REVIEW

Vitamin B_6: Deficiency diseases and methods of analysis

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Abstract: Vitamin B_6 (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 B_6, pyridoxine deficiency, spectrometric methods, chromatographic methods, electrochemical methods, enzymatic methods.

INTRODUCTION

Vitamin B_6 is a unique vitamin that is involved in the metabolism of proteins, lipids and carbohydrates. The metabolism of amino acids requires enzymes that use pyridioxal phosphate as the co-factor or prosthetic group. In the amino acid decarboxylase reaction that leads to the formation of monoamine neurotransmitters, vitamin B_6 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 B_6 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 B_6 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 B_6 generically refers to all these chemically related compounds (Dakshinamurti *et al.,* 2007). However, pyridoxine is the predominantly used form of vitamin B_6 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 B_6 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

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significance in relation to neurological disorders of vitamin B_6 deficiency (Dakshinamurti *et al.,* 2007).

There are difference in the distribution of 3,4-dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophan (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 pyridoxine-deficient rats having considerable loss in PLP and serotonin in relation to vitamin B_6-replete controls (Siow and Dakshinamurti, 1990).

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_6 has been found to improve the cardiovascular function in rats. Treatment of hypertensive vitamin B_6-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_6, 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_6 inhibits oxidative stress due to Cu (II)-ß-amyloid (Aß)-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_6-dependent epilepsy responds to intravenously administered vitamin B_6. The newborns with seizures should be treated with vitamin B_6 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_6-related seizures and their dependency is suppressed by a high-dose treatment of vitamin B_6 (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_6-dependent epilepsy (Footitt *et al,*. 2011). Oxalate, a marker of vitamin B_6 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_6) deficiency diseases

Disease	Dosage/Doses	Reference
Diabetes	-	Abraham *et al*., 2010
B_6 deficiency in neonates	-	Ribeiro *et al*., 2011
Autism	-	Kaluzna *et al*., 2011
Schizophrenia, Alzheimer's disease, Parkinson's disease and epilepsy	-	Disalvo *et al*., 2012
Wilson's disease	-	Kleine *et al*., 2012
Oxaliplatin-induced neurotoxicity	5 mg	Garg and Ackkland, 2011
Pyridoxine-dependent epilepsy (PDE)	-	Bok *et al*., 2010,
B_6 related epilepsy during childhood	-	Wang *et al*., 2007
Doxorubicin-related hand- foot syndrome in gynecologic oncology	200 mg	von Gruenigen *et al*., 2010
Children receiving TB chemotherapy	4.20 mg/kg	Cilliers *et al*., 2010
Intractable epilepsy	50-100 mg	Gospe, 2002
B_6-related seizures	100-400 mg	Ohtahara, 2011
Down syndrome	-	Baggot *et al*., 2008
B_6 dependent seizures	13.5 mg/kg	Goto *et al*., 2001
Polyneuropathy	30 mg	Moriwaki *et al*., 2000
Carpal tunnel syndrome	-	Keniston *et al*., 1997
Neuropathy	1000 mg	Bendich and Choen, 1990

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.

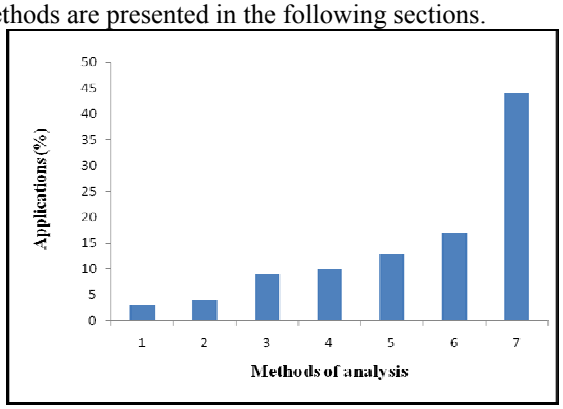

Fig. 1: Applications of analytical methods for the assay of pyridoxine in pharmaceutical preparations and biological fluids. 1. Enzymatic methods 2. electrochemical methods 3. mass spectrometry 4. electrophoresis 5. spectro-fluorimetry 6. spectrophotometry 7. HPLC.

Spectrometric methods

UV spectrophotometric and spectrofluorimetric methods have widely been used for the assay of pyridoxine in binary mixtures, B-vitamin/multivitamin preparations and biological fluids. The analytical parameters for the assay of pyridoxine in these materials in selected methods are reported in table 2.

UV spectrophotometry

UV spectrophotometric methods have been introduced for the assay of binary mixtures of vitamin B_6 and either metoclopramide HCl or meclozine HCl in commercial tablets and syrups. The analysis of these mixtures has been achieved by partial least squares (PLS) and principle component regression (PCR) applied to the zero- and first-order spectra, respectively (El-Gindy, 2003). The analysis of vitamin B_6 and melatonin mixtures using second- and third-derivative UV spectrometry, and the zero-crossing technique, has also been reported. The lower limits of detection at the 95% confidence level are 0.26 µg/ml for pyridoxine and 0.05µg/ml for melatonin. The method has been applied to the assay and vitro-dissolution studies of these compounds in tablet formulation (Surmeian and Aboul-enein, 1998).

An assay method has been developed for the determination of binary mixtures of vitamin B_6 and thiamine in vitamin combination using UV spectrometry and classical least square and newly developed genetic algorithms. The sample data set contain the UV spectra of 30 synthetic mixtures of these vitamins and 10 tablets containing each vitamin. The standard error of calibration and the standard error of prediction for the analytical data have a range of <0.01 and 0.43 µg/ml, respectively. The later values for the tablets are in the range of 2.91 and 11.51 mg/tablets (Ozdemir and Dinc, 2004). The content of pyridoxine HCl in two-component pharmaceutical preparations containing various magnesium compounds has been determined by differential spectrometry. The analysis of the absorption spectra and their first- and second-derivatives indicated the appropriate analytical wavelengths as 290 nm, 302 nm and 308 nm, respectively. The magnesium compounds present included hydroaspartate, lactate and lactogluconate (Muszalska *et al.,* 2011).

Graphical and multivariate calibration-prediction methods have been used to determine pyridoxine and other drugs in different dosage forms in various concentration ranges (Bautista *et al.,* 1996). A new spectrophotometric method has been developed for the assay of vitamin B_1, B_2, B_6 and folic acid using their absorbance measurements and treatment by PLS. The detection limit for vitamin B_6 is 0.45 µg/ml. The method can resolve complex mixtures of the vitamins in the presence of strongly overlapped signals (Aberasturi *et al.*, 2002). A comparison of two spectrometric methods for the determination of vitamin B_1 and B_6 in a vitamin mixture has been made. The A (1%, 1 cm) values of the vitamins have been determined by absorbance measurement at 246.8 and 290.5 nm. The concentrations of both compounds were determined by solving the matrix equations using A (1%, 1 cm) values at the two wavelengths. In another method the determinations have been carried out in the derivative of the ratio spectra at selected wavelengths for the two vitamins (Din *et al.,* 2000). A chemometric assay of vitamin B_6 and isoniazid in tablets by PLS and PCR has been performed. Both calibration models have been established by the relationship between the concentration data matrix and the absorbance data matrix. The recovery results from both methods have 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 B_6 has been found in the range of 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 (B_6) assay

Material	Technique	Wavelength (nm)	Concentration range (μg ml^{-1})	Reference
Vitamin B_6/ melatonin mixture	Spectrophotometry, partial least squares (PLS) calibration and Principle component regression (PCR) analysis	278 and 310	1-24	Sorouraddin *et al*., 2005
Vitamin B_6/ diazepam mixture	Spectrophotometry multiple linear regression and partial least-squares regression analysis	242 and 290	1.4-12.0	Bautista *et al*., 1996
Pyridoxine in B Vitamin mixture	Spectrophotometry partial least squares regression analysis	-	1.01-16.2	Aberasturi *et al*., 2002
Pyridoxine in binary drug mixtures of tablets and syrups	Spectrophotometry, PLS and PCR analysis	Zero and first order UV spectra	-	el-Gindy, 2003
Pyridoxine HCl and thiamine HCl in pharmaceutial preparations	Spectrophotometry Zero order spectra and ratio spectra derivative	Zero order spectra 246.8 and 290.5 ratio spectra derivative B_6 297.8/309.5 and B_1 245.6/257.7	8-40	Din *et al*., 2000
Pyridoxine and melatonin in tablets	Derivative UV spectrophotometry Zero-crossing technique	UV	pyridoxine 2-10 melatonin 0.5-3.5	Surmeian and Aboul-Enein, 1998
B_6 in B vitamin preparations	Spectrophotometry	290	0.50-8.0	Rocha *et al*., 2003
Pyridoxine in water soluble vitamins	Spectrophotometry derivative and multivariate methods	200-500	2.5-90	Mohamed *et al*., 2011
Pyridoxine HCl in pharmaceutical preparations	Differential spectrophotometry	290	-	Muszalska *et al*., 2011
Pyridoxine HCl and thiamine HCl in vitamin preparations	Spectrophotometry least squares and multivariate calibration methods	200-330 at 0.1 nm intervals	8-40	Ozdemir and Dinc, 2004
B_6 in water soluble vitamins	Spectrophtometry, derivative and multivariate method	200-500	2.5-90	Mohammad, 2011
Vitamins B_1, B_6, B_{12}	TLC densitometric method	242, 291, 360	0.1-1.3/spot	Elzanfaly, 2010
Vitamin B_6/ melatonin mixture	Spectrofluorimetry	λex 285, λem 324-550	0.04-4.0	Sorouraddin *et al*., 2005
Pyridoxine, peroxicam mixture	Spectrofluorimetry	λex 290-340, λem 370-560	0.66-8.00 for B6	Abdollahi *et al*., 2006
Pyridoxine in B Vitamins	Spectrofluorimetry	λex 295, λem 385	0.05-1.8 ng/ml	Ruiz-Madina *et al.*, 1998
Pyridoxine in presence of peroxicam	Spectrofluorimetry	λex 315, λem 465	0.66-8.00	Abdollahi *et al*., 2006
Parental nutrition in vitamin B_1, B_2, B_6 mixtures	Spectrofluorimetry	λex 285, λem 324-550	0.01-0.5	Mohammad, 2011
Pyridoxine and metoclopramide in human plasma	Second derivative fluorescence spectroscopy	Delta lambda=80 nm in methanol	0.1-2.0	El-Enany, 2008
Vitamin B_6/ tablets	Spectrophtometry, PLC and PCS methods	200-330	-	Dinc, 2010

Table 3: Analytical parameters for HPLC/ electrophoretic methods of pyridoxine (B_6) assay

Material	Technique	Column	Mobile Phase	Flow Rate ml / min^{-1}	Detection (nm)	Concentr-ation range µg ml^{-1}	Reference
B_6/chondroitin sulphate sodium, allantoin	Ion-pair HPLC	Alltima C_{18}	25 mM $NH_4H_2PO_4$: CAN (95:5,v/v)	0.5	195, 215, 291,371	23-1488	Jin *et al*., 2009
Vitamin B_1 and B_6 in parenteral nutrition	HPLC	Bondapak C_{18}	Methanol-water (27:73, v/v)	0.35	250, 295	20-90	Ribeiro *et al*., 2011
Bnclazine/ tryptophan and vitamin B_6/B_{12} in pharmaceutical formulations	RP-HPLC	RP-C_{18}	Methanol-15 mM phosphate buffer (pH 3.0)- 30 mM H_3PO_4	1.0	290, 280, 360	-	Kuminek *et al*., 2011
Isoniazid/B_6 formulations	HPLC	ZIC-HILIC	1 mM $HCOONH_4$ (pH 6)- CAN (20:80, v/v)	1.0	-	-	Pasakova *et al*., 2011
Pyridoxine in water-soluble vitamins tablets	RP-HPLC	ODS	0.1% HCOOH in water	0.25	Diode assay	-	Chen *et al*., 2009
Pyridoxine HCl Allantoin Chondroitin Sulphate Na in eye drops	Ion- pair HPLC	Alltima C_{18}	25 mM $NH_4H_2PO_4$: CAN (95:5, v/v)		291, 215, 195 nm	23.32-93.28 for B_6	Pengfei *et al*., 2009
Vitamins B_1, B_6, B_{12} in formulations	HPLC	Supelco LC 18 5 µm	0.05 M phosphate buffer:10% methanol: $(CH_3)_3$ NH_2, pH 3.55	1.0	UV	-	Marszall *et al*., 2005
Vitamins B_1, B_6, B_{12} in tablets	RP-HPLC	HYPERS-IL-BDS C18	0.015% $(C_2H_5)_3$ NH_2, pH 2.7: 0.5 M H_2SO_4: ACN	1.0	280, 350 nm	-	Markopou lou *et al*., 2002
Pyridoxine in B vitamin mixtures	RP-HPLC	LC 18, 5 µm	Methanol-phosphate buffer (10:90, v/v, pH 3.55)	1.0	UV	0.99 ng/ml	Lebiedzin ska et al., 2007
Pyridoxine in plasma	Ion-pair RP-HPLC	C 18 (ODS)	Gradient, acetonitrile 0.5-15% in phosphate buffer, pH 2.16	1.0	Fluoresce nce λex 328, λem 393	1.0-19 nmol/L	Bisp et al., 2002
Pyridoxine in multi-vitamin preparations	RP-HPLC	Nova-pack C 18	Methanol-amonium acetate (95:5, v/v)	2.0	285	-	Moreno et al., 2000
Pyridoxine, meclizine and buclizine in dosage formulations	RP-LC	-	Acetonitrile -water (80:20 v/v), pH 2.6	1.0	230	0.03-10	Arayne et al., 2010
Pyridoxine in vitamin B6 forms	HPLC	ZIC-HILIC	1mM $HCOONH_4$-ACN (20:80 v/v)	1.0	UV	-	Pasakova et al., 2011

Table continued…

Table 3 continued,

Material	Technique	Column	Mobile Phase	Flow Rate ml / min-1	Detection (nm)	Concentr-ation range µg ml-1	Reference
Pyridoxine and B_6 vitamers in cerebrospinal fluid	UPLC - tandam mass spectrom-etry	Acquity HSS-T3 UPLC	Buffer (CH_3COOH, C_3F_7COOH,ACN)	-	MS	0.03-5.37 nM	van der Ham *et al*., 2012
Pyridoxine in almonds	HPLC	Inertsil ODS-3	0.05 M KH_2PO_4-methanol (70:30 v/v)	1.0	265	5.0-50.0	Shen *et al*., 2005
B-complex vitamins	Capillary zone electropho-esis (CZE)	Silica capillary	20 mM tetra borate buffer. pH 9.2	-	214	-	Franco *et al*., 2012
Pyridoxine in urine	Capillary electropho-resis (CE)	Silica capillary	50 mM sodium tetraborate buffer, pH 10	-	230	1-5	Solangi *et al*., 2009
Pyridoxine in urine	CE	Monolithic	5 mM phosphate buffer, pH 4	-	UV	-	Wei *et al*., 2010
Pyridoxine and isoniazid in formulations	CE	Silica capillary	50 mM borate buffer, 25 mM SDS, pH 7.8	-	205	1-100	Nemutlu, 2007
Pyridoxine HCl in drinks	Micellar electrokin-etic chromate-graphy		135 mM sodium dodecyl sulphate	-	210 Diode-array detector	0.3-12.5	Okamoto *et al*., 2002

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 B_6 in commercial products using PLS calibration and PCR methods. A spectroflourimetric 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 B_1, B_2 and B_6 in commercial products by synchronous spectroflourimetry 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 metochlopromide and vitamin B_6 in a binary mixture and syrups. The limit of detection for vitamin B_6 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 B_6 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 spectrofluoremetric method has been reported for the simultaneous assay of melatonin and vitamin B_6 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, B_1, B_2, B_6, C and D_3. The ability of this technique to show molecular fragmentation patterns is very helpful in deducing many functions including the

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 planner 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 (Aranda 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 ^oC) 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_6 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_3, 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 acetonitrile-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 B_1, B_6 and B_{12} in commercial formulation 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 B_1, B_6 and hydroxocobolamine 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 (Lebiedzinska *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 B_6, B_{12}, 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, pyridoxial and pyridoxial 5'-phosphate. The best separation of these vitamins has been found using $HCOONH_4$ (pH 6) and ACN (20:80, v/v) and $HCOONH_4$ (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 B_6 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 Electrochormatography (CEC)

A novel CEC technique for the separation and assay of vitamin B analytes including vitamin B_6 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 B_6 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 B_1, B_6 and B_{12} 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 B_6 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 B_6 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 B_6 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 cpm, 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-pyidoxolactone 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 B_6 (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 B_6 is normally present with other B/C vitamins in pharmaceutical preparations, food materials and biological fluids. The analysis of vitamin B_6 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 B_6 and include the UV spectrometric, spectrofluoremetric, 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 B_6 analytes with good recoveries and precision. Vitamin B_6 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 B_6 in complex systems with a high degree of specificity and sensitivity.

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