Journal of Chromatography, 227 (1982) 181—186
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1045

Note

Separation of vitamin B_6 compounds by paired-ion high-performance liquid chromatography

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(First received March 26th, 1981; revised manuscript received July 30th, 1981)

It is well known that vitamin B_6 [i.e. pyridoxine(3-hydroxy-4,5-bis-(hydroxymethyl)-2-methylpyridine, PN) pyridoxal (PL), pyridoxamine (PM) and their phosphorylated derivatives] is required for growth and development of normal and neoplastic tissues, in general, and that pyridoxal-5'-phosphate (PLP) is the coenzyme of many classes of enzymes [1,2]. The principal urinary metabolite of all forms of vitamin B_6 is 4-pyridoxic acid (PIC) which has no biological activity [3]. The vitamin's effects on tryptophan metabolism, i.e. increased xanthurenic acid excretion in the vitamin depleted state after tryptophan loading, with similar observations during pregnancy and in women receiving oral contraceptive pills coupled with observation of low plasma PLP in these two groups of women [4,5] has prompted investigators to seek methods for the separation and quantitation of the vitamin B_6 compounds in biological materials. Vanderslice et al. [6] have recently reviewed vitamin B_6 methodology and concluded that the best method is separation by high-performance liquid chromatography (HPLC). In fact, these workers reported the separation of all vitamin forms including PIC using a thermostated (55°C) anion-exchange column and two buffer systems at pH 10 and pH 2.5. Separation was completed in approximately 115 min. In a more recent report from the same laboratory [7], the separation and quantitation of the vitamin forms was reported using a dual system of two anion-exchange columns thermostated at 50°C and 18°C, respectively, and the two buffer systems, reported previously [6]. Quantitation was effected using a 650/40 Perkin-Elmer fluorescence spectrophotometer. An effective run with the dual column system required approximately 100 min.

In order to study the metabolic interconversions of vitamin B_6 in vivo investigators have employed radioactively labeled pyridoxine precursor and

0378-4347/82/0000—0000/$02.75

ion-exchange (gravity) chromatography as well as high-voltage electrophoresis for separation of the vitamin metabolites [8—12]. Ion-exchange (gravity) chromatography requires long time periods and does not differentiate well between phosphorylated vitamin B_6 forms, metabolites that might coelute, or between overlapping peaks. For instance, recently we synthesized pyridoxine-5′-phosphate (PNP) [13] which eluted as a single peak on a Dowex H^+ column [12] but was further resolved into three peaks upon subsequent chromatography by HPLC. Although the latter method (electrophoresis) requires much less time to run samples it suffers from the fact that only labeled samples of 5 μl can be effectively run and only when all the vitamin B_6 standards are included with each sample in sufficient amounts for further identification following the completed run [9]. The method also does not differentiate PIC from the other vitamers [9]. Because of these and other undesired features of these methods (i.e. use of buffers; during sample concentration buffer salt is also concentrated, which subsequently interferes with the identification of unknown metabolites) we resorted to ion-pairing HPLC for studying vitamin B_6 metabolism in vivo. This communication reports on a relatively quick method based on ion-pairing for the separation and identification of vitamin B_6 tissue metabolites including PIC. The short period required for a run (<40 min) and the relatively easy separation of the vitamers from the ion-pairing agent (thus avoiding salt interference with identification) make the method more unique and advantageous over other procedures.

EXPERIMENTAL

The apparatus is an HPLC Waters Assoc. system equipped with a Model U6K universal liquid injector, Model 440 UV absorbance detector with a 313-nm filter and Model 6000A solvent delivery system. A 30 × 0.39 cm μBondapak C_{18} column, particle size 10 μm (Waters Assoc., Milford, MA, U.S.A.) was used.

Solvents

(A) 10% 2-propanol with 0.09% glacial acetic acid. (B) 10% 2-propanol with 0.09% glacial acetic acid with a mixture of counter-ions (seven-carbon and eight-carbon alkyl chains). The counter-ions were of sodium-1-heptanesulfonate and sodium-1-octanesulfonate at 0.004 *M* concentration.

The solvents were prepared with HPLC water, filtered through 0.45-μm filter and degassed before use.

Standards

The standards used were: pyridoxine hydrochloride, pyridoxal hydrochloride, pyridoxamine dihydrochloride, pyridoxal-5′-phosphate hydrochloride, pyridoxamine-5′-phosphate hydrochloride, 4-pyridoxic acid and 3-hydroxypyridine and were obtained in the purest state available from Chemical Dynamics Corp. (S. Plainfield, NJ, U.S.A.) or from Sigma (St. Louis, MO, U.S.A.). Pyridoxine-5′-phosphate was prepared as described by Peterson and Sober [13], purified by cation-exchange chromatography (Dowex H^+) [12] and further by HPLC.

Standard solutions. Standard solutions of 1 mg/ml were prepared in HPLC water (10 mg of the standard compound were dissolved in 10.0 ml of pure water).

Working standards. The working standard solutions of 20 ng/μl were prepared by diluting 200 μl of the standard solution to 10.0 ml with solvent B.

Procedure

The eight standards were separated using solvents A and B at a flow-rate of 0.7 ml/min. The column was equilibrated by passing three column volumes (ca. 25 ml) of solvent B. An aliquot of the mixture of the eight standards is injected and elution proceeds for the first 5 min using solvent B. At exactly 5 min, by simply switching to the solvent A channel, elution with solvent A is effected for the remainder of the run. The column is then again equilibrated with solvent B and ready for another run.

Extraction of vitamin B_6 metabolites

Vitamin metabolites are extracted using perchloric acid as described previously [8,12]. The sample is neutralized to pH 4.2 with 3 *M* potassium hydroxide, allowed to stand overnight in the cold and afterwards filtered through Whatman No. 1 filter paper. The filtrate is cleared by centrifugation at 25,000 *g* for 16 h and 4°C and (if desired) chromatographed on a Dowex H^+ column [8,12]. Material isolated from this column is further chromatographed, if desired, using HPLC and ion-pairing as described in this report. If necessary, the sample could be concentrated by freeze drying prior to HPLC, however. Albeit, labeled vitamers can directly be chromatographed by HPLC after acid extraction.

RESULTS AND DISCUSSION

Fig. 1 is a typical chromatogram showing the separation of seven vitamin B_6 metabolites and of 3-hydroxypyridine used here as a marker. The order of elution and retention time was as follows: PNP, 3.7 min; PLP, 5.0 min; PIC, 6.7 min; pyridoxamine-5′-phosphate (PMP), 9.8 min; PL, 21.3 min; 3-hydroxypyridine (marker), 24.4 min; PN, 28.8 min; PM, 38.8 min. The entire run requires less than 40 min and can further be shortened by increasing, after the elution of PMP or of PL (a) the flow-rate, (b) the concentration of 2-propanol in solvent A, or both.

Fig. 2 shows linear dose–response graphs for all the vitamin B_6 vitamers including PIC. A 313-nm filter was used to record the absorbance of the metabolites. PIC does not absorb well at 254 nm and requires large amounts for detection. However, at the higher concentrations the PIC peak spreads and interferes with the separation of the phosphorylated forms (PLP and PMP). On the other hand, although PN and PNP themselves do not absorb well at 313 nm and do require large amounts for detection, they nevertheless have non-spreading, sharp peaks which do not interfere with the separation of the other metabolites (Fig. 1).

The advantage of the present method over other procedures is (a) its simplicity, (b) the relatively short time required for each run (<40 min), (c) the

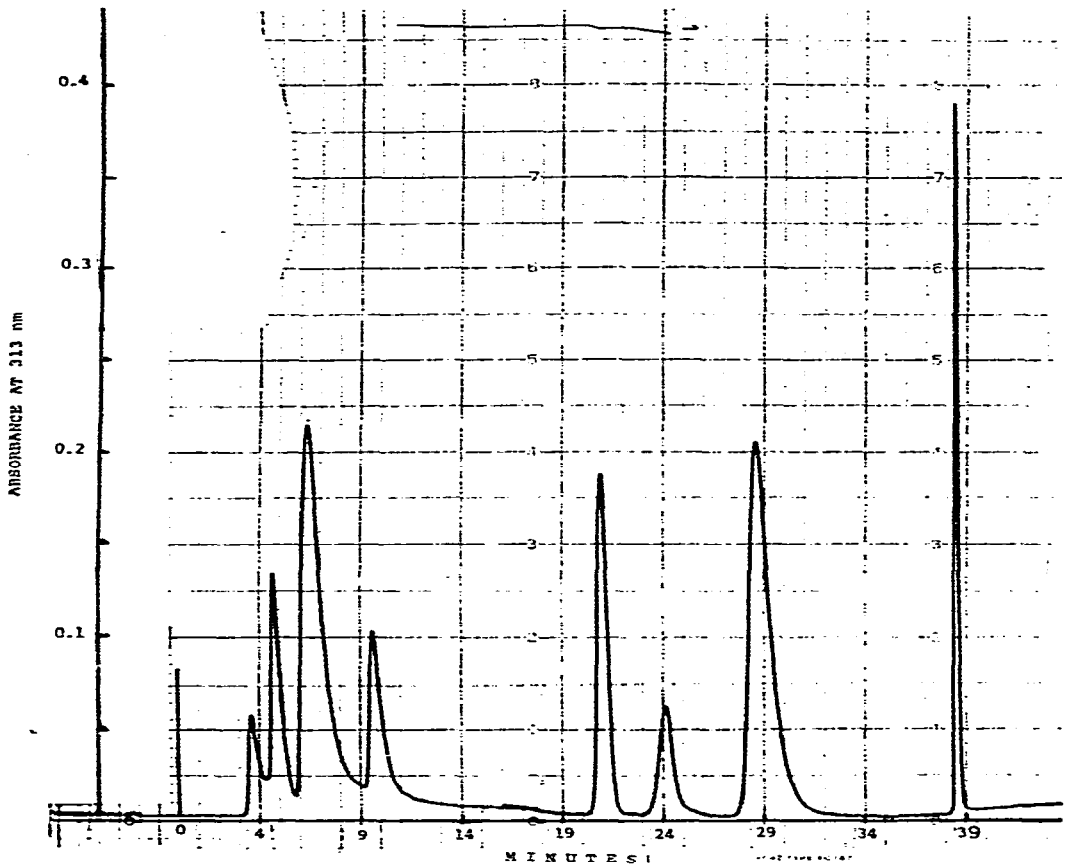

Fig. 1. Separation of vitamin B_6 metabolites and 4-pyridoxic acid by ion-pairing HPLC. Injection time is indicated at time ○. Chart speed was 0.5 cm/min. The order of elution and the retention times were: PNP, 3.7 min; PLP, 5.0 min; PIC, 6.7 min; PMP, 9.8 min; PL, 21.3 min; 3-hydroxypyridine, 24.4 min; PN, 28.8 min; PM, 38.8 min. The amounts of each standard (in the mixture) injected, were: PNP, ca. 6000 ng; PLP, 396 ng; PMP, 400 ng; PIC, 400 ng; 3-hydroxypyridine, 1200 ng; PN, 3000 ng; PL, 400 ng; PM, 140 ng. A typical chromatographic run is shown.

absence of buffers and of salt interference with subsequent metabolite identification, and (d) the ease of separation and subsequent recovery of the metabolites from the alkyl sulfonate counter-ion. As mentioned above, the actual run can also be further shortened. In addition, it would seem feasible that the separation of the vitamers could be quantitated using similar equipment employed by other research workers [6,7] which at this time is not available to us.

As our research endeavor is presently oriented to studying the metabolic transformations of labeled pyridoxine in tumor cells by (subsequent) detection of labeled metabolites attempts to quantitate the vitamers in tissues were not made. Albeit, the synthesis of PIC from labeled pyridoxine precursor by tumor cells [12,14] was recently verified using ion-pairing HPLC. A homogenate was prepared from minimum deviation transplantable Morris hepatoma No. 7777 in 0.05 *M* ammonium formate, pH 7.5 buffer. Samples

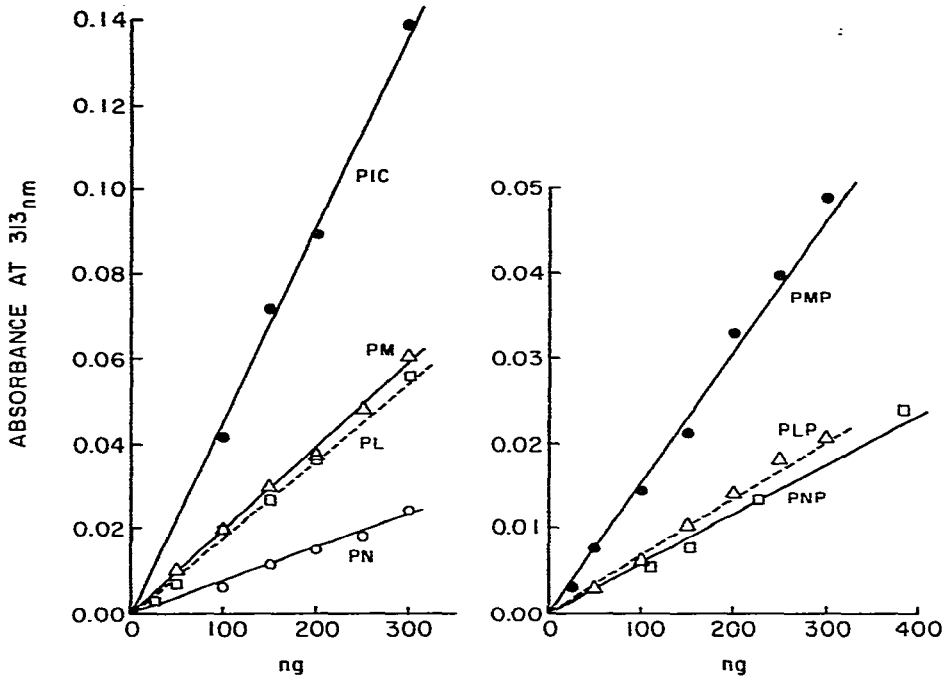

Fig. 2. Typical dose—response curves of PN, PL, PM, PIC, and of the phosphorylated vitamin forms PMP, PLP and PNP. The ordinate (graph on the right) has been expanded. All curves are representative showing a linear relationship between dose injected and absorbance at 313 nm.

were incubated (37°C, 60 min) with shaking in the presence of pyridoxine hydrochloride labeled with tritium at carbon atom No. 6 (Amersham, Arlington Heights, IL, U.S.A.). Vitamin B_6 metabolites were acid extracted [8,12] and chromatographed on Dowex H^+ [12]. Positive identification of PIC was however not possible because of peak overlapping with phosphorylated vitamin forms. Therefore, tubes containing radioactivity were concentrated by freeze-drying and subsequently analyzed by HPLC, as described in this report. Absorbance was continuously monitored at 313 nm. Eluted fractions were collected every 30 sec. Radioactivity was measured in a Tri-Carb liquid scintillation spectrometer, as described previously [12,14]. Fig. 3 demonstrates the positive identification of 4-pyridoxic acid produced by the tumor homogenate (from the labeled pyridoxine precursor) as the radioactivity closely follows the absorbance of the authentic standard. In this instance, the results demonstrate the existence of enzymes in these tumor cells able to effectively utilize pyridoxine subsequently degrading to PIC. Although PIC has been identified as a catabolic vitamin product in rats [10], its presence and importance in hepatoma cells (Fig. 3) to our knowledge has not been reported or assessed.

The HPLC separation technique utilizing heptane- and octanesulfonates as the counter-ions has been successfully employed in studies on vitamin B_6 metabolic transformations by tumor cells [15]. Radioactively labeled precursor was administered to tumor bearing rats and subsequently the vitamers were extracted and separated as described in the Experimental section. The sen-

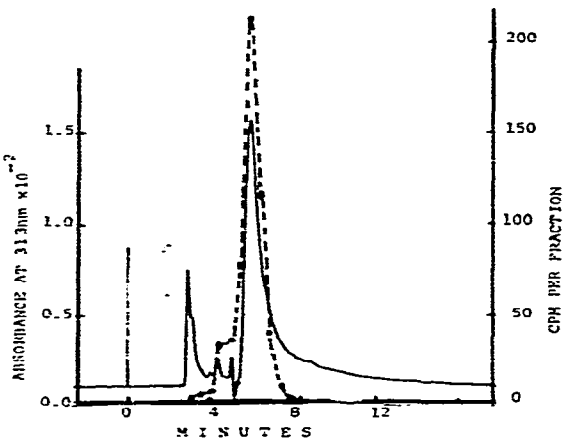

Fig. 3. Identification of radioactively labeled 4-pyridoxic acid (PIC) by ion-pairing HPLC. The radioactivity of the vitamer extracted from the tumor tissue coincides with the absorbance of the authentic PIC sample.

sitivity of the method is indeed extremely high as it is quite possible to detect and measure labeled metabolites even when the counts per minute are quite low. Since radioactivity can be derived only from the administered labeled precursor the method is in addition quite precise especially when the optical density of the unknown labeled vitamin B_6 metabolite coincides with that of the authentic standard sample (i.e. Fig. 3).

ACKNOWLEDGEMENTS

This work was supported by grant 1R01 CA 28140 awarded by the National Cancer Institute, DHEW. Our thanks are due to Dr. Harold P. Morris for a tumor donor animal given to us gratis and for his continued interest and support.

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