).

The biochemical reactions involving pyridoxal-5-phosphate (PLP) as the coenzyme are diverse in nature since more than 140 enzymes are PLP dependent. Pyridoxine has a crucial role in nervous system as the putative neurotransmitters, and the synthesis of other compound such as taurine and sphingolipids are dependent on PLP-dependent enzymes. The involvement of PLP enzymes in the decarboxylation of glutamic acid and 5-hydroxytryptophan (5-HTP) has considerable
significance in relation to neurological disorders of vitamin B<sub>6</sub> deficiency (Dakshinamurti et al., 2007).

There are differences in the distribution of 3,4-dihydroxyphenylalanine (DOPA) and 5-hydroxtryptophan (5-HTP) decarboxylation due to variation in the activity of PLP (Siow and Dakshinamurti, 1985, 1986). The decarboxylation step has been found to be the site of difference between pyridoxine-replete and pyridoxine-deficient rats with regard to the decrease of serotonin. The hypothalamus is one of the parts of the brain of deficient rats with regard to the decrease of serotonin. The difference between pyridoxine-replete and pyridoxine-deficient rats having considerable loss in PLP activity of PLP (Siow and Dakshinamurti, 1985, 1986).

Pyridoxine dependency has been recognized as an inborn abnormality. Infants soon after birth having seizures that are resistant to the commonly used antiepileptic drugs respond only to pharmacological doses of pyridoxine. A pyridoxine-dependent condition has to be considered in all children with intractable epilepsy up to three years of age (Gospe, 2002). The intravenous administration of pyridoxine results in a dramatic cessation of seizures. Vitamin B<sub>6</sub> has been found to improve the cardiovascular function in rats. Treatment of hypertensive vitamin B<sub>6</sub>-deficient rats using dietary pyridoxine has corrected both the deficiency state and the hypertensive condition (Dakshinamurti and Lal, 1992; Dakshinamurti and Dakshinamurti, 2001).

The deficiency of the biological active form of vitamin B<sub>6</sub>, PLP, causes serious neurological complications including convulsions and epileptic encephalopathy. Multifactorial neurological diseases such as Alzheimer’s disease and Parkinson’s disease have been correlated to insufficient levels of the enzyme (Di Salvo et al., 2012). Vitamin B<sub>6</sub> inhibits oxidative stress due to Cu (II)-β-amyloid (Aβ)-peptide (Hashmi et al., 2011).

Pyridoxine has been shown to reduce cisplastin and fluoropyrimidine-related neurotoxicity without compromising the anti-tumor effect (Garg and Ackland, 2011). The vitamin B<sub>6</sub>-dependent epilepsy responds to intravenously administered vitamin B<sub>6</sub>. The newborns with seizures should be treated with vitamin B<sub>6</sub> until epilepsy is completely cured (Bok et al., 2010). In some patients without pyridoxine deficiency epilepsy cannot be controlled without any extra supplement of pyridoxine (Wang and Kuo, 2007). Vitamin B<sub>6</sub>-related seizures and their dependency is suppressed by a high-dose treatment of vitamin B<sub>6</sub> (Ohtahara et al., 2011). The early treatment with pyridoxine is an important factor in the prevention of Wilson’s disease and potentially fatal disease progression in children (Kleine et al., 2012).

An analysis of PLP concentrations in cerebrospinal fluid has shown that lower reference limit for the detection of inborn metabolic errors of PLP loss are: <30 days, 26 nmol/l; 30/days-12 months, 14/nmol/l, 1-2/year, 11 nmol/l; >3 years, 10 nmol/l. Inborn errors resulting in PLP losses below these levels indicate vitamin B<sub>6</sub>-dependent epilepsy (Footitt et al., 2011). Oxalate, a marker of vitamin B<sub>6</sub> deficiency, is increased in the amniotic fluid of fetuses with Down syndrome. This is due to abnormalities in the metabolism of pyridoxine (Baggot et al., 2008). Low levels of folate, cyanocobalamin and pyridoxine are related to the neurological and psychological disorders. In elderly, incident dementia may result from high occurrence of insufficient B vitamins (Selhub et al., 2010). Thus, pyridoxine has a significant role in the

### Table 1: Pyridoxine (vitamin B<sub>6</sub>) deficiency diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Dosage/Doses</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
<td>-</td>
<td>Abraham et al., 2010</td>
</tr>
<tr>
<td>B&lt;sub&gt;6&lt;/sub&gt; deficiency in neonates</td>
<td>-</td>
<td>Ribeiro et al., 2011</td>
</tr>
<tr>
<td>Autism</td>
<td>-</td>
<td>Kaluzna et al., 2011</td>
</tr>
<tr>
<td>Schizophrenia, Alzheimer’s disease, Parkinson’s</td>
<td>-</td>
<td>Disalvo et al., 2012</td>
</tr>
<tr>
<td>disease and epilepsy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wilson’s disease</td>
<td>-</td>
<td>Kleine et al., 2012</td>
</tr>
<tr>
<td>Oxaliplatin-induced neurotoxicity</td>
<td>5 mg</td>
<td>Garg and Ackland, 2011</td>
</tr>
<tr>
<td>Pyridoxine-dependent epilepsy (PDE)</td>
<td>-</td>
<td>Bok et al., 2010</td>
</tr>
<tr>
<td>B&lt;sub&gt;6&lt;/sub&gt; related epilepsy during childhood</td>
<td>-</td>
<td>Wang et al., 2007</td>
</tr>
<tr>
<td>Doxorubicin-related hand-foot syndrome in</td>
<td>200 mg</td>
<td>von Gruenigen et al., 2010</td>
</tr>
<tr>
<td>gynecologic oncology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Children receiving TB chemotherapy</td>
<td>4.20 mg/kg</td>
<td>Cilliers et al., 2010</td>
</tr>
<tr>
<td>Intractable epilepsy</td>
<td>50-100 mg</td>
<td>Gospe, 2002</td>
</tr>
<tr>
<td>B&lt;sub&gt;6&lt;/sub&gt;-related seizures</td>
<td>100-400 mg</td>
<td>Ohtahara, 2011</td>
</tr>
<tr>
<td>Down syndrome</td>
<td></td>
<td>Baggot et al., 2008</td>
</tr>
<tr>
<td>B&lt;sub&gt;6&lt;/sub&gt;-dependent seizures</td>
<td>13.5 mg/kg</td>
<td>Goto et al., 2001</td>
</tr>
<tr>
<td>Polyneuropathy</td>
<td>30 mg</td>
<td>Moriwaki et al., 2000</td>
</tr>
<tr>
<td>Carpal tunnel syndrome</td>
<td>-</td>
<td>Keniston et al., 1997</td>
</tr>
<tr>
<td>Neuropathy</td>
<td>1000 mg</td>
<td>Bendich and Choen, 1990</td>
</tr>
</tbody>
</table>

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proper functioning of physiological system and its deficiency may cause several disorders related to the nervous, immune and endocrine systems. A summary of various pyridoxine deficiency diseases has been presented in table 1.

**METHODS OF ANALYSIS**

Various analytical methods have been used for the assay of pyridoxine in pharmaceutical preparations, food materials and biological fluids. The frequency of the applications of these methods is shown in Fig. 1 and the methods are presented in the following sections.

![Graph: Applications of analytical methods for the assay of pyridoxine in pharmaceutical preparations and biological fluids. 1. Enzymatic methods 2. electrochemical methods 3. mass spectrometry 4. electrophoresis 5. spectrofluorimetry 6. spectrophotometry 7. HPLC.](image)

**UV spectrophotometry**

UV spectrophotometric methods have been introduced for the assay of pyridoxine in pharmaceutical preparations. A linear relationship for vitamin B6 has been found in the range 100.0-100.7%. The methods have been applied to routine determination of the commercial products (Dinc et al., 2010).

A multi-commuted flow system has been developed for the spectrometric assay of pyridoxine in pharmaceutical preparations. A linear relationship for vitamin B6 has been found in the range 0.1-0.9 µmol/l at 99.7% confidence level with recoveries between 95.6 and 100% (Rocha et al., 2003).

The derivative and multivariate spectrometric methods have been applied to the determination of binary, ternary and quaternary mixtures of water-soluble vitamins containing thiamine HCl, pyridoxine HCl, riboflavin and cyanocobalamin. Both methods involve absorbance measurements in a wide range of UV/visible wavelengths.
Table 2: Analytical parameters for spectrometric methods of pyridoxine (B₆) assay

<table>
<thead>
<tr>
<th>Material</th>
<th>Technique</th>
<th>Wavelength (nm)</th>
<th>Concentration range (µg ml⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₆/ melatonin mixture</td>
<td>Spectrophotometry, partial least squares (PLS) calibration and Principle component regression (PCR) analysis</td>
<td>278 and 310</td>
<td>1-24</td>
<td>Sorouraddin et al., 2005</td>
</tr>
<tr>
<td>Vitamin B₆/ diazepam mixture</td>
<td>Spectrophotometry multiple linear regression and partial least-squares regression analysis</td>
<td>242 and 290</td>
<td>1.4-12.0</td>
<td>Bautista et al., 1996</td>
</tr>
<tr>
<td>Pyridoxine in B Vitamin mixture</td>
<td>Spectrophotometry partial least squares regression analysis</td>
<td></td>
<td>1.01-16.2</td>
<td>Aberasturi et al., 2002</td>
</tr>
<tr>
<td>Pyridoxine in binary drug mixtures of tablets and syrups</td>
<td>Spectrophotometry, PLS and PCR analysis</td>
<td></td>
<td>Zero and first order UV spectra</td>
<td>el-Gindy, 2003</td>
</tr>
<tr>
<td>Pyridoxine HCl and thiamine HCl in pharmaceutical preparations</td>
<td>Spectrophotometry Zero order spectra and ratio spectra derivative</td>
<td>Zero order spectra 246.8 and 290.5 ratio spectra derivative B₆ 297.8/309.5 and B₁ 245.6/257.7</td>
<td>8-40</td>
<td>Din et al., 2000</td>
</tr>
<tr>
<td>Pyridoxine and melatonin in tablets</td>
<td>Derivative UV spectrophotometry Zero-crossing technique</td>
<td>UV</td>
<td>pyridoxine 2-10 melatonin 0.5-3.5</td>
<td>Surmeian and Aboul-Enein, 1998</td>
</tr>
<tr>
<td>B₆ in B vitamin preparations</td>
<td>Spectrophotometry</td>
<td>290</td>
<td>0.50-8.0</td>
<td>Rocha et al., 2003</td>
</tr>
<tr>
<td>Pyridoxine in water soluble vitamins</td>
<td>Spectrophotometry derivative and multivariate methods</td>
<td>200-500</td>
<td>2.5-90</td>
<td>Mohamed et al., 2011</td>
</tr>
<tr>
<td>Pyridoxine HCl in pharmaceutical preparations</td>
<td>Differential spectrophotometry</td>
<td>290</td>
<td>-</td>
<td>Muszalska et al., 2011</td>
</tr>
<tr>
<td>Pyridoxine HCl and thiamine HCl in vitamin preparations</td>
<td>Spectrophotometry least squares and multivariate calibration methods</td>
<td>200-330 at 0.1 nm intervals</td>
<td>8-40</td>
<td>Ozdemir and Dinc, 2004</td>
</tr>
<tr>
<td>B₆ in water soluble vitamins</td>
<td>Spectrophotometry, derivative and multivariate method</td>
<td>200-500</td>
<td>2.5-90</td>
<td>Mohammad, 2011</td>
</tr>
<tr>
<td>Vitamins B₁, B₆, B₁₂</td>
<td>TLC densitometric method</td>
<td>242, 291, 360</td>
<td>0.1-1.3/spot</td>
<td>Elzanfaly, 2010</td>
</tr>
<tr>
<td>Vitamin B₆/ melatonin mixture</td>
<td>Spectrofluorimetry</td>
<td>λex 285, λem 324-550</td>
<td>0.04-4.0</td>
<td>Sorouraddin et al., 2005</td>
</tr>
<tr>
<td>Pyridoxine, peroxicam mixture</td>
<td>Spectrofluorimetry</td>
<td>λex 290-340, λem 370-560</td>
<td>0.66-8.00 for B₆</td>
<td>Abdollahi et al., 2006</td>
</tr>
<tr>
<td>Pyridoxine in B Vitamins</td>
<td>Spectrofluorimetry</td>
<td>λex 295, λem 385</td>
<td>0.05-1.8 ng/ml</td>
<td>Ruiz-Madina et al., 1998</td>
</tr>
<tr>
<td>Pyridoxine in presence of peroxicam</td>
<td>Spectrofluorimetry</td>
<td>λex 315, λem 465</td>
<td>0.66-8.00</td>
<td>Abdollahi et al., 2006</td>
</tr>
<tr>
<td>Parental nutrition in vitamin B₁, B₆, B₆</td>
<td>Spectrofluorimetry</td>
<td>λex 285, λem 324-550</td>
<td>0.01-0.5</td>
<td>Mohammad, 2011</td>
</tr>
<tr>
<td>Pyridoxine and metoclopramide in human plasma</td>
<td>Second derivative fluorescence spectroscopy</td>
<td>Delta lambda=80 nm in methanol</td>
<td>0.1-2.0</td>
<td>El-Enany, 2008</td>
</tr>
<tr>
<td>Vitamin B₆/ tablets</td>
<td>Spectrophotometry, PLC and PCS methods</td>
<td>200-330</td>
<td>-</td>
<td>Dinc, 2010</td>
</tr>
</tbody>
</table>
Table 3: Analytical parameters for HPLC/ electrophoretic methods of pyridoxine (B$_6$) assay

<table>
<thead>
<tr>
<th>Material</th>
<th>Technique</th>
<th>Column</th>
<th>Mobile Phase</th>
<th>Flow Rate ml / min$^{-1}$</th>
<th>Detection (nm)</th>
<th>Concentration range µg ml$^{-1}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B$_6$/chondroitin sulphate sodium, allantoin</td>
<td>Ion-pair HPLC</td>
<td>Alltima C$_{18}$</td>
<td>25 mM NH$_4$H$_2$PO$_4$: CAN (95:5,v/v)</td>
<td>0.5</td>
<td>195, 215, 291, 371</td>
<td>23-1488</td>
<td>Jin et al., 2009</td>
</tr>
<tr>
<td>Vitamin B$_1$ and B$_6$ in parenteral nutrition</td>
<td>HPLC</td>
<td>Bondapak C$_{18}$</td>
<td>Methanol-water (27:73, v/v)</td>
<td>0.35</td>
<td>250, 295</td>
<td>20-90</td>
<td>Ribeiro et al., 2011</td>
</tr>
<tr>
<td>Bnclazine/ tryptophan and vitamin B$<em>6$/B$</em>{12}$ in pharmaceutical formulations</td>
<td>RP-HPLC</td>
<td>RP-C$_{18}$</td>
<td>Methanol-15 mM phosphate buffer (pH 3.0)- 30 mM H$_3$PO$_4$</td>
<td>1.0</td>
<td>290, 280, 360</td>
<td>-</td>
<td>Kuminek et al., 2011</td>
</tr>
<tr>
<td>Isoniazid/B$_6$ formulations</td>
<td>HPLC</td>
<td>ZIC-HILIC</td>
<td>1 mM HCOONH$_4$ (pH 6)- CAN (20:80, v/v)</td>
<td>1.0</td>
<td>-</td>
<td>-</td>
<td>Pasakova et al., 2011</td>
</tr>
<tr>
<td>Pyridoxine in water-soluble vitamins tablets</td>
<td>RP-HPLC</td>
<td>ODS</td>
<td>0.1% HCOOH in water</td>
<td>0.25</td>
<td>Diode assay</td>
<td>-</td>
<td>Chen et al., 2009</td>
</tr>
<tr>
<td>Pyridoxine HCl Allantoin Chondroitin Sulphate Na in eye drops</td>
<td>Ion-pair HPLC</td>
<td>Alltima C$_{18}$</td>
<td>25 mM NH$_4$H$_2$PO$_4$: CAN (95:5, v/v)</td>
<td>2.0</td>
<td>291, 215, 195 nm</td>
<td>23.32-93.28 for B$_6$</td>
<td>Pengfei et al., 2009</td>
</tr>
<tr>
<td>Vitamins B$_1$, B$<em>6$, B$</em>{12}$ in formulations</td>
<td>HPLC</td>
<td>Supelco LC 18 5 µm</td>
<td>0.05 M phosphate buffer:10% methanol: (CH$_3$)$_3$ NH$_2$, pH 3.55</td>
<td>1.0</td>
<td>UV</td>
<td>-</td>
<td>Marszall et al., 2005</td>
</tr>
<tr>
<td>Vitamins B$_1$, B$<em>6$, B$</em>{12}$ in tablets</td>
<td>RP-HPLC</td>
<td>HYPER-IL-BDS C18</td>
<td>0.015% (C$_2$H$_5$)$_3$ NH$_2$, pH 2.7: 0.5 M H$_2$SO$_4$: ACN</td>
<td>1.0</td>
<td>280, 350 nm</td>
<td>-</td>
<td>Markopoulou et al., 2002</td>
</tr>
<tr>
<td>Pyridoxine in B vitamin mixtures</td>
<td>RP-HPLC</td>
<td>LC 18, 5 µm</td>
<td>Methanol-phosphate buffer (10:90, v/v, pH 3.55)</td>
<td>1.0</td>
<td>UV</td>
<td>0.99 ng/ml</td>
<td>Lebiedzinska et al., 2007</td>
</tr>
<tr>
<td>Pyridoxine in plasma</td>
<td>Ion-pair RP-HPLC</td>
<td>C 18 (ODS)</td>
<td>Gradient, acetonitrile 0.5-15% in phosphate buffer, pH 2.16</td>
<td>1.0</td>
<td>Fluorescence λex 328, λem 393</td>
<td>1.0-19 nmol/L</td>
<td>Bispe et al., 2002</td>
</tr>
<tr>
<td>Pyridoxine in multi-vitamin preparations</td>
<td>RP-HPLC</td>
<td>Nova-pack C 18</td>
<td>Methanol-ammonium acetate (95:5, v/v)</td>
<td>2.0</td>
<td>285</td>
<td>-</td>
<td>Moreno et al., 2000</td>
</tr>
<tr>
<td>Pyridoxine, meclizine and buclizine in dosage formulations</td>
<td>RP-LC</td>
<td>-</td>
<td>Acetonitrile -water (80:20 v/v), pH 2.6</td>
<td>1.0</td>
<td>230</td>
<td>0.03-10</td>
<td>Arayne et al., 2010</td>
</tr>
<tr>
<td>Pyridoxine in vitamin B6 forms</td>
<td>HPLC</td>
<td>ZIC-HILIC</td>
<td>1mM HCOONH$_4$: ACN (20:80 v/v)</td>
<td>1.0</td>
<td>UV</td>
<td>-</td>
<td>Pasakova et al., 2011</td>
</tr>
</tbody>
</table>

*Table continued...*
The recovery ranges from 96.1-101.2% for derivative method and 97.0-101.9% for multivariate method (Mohamed et al., 2011).

The determination of pyridoxine HCl in multivitamin preparations using colorimetric, spectrometric absorbance difference and multicomponent spectrometric methods has been carried out. The colorimetric method based on the reaction of pyridoxine HCl with chlorimide reagent and measurement of absorbance at 650 nm has been found to be more accurate than the other methods (Usmanghani et al., 1980).

Spectrofluorimetry
Sorouraddin et al. (2005) have carried out spectrofluorimetric and spectrophotometric assay of melatonin and vitamin B₆ in commercial products using PLS calibration and PCR methods. A spectrofluorimetric method for the assay of pyridoxine and piroxicam involves solid-phase extraction for the separation of the two compounds followed by fluorometric determination (Escandara et al., 2002). The assay of vitamin B₃, B₅ and B₆ in commercial products by synchronous spectrofluorimetry has been performed. The detection limits for pyridoxine are in µg quantities (Garcia et al., 2001). A highly sensitive second-derivatives synchronous fluorimetric method has been applied to the assay of metochlopramide and vitamin B₆ in a binary mixture and syrups. The limit of detection for vitamin B₆ is 0.007 µg/ml and the limit of quantification is 0.02 µg/ml. The assay of these drugs has been carried out in human plasma with recoveries of 90% (El-Enany, 2008).

The development of a flow-through optosensor for the assay of vitamin B₆ has been reported. The sensor is used in conjunction with a monochannel flow-injection analysis system and detection at 385 nm. The sensor gives linear response in a wide range of concentration at the ng level. The RSD for ten assays is less than 0.75% for 0.2-1.0 ml samples (Ruiz-Madina et al., 1998).

Pyridoxine HCl and riboflavin (pH 6, acetate buffer) have been determined by a sensitive fluorimetric method. The RSD of the method ranges from 0.46-1.02% with recoveries of 97.6-101.2% (Mohamed et al., 2011). A net analyte signal standard addition spectrofluorimetric method has been reported for the simultaneous assay of melatonin and vitamin B₆ in pharmaceuticals. The method can determine the analyte in the presence of interfering substances (Asadpour-Zeynali and Bastami, 2010).

Mass spectrometry
Laser depletion mass spectrometry (LDMS) alone and in combination with liquid chromatography has been used for the assay of vitamins in commercial products. These include vitamins A, B₃, B₅, B₆, C and D₃. The ability of this technique to show molecular fragmentation patterns is very helpful in deducing many functions including the

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**Table 3 continued**

<table>
<thead>
<tr>
<th>Material</th>
<th>Technique</th>
<th>Column</th>
<th>Mobile Phase</th>
<th>Flow Rate ml/min</th>
<th>Detection (nm)</th>
<th>Concentration range µg/ml</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxine and B₆ vitamers in cerebrospinal fluid</td>
<td>UPLC - tandem mass spectrometry</td>
<td>Acquity HSS-T3 UPLC</td>
<td>Buffer (CH₃COOH, C₃F₆COOH,ACN)</td>
<td>-</td>
<td>MS</td>
<td>0.03-5.37 nM</td>
<td>van der Ham et al., 2012</td>
</tr>
<tr>
<td>Pyridoxine in almonds</td>
<td>HPLC</td>
<td>Inertsil ODS-3</td>
<td>0.05 M KH₂PO₄ methanol (70:30 v/v)</td>
<td>1.0</td>
<td>265</td>
<td>5.0-50.0</td>
<td>Shen et al., 2005</td>
</tr>
<tr>
<td>B-complex vitamins</td>
<td>Capillary zone electrophoresis (CZE)</td>
<td>Silica capillary</td>
<td>20 mM tetra borate buffer, pH 9.2</td>
<td>-</td>
<td>214</td>
<td>-</td>
<td>Franco et al., 2012</td>
</tr>
<tr>
<td>Pyridoxine in urine</td>
<td>Capillary electrophoresis (CE)</td>
<td>Silica capillary</td>
<td>50 mM sodium tetraborate buffer, pH 10</td>
<td>-</td>
<td>230</td>
<td>1-5</td>
<td>Solangi et al., 2009</td>
</tr>
<tr>
<td>Pyridoxine in urine</td>
<td>CE</td>
<td>Monolithic</td>
<td>5 mM phosphate buffer, pH 4</td>
<td>-</td>
<td>UV</td>
<td>-</td>
<td>Wei et al., 2010</td>
</tr>
<tr>
<td>Pyridoxine and isoniazid in formulations</td>
<td>CE</td>
<td>Silica capillary</td>
<td>50 mM borate buffer, 25 mM SDS, pH 7.8</td>
<td>-</td>
<td>205</td>
<td>1-100</td>
<td>Nemutlu, 2007</td>
</tr>
<tr>
<td>Pyridoxine HCl in drinks</td>
<td>Micellar electrokinetic chromatography</td>
<td></td>
<td>135 mM sodium dodecyl sulphate</td>
<td>-</td>
<td>210</td>
<td>Diode-array detector</td>
<td>0.3-12.5</td>
</tr>
</tbody>
</table>
analysis of vitamins (McMahon, 1985). A method has been reported for the assay of B-complex vitamins in multi-vitamin, multi-mineral products using liquid chromatography and mass spectrometry in the multiple reaction modes (LC/UV/MS-MRM). The samples do not need any treatment prior to analysis (Chen and Woulf, 2007). The content of selected B-complex vitamins including pyridoxine in vitamin supplements is assayed by RP-liquid chromatography-isotope dilution mass spectrometry. The determination of the vitamins has been carried out with a gradient elution and MS/MS detection. The vitamin contents are determined by a comparison of ratios of reciprocal peaks at different masses of the vitamins (Chen et al., 2007).

A new rapid assay of vitamin B_2, B_3, B_6, caffeine and taurine in energy drinks by planar chromatography electro-spray ionization mass spectrometry (ESIMS) has been reported. After chromatographic separation multi-wavelength scanning is performed by fluorescence measurements at 340 nm with excitation at 313 nm for pyridoxine. The recoveries of the vitamins are in the range of 81-106% with a RSD of 0.8-1.5%. Mass confirmation of the vitamins has been achieved by a single quadrupole MS in positive electro-spray ionization mode (Araldi and Morlock, 2006).

The degradation of pyridoxine has been studied using gas analysis-Li^+ ion attachment mass spectrometry. The formation of pyridoxal and o-quinone methide has been observed on the solid-phase degradation of pyridoxine. The life time (t_90, 25 °C) of pyridoxine in nitrogen has been obtained as 1.7x10^2 years by EGA-IAMS (Juhasz et al., 2012). The assay of pyridoxine in a nutritional formula powder has been carried out by LC/IDMS and applied to the study of the stabilities of these vitamins in the powder (Goldschmidt et al., 2010).

van der Ham et al. (2012) have quantified pyridoxine vitamers in biological samples using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Positive ESI has been applied to observe the transitions m/z 170.1→134.1 for pyridoxine and other B_6 vitamers. The concentration range of the vitamin is within 0.03-5.37 nM. The method is suitable for routine analysis using small volumes of samples.

**Chromatographic methods**

Chromatographic methods have found extensive application in the assay of pyridoxine in pharmaceutical formulations. The analytical parameters used for a number of HPLC/electrophoretic methods are given in table 3.

**Thin Layer Chromatography (TLC)**

A TLC densitometric assay of vitamins B_1, B_2 and B_12 in tablets has been carried out using a mobile phase consisting of chloroform: ethanol: water: acetic acid (2: 8: 2: 0.5, v/v). The spots are detected at 242, 291 and 360 nm for thiamine HCl, pyridoxine HCl and cyanocobalamin, respectively. The assay has been performed in the range of 0.1-3.5 µg/spot for all the vitamins (Elzanfaly et al., 2010). A HP-TLC method has been used for stability assay for thiamine and pyridoxine in aged pharmaceutical preparations (Such et al., 1980).

**High Performance Liquid Chromatography (HPLC)**

A large number of HPLC assays have been reported for the determination of pyridoxine in combination with other drugs and B/C vitamins in pharmaceutical preparations, food material and biological fluids. The details of the important HPLC methods are provided in this section.

A HPLC method has been developed for the determination of PLP in biological samples. The loss of PLP is a risk factor for coronary artery disease and is a guide for patient care. The method is rapid and reliable for routine analysis. The standard samples should be protected from light prior to analysis (Deitrick et al., 2001). A reversed phase ion-pair liquid chromatographic determination of vitamins B_1, B_2, B_6 and C has been reported. The analysis of the vitamins is carried out within 20 min. A C_18 column and a mobile phase of methanol: water (15:85, v/v) gives satisfactory results. The peaks are eluted in the order: vitamins B_1, B_2, B_6 and C. Recoveries of the 5 vitamins are in the range of 98.2-102.0%, with confidence limits, ±3 SD, as 1.0-5.5% (Lam et al., 1984).

The automated determination of pyridoxine and other B-vitamins in tablets by RP_18-HPLC has been achieved. The RSD for pyridoxine is 1.6% and the mean recovery is 95.2-103.9% (Holler et al., 2003). A RP-LC method with an amide stationary phase for the assay of B-vitamins has been described. Analysis has been carried out using acetoni-trile-phosphate buffer as mobile phase and a PD detector. Nine vitamins including pyridoxine and other B-vitamins in different nutritional products have been determined (Vinas et al., 2003). Jin et al. (2009) reported an ion-pair HPLC assay for the determination of pyridoxine HCl in eye drop dosage forms with recoveries of 99.01-101.92%.

An isocratic HPLC assay has been used for the determination of pyridoxine kinase activity in biological samples. The products of kinase activity are separated by HPLC and determined spectrometrically (Argoudelis, 1990). A rapid HPLC method for the quality control of commercial products and biological samples containing antihistaminic drugs and pyridoxine has been reported. The recoveries of more than 97.8% have been achieved (Arayne et al., 2010). The stability of vitamins B_1, B_2, B_6 and C in TPN mixtures has been studied by an ion-pair HPLC method using diode array detection for pyridoxine and thiamine (Ribeiro et al., 2011).
The assay of vitamins B1, B6 and B12 in commercial formulations has been achieved by HPLC using isocratic elution with UV and coulometric detection. The limit of detection of pyridoxine is 2.7 ng/ml and the recovery is 99.6-102.7% (Marszall et al., 2005). An optimized RP-HPLC assay of vitamins B1, B6 and hydroxocobalamin chloride in tablets using gradient elution has been performed. The detection for pyridoxine HCl is carried out at 280 nm. The excipients in tablets showed no interference in the assay of the vitamins (Markopoulou et al., 2002). Several other methods for the simultaneous assay of pyridoxine and other vitamins in foods (Lebedzinska et al., 1980; Gregory and Feldstein et al., 1985; Agostini and Godoy, 1997; Kall, 2003; Zafra-Gomez et al., 2006; Blake, 2007), multivitamin blends (Krichmeier and Upton, 1978), pharmaceutical formulations (Moreno and Salvado; 2000; Din et al., 2000) and in plasma (Edwards et al., 1989; Bisp et al., 2002; El-Gindy, 2003), and serum (Rybak and Pfeiffer, 2004) are reported. The assay of vitamins B6, B12, buclizine and tryptophan by HPLC in pharmaceutical formulations has been performed. The RSD values for intra-day and inter-day precision are below 1.82 and 0.63%, respectively, and recoveries range from 98.11 to 101.95% (Kuminek et al., 2011).

Different stationary phases for HPLC-UV have been used for the determination of polar vitamins including pyridoxine, pyridoxal and pyridoxal 5'-phosphate. The best separation of these vitamins has been found using HCOONH4 (pH 6) and ACN (20:80, v/v) and HCOONH4 (pH 3) and ACN (40:60, v/v) as mobile phase and a ZIC-HILIC column. The LC-MS has shown that ZIC-HILIC column is suitable for the assay of vitamin B6 forms (Pasakova et al., 2011).

The water-soluble B-complex and vitamin C present in multivitamin/multimineral dietary supplements have been determined by RP-HPLC-diode array/fluorescence detectors and the MS method. It involves the use of gradient elution, i.e. after 5 min isocratic elution at 100% A (0.1% formic acid in water), and a linear gradient to 50% A and 50% B (0.1% formic acid in acetonitrile) at 15 min (Chen et al., 2009). The B-complex vitamins thiamine, riboflavin, pantothenic acid, and pyridoxine have been separated by a HPLC method using FTIR detector followed by their quantitative determination in mixtures (Li and Brown, 2003).

Capillary Zone Electrophoresis (CZE)/Capillary Electrochromatography (CEC)

A novel CEC technique for the separation and assay of vitamin B analytes including vitamin B6 has been used employing a methacrylate based column. The method has been validated and the linearity curves established with correlation coefficients more than 0.997 and good recovery of the material. The method is used for the assay of pyridoxine in urine samples (Wei et al., 2010). Micellar electrokinetic capillary chromatography (MEKC) has been applied to the assay of isoniazid and vitamin B6 in commercial preparations. The RSD of the method ranges from 0.54 - 2.27% for intra-day precision and from 0.65 - 2.69% for inter-day precision (Nemutlu et al., 2007). MECK has been employed to assay 2-aminoethanesulfonic acid, anhydrous caffeine, thiamine, riboflavin and pyridoxine in a vitamin enriched drink. The separated compounds are detected at 210 nm. Recoveries and precisions of the method are 99.0-101.2%, and 0.4-2.5% RSD, respectively (Okamoto et al., 2002).

An in-capillary enzyme reaction has been applied to assay thiamine, riboflavin, niacinamide and pyridoxine in a vitamin-enriched drink by MEKC. Good linear relationships have been found with correlation coefficient >0.999. The recoveries and precisions ranges are 99.3-101.8%, and 0.1-2.5% RSD, respectively. The results show that this method is suitable for the assay of B vitamins in pharmaceuticals (Okamoto et al., 2003).

The simultaneous assay of seven drugs including vitamins B1, B6 and B12 in pharmaceuticals and urine samples has been carried out by CZE. Calibration plots are linear over at least three orders of magnitude of the analyte concentrations. The RSD of the method is 0.5-2.4% and the recovery of the analyte is >99% (Solangi et al., 2009). Six B-complex vitamins (thiamine, riboflavin, niacinamide, pantothenic acid, pyridoxine and cyanocobalamin) in vitamin supplements have been determined by CZE. The RSDs of the method has been found as 1.08-3.68% (intra-day precision) and 1.26-3.5% (inter-day precision). The method is fast, accurate, simple and inexpensive for the quantitative determination of vitamins (Schreiner et al., 2003).

Electrochemical Methods

Pyridoxine and cyanocobalamin have simultaneously been determined in pharmaceutical preparations by square wave voltammetry with glassy carbon electrode coupled to multivariate calibration tools. Recoveries of the vitamins range from 96.4-100.2% (Hernandez et al., 2003). The voltammetric measurement of vitamin B6 at a carbon paste electrode modified with vanadyl(IV)-Salen complex has been carried out. The method is based on electrochemical oxidation of pyridoxine by cyclic voltammetry. A linear sweep response for vitamin B6 has been found in the range of 4.5x10^-4 to 3.3x10^-3 M with a detection limit of 3.7x10^-5 M. Thiamine has been found to interfere with the analytical signal (Marcos et al., 2004). A flow injection amperometric assay of vitamin B6 by electrocatalytic oxidation at a Prussian blue non-particle-modified carbon ceramic electrode has been reported. The sensor exhibits good linear response for pyridoxine in the range of 5-69 µM and the detection limit of 0.51 µM (Razmi and Rezaei, 2010).
**Enzymatic assay methods**

A radioenzymatic analytical method for the determination of pyridoxal-5'-phosphate (PLP) has been reported. It is based on the incubation of (10^6 cpn, spec. acty. 1.88 Ci/mol) in the presence of the apo-enzyme tyrosine decarboxylase (EC 4.1.1.25) and PLP in 0.1 M phosphate buffer (pH 5.5) at 37°C for 60 min. The decarboxylated metabolite, [3H]-tyramine, is extracted into ethyl acetate and the tritium radioactivity in the sample is determined by liquid scintillation counting. Detection limit of PLP is 0.5 nM. The method is specific and concentrations of PLP in plasma without previous de-proteinisation of the samples can be determined (Camp et al., 2006).

A simultaneous enzymatic assay method for the determination of pyridoxine analogues and pyridoxine-ß-glucoside in human urine has been developed. These compounds are converted enzymatically to a highly fluorescent 4-pyridoxolactone that is analyzed by an isocratic HPLC method. The substances determined included pyridoxine, pyridoxal, pyridoxamine, 4-pyridoxic acid, pyridoxal-5-phosphate and pyridoxine-ß-glucoside (Yagi et al., 2010).

**CONCLUSION**

Vitamins including B₆ (pyridoxine) are micronutrients that are essential for the transformation of energy and regulation of metabolism. A lack of these compounds in diet leads to the manifestations of deficiency diseases. Pyridoxine deficiency in humans largely affects the nervous system resulting in behavioral changes and convulsive seizures. Treatment with pyridoxine is used to improve the deficiency state.

Vitamin B₆ is normally present with other B/C vitamins in pharmaceutical preparations, food materials and biological fluids. The analysis of vitamin B₆ in complex systems requires specific and sensitive methods for its determination. The methods used are based on the spectral characteristics, electrochemical properties and chromatographic behavior of vitamin B₆ and include the UV spectrometric, spectrofluorometric, mass spectrometric, electrochemical, chromatographic, electrophoretic and enzymatic methods. All these methods have been applied to the analysis of pyridoxine in these materials with a variable degree of sensitivity.

UV-spectrometry of binary mixture of pyridoxine and other drugs suffers from the disadvantage of interference from minor impurities. This has been overcome by the application of PLS and PCR methods. The graphical and multivariate calibration/ prediction methods can resolve complex mixtures in µg quantities in the presence of overlapping signals. However, spectrofluorometry has the advantage of determining ng quantities of vitamins mixtures in pharmaceutical preparations.

Mass spectrometer techniques (LDMS, MS/MS, ESIMS, LC/UV/MS-MRM, LC/IDMS, UPLC-MS/MS) have found greater applications in the structural and analytical studies of B-complex vitamins. These techniques have also been used to evaluate the stability of pyridoxine in pharmaceutical materials.

The HPLC is the most widely used technique for vitamin analysis in pharmaceutical preparations, food materials and biological fluids. It has the advantage of resolution of the vitamin mixtures followed by determination at submicrogram level. The sensitivity of the technique is greater with fluorescence detector compared to that of the UV and FTIR detectors. Capillary zone electrophoresis, capillary electro-chromatography and micellar electrokinetic capillary chromatography have also been employed for the assay of vitamin B₆, analytes with good recoveries and precision. Vitamin B₆ vitamers could specially be determined by enzymatic assay voltammetry involves electro-excitation of pyridoxine with a high sensitivity, however, thiamine has been found to interfere with the method. The LC-MS combination methods have facilitated the separation and determination of individual compounds in vitamin preparations. Further progress in these methods would enable the resolution and determination of vitamin B₆ in complex systems with a high degree of specificity and sensitivity.

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