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Note

Post-column derivatization of vitamin B_6 using 2,6-dibromoquinone-4-chlorimide

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The analysis of vitamin B_6 is of considerable importance in various fields such as nutrition science, pharmaceutics and clinical chemistry. The introduction of high-performance liquid chromatography (HPLC) for the analysis of vitamin B_6 made its rapid separation and quantitation possible. Many authors have indicated the advantages of HPLC for vitamin B_6 analysis[1–7].

As conventional HPLC detection by ultraviolet absorbance is affected by impurities in the sample, it is necessary to separate vitamin B_6 completely from other impurities, which is time consuming, and/or remove them by extraction before injection.

Automatic derivatization of vitamin B_6 after elution from the column, which gives high selectivity together with high sensitivity, reduces the analysis time and makes sample preparation easier. 2,6-Dibromoquinone-4-chlorimide, a conventional colour reagent for use with pyridoxine hydrochloride in spectrophotometry, which gives an absorption maximum at 650 nm in the presence of ammonia solution, has been applied to the post-column derivatization of vitamin B_6. Hydrochloride forms of pyridoxine, pyridoxal, pyridoxamine and 4-pyridoxic acid were separated by the reversed-phase ion-pair chromatography, derivatized and selectively detected using by this system.

EXPERIMENTAL

An LC-3A liquid chromatograph equipped with a SPD-1A spectrophotometric detector, SIL-1A sample injector, CTO-2A column oven, two PRP-1 pumps as the reagent pump, a reaction coil made of stainless-steel tubing of various lengths and of 0.5 mm I.D. and a UV-240 spectrophotometer were used (all products from Shimadzu, Kyoto, Japan).

The column (25 cm × 4.6 mm I.D.) contained Zorbax C 8 of particle size 5 μm (DuPont Company, Wilmington, DE, U.S.A.). The mobile phase was distilled water containing 5 m*M* of sodium perchlorate and 5 or 10 m*M* of sodium 1-hexanesulphonate, which is an ion-pair reagent. The pH of the mobile phase was adjusted to 2.5 with perchloric acid, and 10–20% of methanol was added in order to adjust the retention time of vitamin B_6.

Standard samples were prepared by dissolving pyridoxine hydrochloride, pyr-

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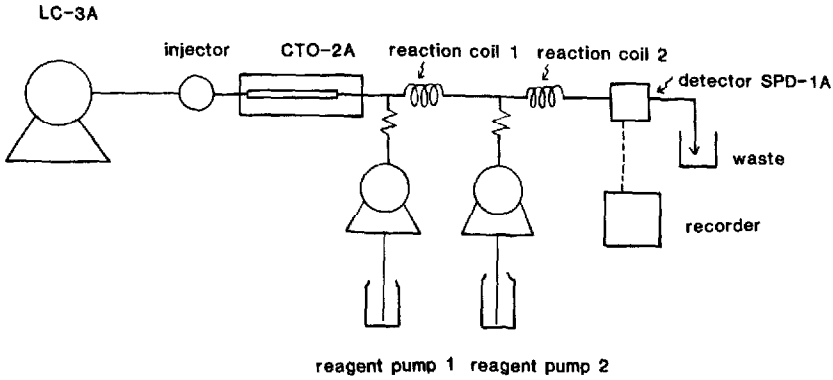

Fig. 1. Flow diagram of the system. Pump 1: 2,6-dibromoquinone-4-chlorimide (colour reagent). Pump 2: ammonia solution (2.5%).

idoxal hydrochloride (Tokyo Kasei, Tokyo, Japan), pyridoxamine dihydrochloride (P-L Biochemical, Milwaukee, WI, U.S.A.), deoxypyridoxine hydrochloride (Wako, Osaka, Japan) and 4-pyridoxic acid, pyridoxamine 5′-phosphate, pyridoxal 5′-phosphate (Sigma, St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Fig. 1 shows the flow diagram of the system. Vitamin B_6 compounds are separated on the Zorbax C_8 column by reversed-phase ion-pair chromatography and

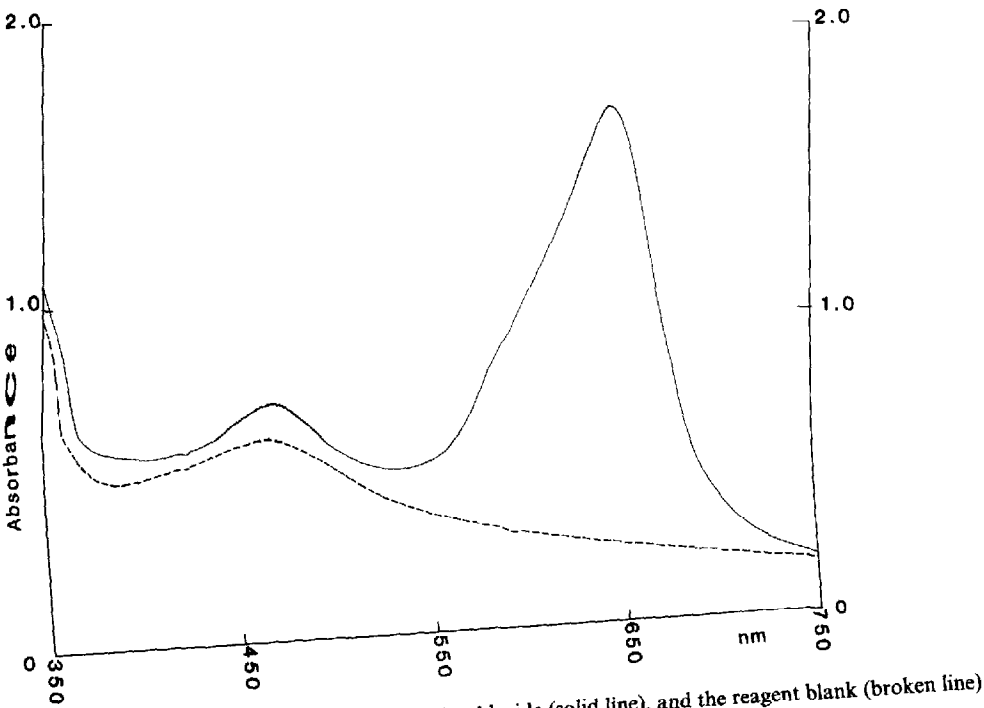

Fig. 2. Absorption spectrum of pyridoxine hydrochloride (solid line), and the reagent blank (broken line)

mixed with the colour reagent in reaction coil 1. The colour reagent is pumped to the reaction coil 1 by pump 1; 2.5% ammonia solution pumped by pump 2 is then added to the mixture and reacted in coil 2. The reaction product is detected by the SPD-1A detector at a wavelength of 650 nm.

Fig. 2 shows the absorption spectrum of pyridoxine hydrochloride (solid line) and the reagent blank (broken line). A 10-μl volume of a solution of pyridoxine hydrochloride at a concentration of 1.03 mg/ml (in a blank test, 10 μl of distilled water) and 100 μ of 25% ammonia solution were poured into 2 ml of reagent solution (50 mg dissolves in 200 ml of ethanol) in the cuvette, and the absorbance spectrum was obtained with the UV-240 spectrophotometer. As shown in Fig. 2, pyridoxine hydrochloride has an absorption maximum at 650 nm, but reagent blank solution also has almost one sixth of this absorbance at this wavelength, which causes a rise of the baseline.

In order to obtain the spectrum, 25% ammonia solution was poured into the cuvette, following the original method adopted in the Japanese Pharmacopoeia[8]. However, in this system, in order to avoid harmful ammonia vapour, a 2.5% concentration of ammonia solution was used as a regent, pumped by pump 2. The difference in the ammonia concentration can be ignored (measured with the UV-240; results not shown); we used 2.5% ammonia solution so as to make handling easier.

The effect of reagent concentration, length and temperature of reaction coil and flow-rate of the reagent on the peak height of pyridoxine hydrochloride was studied. The optimal conditions obtained from these experiments are presented in Table I.

The increase in peak height with increase in concentration or flow-rate attains a plateau at a concentration of 0.5 mg/ml or at a flow-rate of 1.4 ml/min. Concentrations higher than 0.5 mg/ml do not give any further increase in peak height and cause baseline fluctuations. A decrease in peak height, caused by dilution owing to the increase in flow-rate, did not occur in the range of flow-rates examined (0.2–1.8 ml/min). Temperatures of the reaction coil higher or lower than room temperature (25°C) resulted in smaller peak heights.

The calibration graph obtained under the optimal conditions is linear over the range of 50–1000 ng injected, and 10 ng of pyridoxine hydrochloride is the lower limit that can be identified as a peak. As the reagent blank shows absorption at the

TABLE I

OPTIMAL CONDITIONS

Mobile phase: 10 m*M* sodium 1-hexanesulphonate, 5 m*M* sodium perchlorate, 10% methanol (pH 2.5, adjusted with perchloric acid); flow-rate, 0.8 ml/min. Injection volume: 10 μl of an aqueous solution of pyridoxine hydrochloride (1.0 mg/ml). Pump 1, 0.8 ml/min and pump 2 1.2 ml/min for reagent concentration experiments; pump 1 1.4 ml/min and pump 2 1.0 ml/min for other experiments.

Parameter	*Optimal value*
Reagent concentration	0.5 mg/ml
Flow rate of pump 1	1.4 ml/min
Coil length (coil 1)	2 m (I.D. 0.5 mm; coil 2 is fixed to 2 m)
Reaction temperature	25°C

TABLE II

PEAK AREAS CORRESPONDING TO AN INJECTED AMOUNT OF 500 ng OF EACH COMPOUND

Compound	*Peak area ($\mu V \cdot sec$)*	*Compound*	*Peak area ($\mu V \cdot sec$)*
Pyridoxine hydrochloride	8201	Pyridoxamine 5'-phosphate	13917
Pyridoxal hydrochloride	11154	Pyridoxal 5'-phosphate	1616
Pyridoxamine dihydrochloride	9415	Catechol	1615
Deoxypyridoxine hydrochloride	10560	*o*-Xylenol	415
4-Pyridoxic acid	4036	*p*-Aminophenol	1016

wavelength used, pulsation of the reagent pump causes vibration of the baseline, so identification of peaks representing less than 10 ng is difficult.

Peak areas corresponding to injected amounts of 500 ng of five compounds of vitamin B_6, 4-pyrioxic acid (metabolite of vitamin B_6), deoxypyridoxine (used as an internal standard) and catechol, *o*-xylenol and *p*-aminophenol which may interfere with the detection are presented in Table II.

4-Pyridoxic acid, which is an excretion metabolite of vitamin B_6, has almost half the sensitivity of vitamin B_6 and pyridoxal 5'-phosphate is less sensitive than other vitamin B_6 compounds. Phenols have much lower sensitivity than vitamin B_6 and will not interfere with the determination of vitamin B_6 even if they are present in the sample.

Fig. 3 shows the chromatogram of three compounds of vitamin B_6 and 4-pyridoxic acid. Because of the separation selectivity of reversed-phase ion-pair chromatography[7], the four compounds are well separated and were eluted within 14 min.

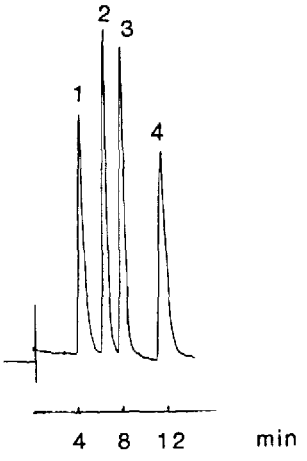

Fig. 3. Chromatogram of three compounds of vitamin B_6 and 4-pyridoxic acid. The mobile phase does not contain methanol; other conditions as in Table I. Amounts injected were 5.25 μg of pyridoxine hydrochloride, 5.2 μg of pyridoxal hydrochloride, 9.9 μg of 4-pyridoxic acid and 4.7 μg of pyridoxamine dihydrochloride; attenuation 0.64 a.u.f.s. Column temperature, 45°C. Peaks: 1 = 4-pyridoxic acid; 2 = pyridoxal hydrochloride; 3 = pyridoxine hydrochloride; 4 = pyridoxamine dihydrochloride.

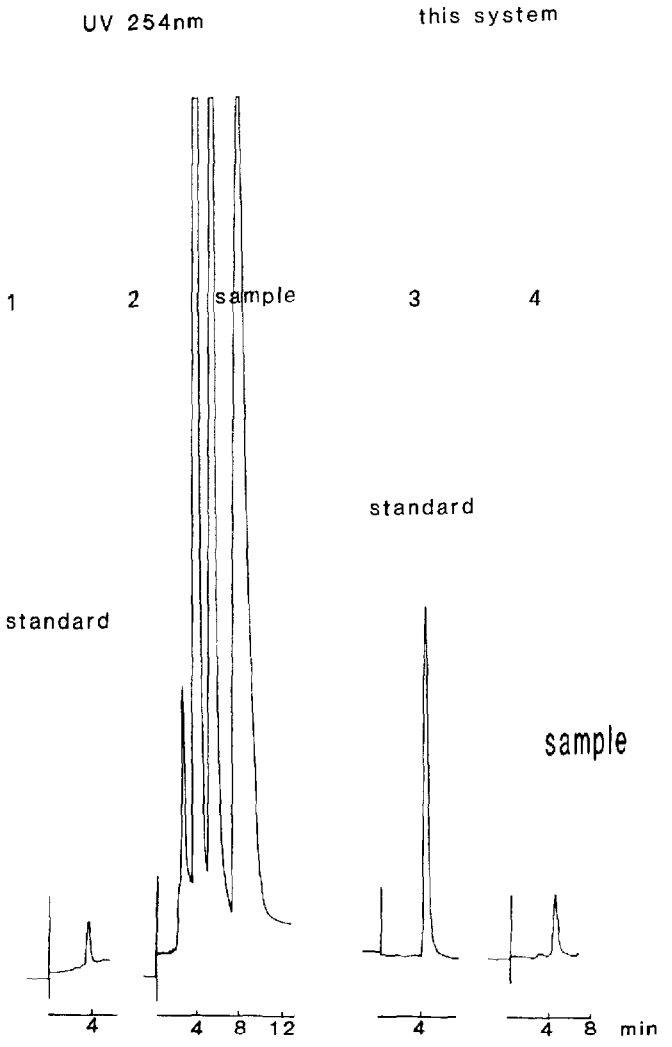

Fig. 4. Application of the system to the analysis a vitamin B tablet. The mobile phase contains 5 m*M* sodium 1-hexanesulphonate, 5 m*M* sodium perchlorate and 20% methanol (pH 2.5). Flow-rate, 0.8 ml/min; pumps 1 and 2, 1.0 and 1.2 ml/min, respectively. Pyridoxine hydrochloride: 0.95 mg/ml; 5.0 μl injected. Vitamin B tablet: 34.1 mg/ml, 100 μl injected. Attenuation 0.64 a.u.f.s.

Fig. 4 shows the application of this system to the analysis of a vitamin B tablet; the two chromatograms on the left are examples of detection by conventional UV (254 nm) absorption, and the two on the right are examples of the present system. The present system is almost seven times more sensitive than UV (254 nm) detection (see chromatograms 1 and 3). In the analysis of a vitamin B tablet, using UV (254 nm) detection (chromatogram 2) it is difficult to indentify pyridoxine hydrochloride because of interfering substances (other vitamin B compounds, caffeine or impurities sensitive at the wavelength used), but in the present system, no peak except pyridoxine hydrochloride is eluted, which indicates the high selectivity.

Hence when analysing one vitamin B_6 compound, it is not necessary to separate it from other interfering compounds because of the high selectivity; this system

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