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Short communication

Use of chlorite to improve HPLC detection of pyridoxal 5′-phosphate

Karen L. Ericson*, J. Dennis Mahuren, Yvonne M. Zubovic, Stephen P. Coburn

Indiana University-Purdue University, Fort Wayne, 2101 East Coliseum Blvd., Fort Wayne, IN 46805-1499, USA

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Abstract

The sensitivity of fluorescent detection of the biologically active form of Vitamin B-6, pyridoxal 5′-phosphate (PLP), in biological samples has been improved approximately four-fold by adopting chlorite as a post-column derivatization reagent (instead of bisulfite) in high-performance liquid chromatography (HPLC) separation. Chlorite oxidizes PLP to the more fluorescent 4-pyridoxic acid 5′-phosphate, and avoids the toxicity and heating of the cyanide procedure. Detection of another major metabolite, 4-pyridoxic acid (4-PA), is not effected. Detection of pyridoxal (PL) is slightly lowered due to eluting at a lower pH.
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1. Introduction

Pyridoxal 5′-phosphate (PLP) is the biologically active and least fluorescent of the seven B-6 vitamers; the other primary vitamers in plasma include 4-pyridoxic acid (4-PA) and pyridoxal (PL). Derivatization of PLP is required for fluorescent detection by HPLC separation methods (cation-exchange or reversed phase); generally utilized methods for derivatization include bisulfite [1], cyanide methods [2,3] and semicarbazide [4]. The chlorite oxidation technique of Lindgren and Nilsson [5] offers the potential to convert PLP to the more fluorescent 4-pyridoxic acid 5′-phosphate while avoiding the heating and toxicity of the cyanide procedure. This work has been verified by Rybak and Pfeiffer [6].

2. Experimental

The post-column chlorite reagent should be made fresh daily by adding 10 mg sodium chlorite ($NaClO_2$; Aldrich Chemical) per mL deionized water; the solution should be clear and colorless. If needed, 0.5 mg sulfamic acid (NH_2SO_3H; Aldrich Chemical) per mL reagent may be added as a chlorine scavenger to minimize baseline shift. If the solution is yellow (with or without sulfamic acid), it will not be effective and should be re-made. Flow rate for the post-column reagent is 1.8 mL/h as delivered by Harvard Apparatus Syringe Pump, Model 975 (South Natick, MA). The detection of vitamers is enhanced if the mixing coil following delivery of the post-column reagent is at least 100 μL. The presence of the post-column reagent in the eluent can be verified by formation of a yellow color when the eluent is mixed with aqueous 1 M potassium iodide.

3. Results/discussion

In 10 standard runs containing 19 ng PLP (304 nmol/L), 10 ng 4-PA (223 nmol/L) and 11 ng PL (210 nmol/L), the average signal-to-noise ratio (S/N) for PLP was 572:1 for the chlorite oxidation and 142:1 for the bisulfite oxidation; the S/N for chlorite was 4.03 times higher than the bisulfite. The S/N ratios for 4-PA were 435:1 and 303:1 (chlorite S/N was 1.43 that of bisulfite) and for PL were 55:1 and 101:1 (chlorite S/N was 0.54 that of bisulfite).

Using one normal (unsupplemented) human serum, the average PLP concentration in five assays was 90 nmol/L

* Corresponding author. Tel.: +1 260 481 5427; fax: +1 260 481 6070.
E-mail address: ericsonk@ipfw.edu (K.L. Ericson).

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with chlorite and 78 nmol/L with bisulfite derivatization (S.D. = 8.5 nmol/L; CV = 10.2%), however, the S/N ratio was four- to five-fold higher with the chlorite method, supporting the premise that lower PLP levels could be detected with the chlorite method. In five additional runs of the same normal human plasma, spiking the sample with an additional 45 nmol/L PLP (134 nmol/L total) and using the chlorite derivatization method, the recovery rate was 105% (S.D. = 4.2%; CV = 3.1%). The same samples were spiked with 56 nmol/L 4-PA and 53 nmol/L PL for recovery rates for 4-PA of 100% (S.D. = 5%; CV = 8.9%) and for PL of 95% (S.D. = 6%; CV = 11.3%).

Eleven additional, random human samples (concentration ranges: PLP = 16–334 nM; 4-PA = 6–190 nM; PL = 39–274 nM) were analyzed with both the chlorite and bisulfite methods. Because the S/N ratio for each method is clear-cut, peak area can be used to calculate vitamer concentration, based upon peak area for standard concentration. Designating the bisulfite method as the "standard" method and the chlorite method and the "experimental" method, results of peak area for each method (peak area being directly proportional to analyte concentration) were compared utilizing the Deming regression ([experimental method] versus [standard method]). The Deming regression was used to note if any clinical or statistical difference existed in vitamer detection (as measured by peak area) of standards or samples. Sample peak areas were changed with the different methods, but the standard peak areas were altered to the same degree, ultimately giving the same analyte concentration by either method. The regression equation for PLP had a slope of 4.0376 with a 95% confidence interval of 1.4337 to 6.6415; for 4-PA the slope was 1.0620 (with a 95% confidence interval of 0.6581 to 1.4659), and the regression for PL had a slope of 0.6712 with 95% confidence interval of −0.1975 to 1.5399.

These statistical results support the approximate four-fold increase in sensitivity of the chlorite method over the bisulfite method for PLP, equal sensitivity for 4-PA, and, while slightly less sensitive for PL, the 95% confidence interval indicates that this sensitivity alteration for PL is probably not clinically significant. This lowered sensitivity of the chlorite method for PL may be due to the lower pH of the unbuffered chlorite reagent compared to buffered bisulfite reagent; lower pH lessens the fluorescence of PL. These results were corroborated by the Bland & Altman plot ([standard − experimental] versus [average result of the two methods]) plots for the same sample peak area results.

Representative chromatograms of both bisulfite and chlorite standard runs are shown in Fig. 1. The detection limit for PLP in bisulfite-derivatized samples was previously reported as 0.3 ng injected (4.8 nmol/L plasma) [1]. Using this chlorite derivatization with our published cation-exchange method [1], the detection limit for PLP is now about 0.1 ng injected or 1.6 nmol/L plasma. Samples that would have low (but detectable) PLP concentration by the chlorite methods would be likely undetectable by the bisulfite method. Our PLP detection limit using chlorite is comparable to the 2.1 nmol/L recently reported by Talwar et al., using semicarbazide derivatization [4].

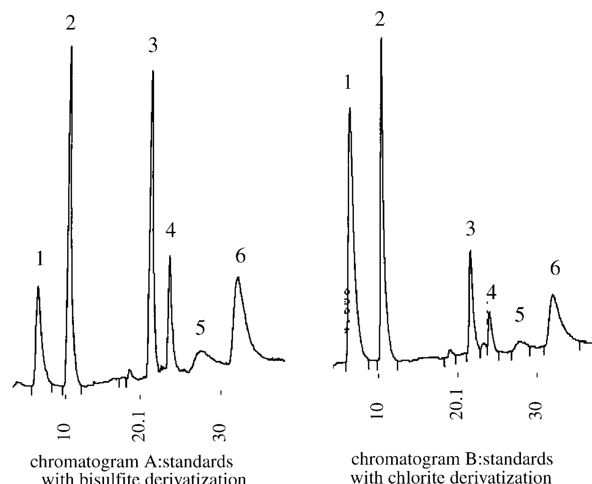

Fig. 1. Chromatogram of B_6 vitamer standards comparing bisulfite vs. chlorite derivatization using cation-exchange chromatography: peak 1, pyridoxal phosphate; peak 2, pyridoxic acid; peak 3, pyridoxamine phosphate hydrochloride; peak 4, pyridoxal hydrochloride; peak 5, pyridoxine hydrochloride; and peak 6, pyridoxamine hydrochloride. Other small peaks shown are solvent change peaks.

While we employ this cation-exchange HPLC method with Vydac 401 TP resin, which is no longer available, we have found this chlorite post-column reagent provides comparable results utilizing Phenosphere strong cation-exchange (SCX) silica-based material, 80 Å pore size, 10 μm particle size, 250 mm × 4.6 mm column (Phenomenex, Torrance, California, USA) with a guard column 50 mm × 4.6 mm of the same material.

In the cyanide derivatization method [2,3], pyridoxal (PL) is oxidized to 4-pyridoxolactone; unfortunately, the lactone's fluorescence is maximal at pH 9–10 and markedly decreases at lower pH [7]. This might explain why acidic, isocratic cyanide methods fail to detect PL [2,3]. In our cation-exchange procedure [1], pyridoxal elutes about pH 6, while chlorite oxidation of PL would require a lower pH. However, even though pyridoxal is not oxidized in our cation-exchange method, our method routinely quantitates pyridoxal in human plasma at this lower pH; it is not clear why the reverse-phase method of Bisp et al. [8], employing bisulfite derivatization, had only 61% recovery for pyridoxal.

4. Conclusion

The sensitivity of detection of pyridoxal 5′-phosphate in biological samples has been improved approximately four-fold by adopting chlorite as a post-column derivatization reagent which oxidizes the PLP to the more fluorescent 4-pyridoxic acid 5′-phosphate.

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References

[1] J.D. Mahuren, S.P. Coburn, J. Nutr. Biochem. 1 (1990) 659.
[2] C.J. Bates, K.D. Pentieva, N. Matthews, A. MacDonald, Clin. Chim. Acta 280 (1999) 101.
[3] A.L. Bailey, A.J. Wright, S. Southon, Eur. J. Clin. Nutr. 53 (1999) 448.
[4] D. Talwar, T. Quasim, D.C. McMillan, J. Kinsella, C. Williamson, D.S. O'Reilly, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 792 (2003) 333.
[5] B.O. Lindgren, T. Nilsson, Acta. Chem. Scand. 27 (1973) 888.
[6] M. Rybak, C. Pfeiffer, Anal. Biochem. 333 (2004) 336.
[7] Z. Tamura, S. Takanashi, Methods Enzymol. 18A (1970) 471.
[8] M.R. Bisp, B.M. Vakur, E.M. Heinsvig, M.A. Kall, E. Nexo, Anal. Biochem. 305 (2002) 82.