Vitamin B6 HPLC Fluorescence Response Enhanced by Post Column Photochemical UV Irradiation

Henry Joshua
Aura Industries Inc., 545 8th Ave., New York, NY, 10018, USA, 212-290-9190
(Joshua@aura-inc.com)

Abstract
A simple, reliable, sensitive and selective high performance liquid chromatographic method for the determination of vitamin B6 has been developed using post-column photolytic derivatization with 245 nanometer uv light. The method allows for the confirmation of analyte identity by the simple expedient of performing the analysis with and without irradiation. The simplicity of the apparatus (no moving parts) and technique provides significant advantages over methods using post-column chemical derivatization.

Introduction
Vitamin B6 whose primary biologically active form is pyridoxal 5'-phoshate (PLP) is a coenzyme in numerous biologically vital processes. Sensitive quantitative analysis is important to determine the level of PLP in serum and milk. The fluorescence method of detection is quite sensitive for compounds with natural fluorescence. PLP in its native form has little if any fluorescence. Rybak and Pfeiffer (1) have reported a method for the clinical analysis of PLP and 4-pyridoxic acid in human serum by reverse-phase HPLC with chlorite postcolumn derivatization. Gatti and Gioia (2) reported that PLP fluorescence response was enhanced by post-column photochemical conversion. We now confirm the use of oxidative photolytic post column derivatization using PHRED™ (photochemical reactor for enhanced detection)(Fig. 1) to enhance the fluorescence response of PLP.

Discussion
There are important advantages of the PHRED™ method over the Rybak method. The PHRED™ method does not require a second pump for the reagent delivery, does not require a thermostated reactor coil with its oven, and does not require a corrosive and odoriferous chemical reagent (bleach). PHRED™ is a compact unit connected between the outlet of a HPLC column and the detector (Fig. 2). It is widely used in many applications such as the analysis of aflatoxins (3,4).

Materials and methods
Chromatography was performed using a MetaChem in-line solvent degasser, a Hitachi L7100 HPLC pump, a VALCO motorized injection valve with a 10 microliter loop, a Supelco Discovery HS C18 3Micron, 4,6mm ID x 15 cm long HPLC column thermostated at 40 degrees

Centigrade with a CJB 10 column jacket (Aura Industries, Inc.) with a photochemical reactor PHRED™ (Aura Industries, Inc., NY, NY) equipped with a 254 nm UV lamp and a knitted reactor coil, 0.25 mm ID x 25 meter long (KRC 25-25, Aura Industries, Inc.) an Applied Biosystems 980 Fluorescence detector (325nm excitation. >417 nm emission) and a SRI data acquisition and "Peak Simple" integration system.

Eluent: 50 mM sodium phosphate (pH 3.4) containing 0.2 % acetonitrile at 0.8 ml/min. Injection solution : 1 millimolar PLP (Sigma –Aldrich).

Results

We compared 2 chromatograms: one with the UV lamp on (Fig. 3) and one with the lamp off (Fig. 4). Both were otherwise identical. The chromatogram with the light off had only small peaks at 8.79 and 10.71 minutes with integrated areas of 74.35 and 17.61 respectively. The chromatogram with UV light on in contrast had a very substantial peak at 10.67 min with an integrated area of 20595. Thus the 10.70 minute peak in the light off chromatogram (Fig. 4) has increased 1169 fold due to the photolysis as shown in the light on chromatogram (Fig. 3). We can estimate the limit of detection (LOD) considering that the noise level is circa .25 millivolt and the LOD should consequently be 0.75 millivolt. Since a 10 microliter injection of a 1 millimolar solution gives a response with a peak height of 708 millivolt the LOD should be circa 0.1 micromolar. Using larger injections would increase the sensitivity of the analysis.

Conclusions

Post column oxidative photolytic derivatization of PLP allows for a highly sensitive and selective method of analysis without the need of chemical derivatization.

Henry Joshua, Ph.D.
Phone: 212-290-9190
Fax: 212-290-9191
Website: www.aura-inc.com

Aura Industries, Inc.
545 Eighth Ave., Suite 5 W
New York, NY 10018
E-mail: info@aura-inc.com

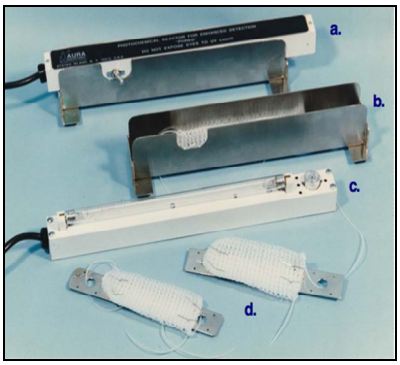

Figure 1. a. Aura Industry PHRED™ Photochemical Reactor for Enhanced Detection unit. b. Stainless steel bottom casing with knitted reactor coil inside. c. Lamp holder outfitted with a 254 nm low-pressure mercury lamp. d. 2 polished support plates displayed with attached knitted reactor coils which when assembled fit inside the stainless steel bottom casing (b) and the lamp holder (c) fits snuggly on top to reduce excess radiation to form a completed unit (a).

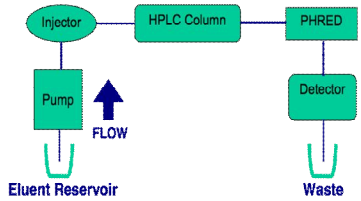

Figure 2. Schematic of Post-Column Photochemical Derivatization Set-Up

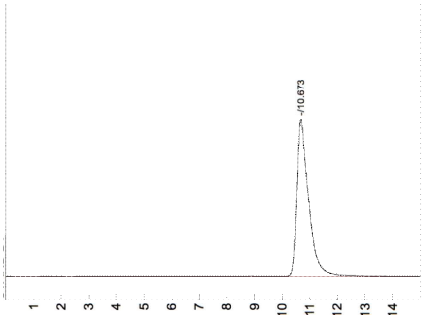

Figure 3. Chromatogram of Vit B6 with PHRED light on. (Scale 2002 millivolt)

Henry Joshua, Ph.D.
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Fax: 212-290-9191
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E-mail: info@aura-inc.com

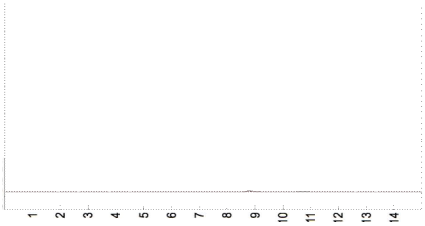

Figure 4. Chromatogram of Vit B6 with PHRED light off. (Scale 2002 millivolt)

Literature cited

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