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Note

Analysis of vitamin B_6 vitamers in plasma by cation-exchange high-performance liquid chromatography

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In recent years, high-performance liquid chromatography (HPLC) has found increasing application in the analysis of vitamin B_6 vitamers, namely pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), their phosphorylated analogues and pyridoxic acid (PA) [1–6]. Of the published ion-exchange HPLC methods for simultaneous analysis of all major forms of vitamin B_6 in human plasma [1, 2], the cation-exchange method of Coburn and Mahuren [1] offers the advantages of shorter analysis times and single-column operation over anion-exchange [2]. However, as the authors point out, the internal standard originally reported in this method [1] (5-chloroanthranilic acid) is not optimal as it must be added just prior to the HPLC analysis. It is thus used only to monitor the quantitation of the HPLC system and cannot be left to stand in the sample for an extended period of time [1].

The aims of the present study were (a) to evaluate the cation-exchange HPLC assay published by Coburn and Mahuren [1] using 4′-deoxypyridoxine 5′-phosphate as an internal standard, (b) to compare the accuracy of this method of pyridoxal 5′-phosphate (PLP) analysis with the established tyrosine decarboxylase apo-enzyme method [8] and (c) to report on various problems encountered during the development of this assay in our laboratory, in particular the importance of the method of blood collection and the effects of storage of standard mixtures of the vitamin B_6 vitamers.

EXPERIMENTAL

Chemicals

Crystalline vitamin B_6 standards [PN·HCl , PM·2HCl, PL·HCl, pyridoxamine 5′-phosphate hydrochloride (PMP·HCl), PA, PLP and 4′-deoxypyridox-

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ine 5′-phosphate (DPNP; used as internal standard] were purchased from Sigma (St. Louis, MO, U.S.A.). Analytical-grade hydrochloric acid was from Riedel-de-Haën (Seelze, F.R.G.) and pro analysi grade orthophosphoric acid and buffer salts were from Merck (Darmstadt, F.R.G.). Prior to use, distilled water was passed through a Milli-Q water purification system from Millipore (Bedford, MA, U.S.A.).

Methods

The HPLC assay system with post-column derivatization, based on the method of Coburn and Mahuren [1], and the preparation of vitamer standard solutions have been previously described [7], as has the analysis of plasma PLP by the tyrosine decarboxylase apo-enzyme method [8].

Samples

Plasma samples were prepared for cation-exchange HPLC analysis as follows. To plasma (0.5 ml) were added 0.01 *M* hydrochloric acid (0.25 ml) and internal standard solution (20 μl of water solution of DPNP equivalent to 125 ng of DPNP). Plasma proteins were precipitated with 40% trichloroacetic acid (0.25 ml). After standing in the dark for 5 min the precipitate was spun down at 8700 *g* in a Microfuge centrifuge (Beckman, Palo Alto, CA, U.S.A.). The supernatant was withdrawn, extracted twice with diethyl ether (8 ml), purged with nitrogen to remove excess diethyl ether and injected (50–500 μl) onto the HPLC column.

The HPLC assay levels for PLP were compared with those obtained using the tyrosine decarboxylase apo-enzyme method. Of the 111 individual plasma samples analysed, 84 samples were drawn from children with the nephrotic syndrome on B_6 supplements [four males, five females, age 7.9 ± 3.0 years (mean $\pm$S.D.)] and 27 samples were drawn from normal healthy laboratory volunteers [thirteen males, fourteen females, age 30.8 ± 9.9 years (mean $\pm$S.D.)]. None of the normal volunteers was taking vitamin supplements or oral contraceptives. Vitamer levels found in these normal plasmas were compared with normal values reported by other workers.

RESULTS AND DISCUSSION

The HPLC method of Coburn and Mahuren [1] was found to be a practical method for analysis of vitamin B_6 compounds in human plasma. However, the use of an internal standard to monitor sample clean-up, extraction and HPLC quantitation was considered preferable to their use of 5-chloroanthranilic acid to monitor the HPLC stage only. 4′-Deoxypyridoxine 5′-phosphate was selected as it satisfied a number of criteria for use as an internal standard, i.e. it has similar chemical structure and fluorescent properties to the B_6 vitamers of which it is a non-naturally occurring analogue; it elutes in a clear region of the chromatogram between the other peaks of interest; and it does not react with other components. Of the other compounds considered, 4′-deoxypyridoxine was found to co-elute with PM on certain columns, and 3-hydroxypyridine, used by Vanderslice et al. [2] for anion-exchange HPLC, was not fully resolved from PN.

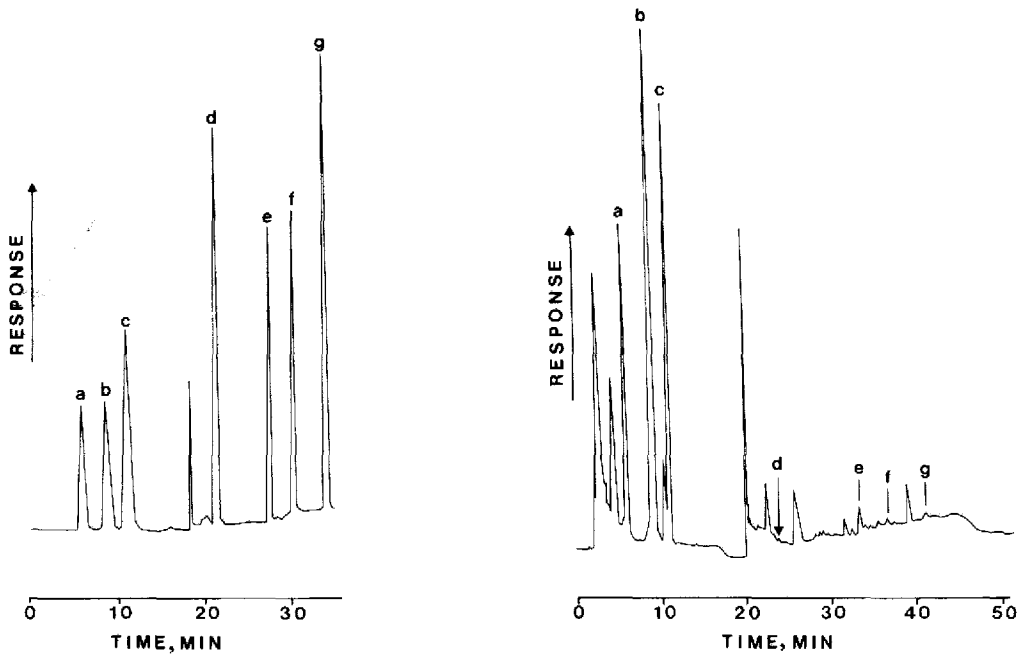

Fig. 1. Chromatographic separation of vitamin B_6 standards (500-μl injection of 4 ng of each vitamer per ml of solution). Peaks: a = PLP; b = DPNP; c = PA; d = PMP; e = PL; f = PN; g = PM.

Fig. 2. Chromatographic analysis of a control plasma sample (300-μl injection of plasma extract). Peak identification as in Fig. 1.

Fig. 1 shows a typical chromatogram of a standard solution and Fig. 2 that of a plasma sample. Reproducibility and recovery data are shown in Table I. The coefficient of variation (C.V.) of all vitamer standards in the HPLC assay was

TABLE I

REPRODUCIBILITY AND RECOVERY DATA FOR THE ASSAY OF PLASMA B_6 VITAMERS

Vitamer	C.V. of response factor for standards* (%)	Plasma level in healthy volunteer (ng/ml)	C.V. for analysis** (%)		Recovery of spike§ (%)
			Unspiked plasma	Spiked plasma***	
PLP	2.4	10.3	3.1	2.6	94.1 ± 9.5
PA	3.2	8.1	2.2	2.0	96.5 ± 8.2
PMP	2.2	0.3	12	1.9	109.1 ± 8.3
PL	2.2	1.9	12	3.6	96.2 ± 9.4
PN	2.3	Not detected	–	2.9	103.8 ± 9.2
PM	2.3	0.3	11	3.2	105.7 ± 10.6

*2 ng of each vitamer per injection.
**Six replicate injections of individually extracted samples.
***Spiked at the 8 ng/ml level in plasma.
§Recovery (mean ± C.V) for ten normal plasma samples spiked with 8 ng of each vitamer per ml.

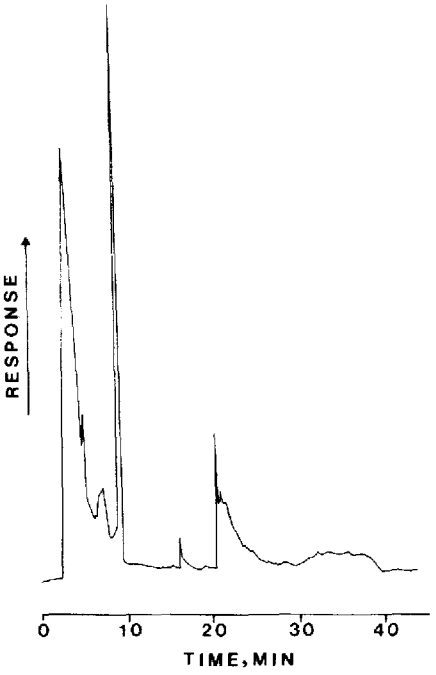

Fig. 3. Chromatogram of the water extract of blood collection tube containing tripotassium EDTA.

3% or better for six replicate 500-μl injections of individual standard solutions containing 4 ng of each vitamer per ml. Reproducibility of a plasma sample analysis is good for the main components (PLP and PA) but poorer for the minor

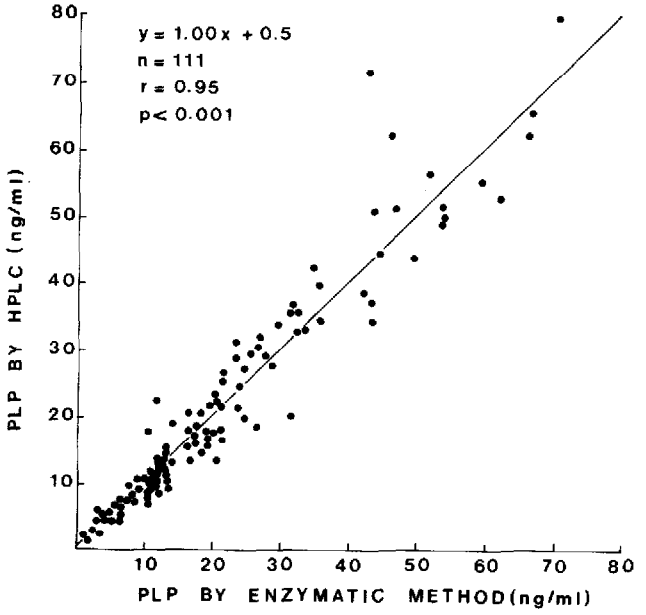

Fig. 4. Comparison of tyrosine decarboxylase enzymatic and HPLC analyses of plasma PLP.

TABLE II

COMPARISON OF PLASMA OR SERUM VITAMIN B_6 ANALYSES BY HPLC

Values are mean ± S.D.; values in parentheses represent range.

Vitamer	Concentration (ng/ml)			
	Present study (n=27)	Coburn and Mahuren [1] (n=38)	Lui et al. [3] (n=9)	Vanderslice et al. [2] (n=2)
PLP	12.5±6.2 (27.5-3.0)	14.1±6.4	7.3±4.2	18.3±8.6
PA	5.4±3.8 (18.5-1.6)	9.0±3.5	10.5±3.6	Not determined
PMP	0.1±0.2 (0.8-0.0)	2.0±2.0	0.1±0.1	7.7±2.5
PL	1.5±1.2 (7.3-0.7)	3.8±1.7	2.1±0.8	Not detected
PN	0.1±0.3 (0.9-0.0)	3.2±5.6	0.4±0.3	30.4±8.6
PM	0.1±0.1 (0.4-0.0)	0.3±0.3	0.1±1.7	1.0±1.3

ones (PMP, PL, PN and PM). This, however, is a result of the low levels of these components as shown by the improvement in precision when analysing a spiked plasma sample (Table I). With a signal-to-noise ratio of 2, the sensitivity of the assay is ca. 0.5 ng of each vitamer per ml of plasma.

During the development of this assay in our laboratory, a number of problems were encountered. Firstly, it was noted that the standards containing both aldehyde (PLP, PL) and amine (PMP, PM) forms of vitamin B_6 could not be stored because interactions occurred between the components. It was necessary to store all the vitamin B_6 compounds individually and to prepare the compound standard for HPLC analysis on the same day as the assay [7]. Another problem initially encountered was the presence at the start of the chromatogram of relatively highly fluorescent impurities, which interfered with the quantification of PLP. The extent to which these arose from native plasma compounds or from extraneous impurities was investigated.

Fig. 3 shows the chromatogram of a water-wash (acidified prior to injection) of a normal blood collection tube [VAC-U-TEST® (Radem Laboratory Equipment, Johannesburg, South Africa)] lavender stopper with tripotassium EDTA solution, Cat. No. LA0505). The considerable impurities presumably arise during tube sterilization, as normal EDTA solutions show no such peaks. The presence in blood collection tubes of compounds interfering in ion-exchange chromatography has been noted previously on amino acid analysis [9]. A water extract of a collection tube containing lithium heparin (VAC-U-TEST, green stopper, Cat. No. 1610010G) also gave considerable interference, whereas the silicone-coated tube with no additive (VAC-U-TEST, red stopper, Cat. No. RE 0505) showed no interference. Blood samples for vitamin B_6 analysis by HPLC were subsequently collected in these silicone-coated tubes, to which 5 mg of solid disodium EDTA had been added as an anticoagulant.

A number of column-treated problems have been experienced, such as changes in peak shape with time and rapid column deterioration. Allowing for initial equilibration and calibration at the start of each day, an average of three samples can be analysed in duplicate per day. Under these conditions, the HPLC columns lasted only ca. two months, during which the retention times of components,

especially PLP and PA, decreased considerably. Hence the resolution of the early eluting vitamers and impurities became difficult to maintain. This short column life remains one drawback of the method.

Once the assay with internal standard and correct sampling procedure had been established, the accuracy of the method regarding PLP values was assessed by simultaneously comparing these values with those obtained by the conventional tyrosine decarboxylase apo-enzyme method. Regression analysis of 111 observations (Fig. 4) gave a correlation coefficient (r) of 0.95 ($P < 0.001$) and a linear regression equation of (HPLC PLP) = 1.00 (enzymatic PLP) + 0.5. These independent methods agree well, in view of the known difficulties of vitamin B_6 analysis.

Further evaluation of the method was performed by comparison of vitamin B_6 vitamer levels in plasma taken from 27 normal healthy volunteers in our laboratory with the previously published values [1–3] for HPLC analysis of plasma vitamers (Table II). The results of this study compare well with previously established values. The high level of PN reported by Vanderslice et al. [2] would appear to be spurious [1].

In conclusion, this sensitive HPLC assay for vitamin B_6 compounds in plasma should aid greatly in the study of the metabolism of this vitamin in normal and in disease states.

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