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Note

High-performance liquid chromatographic determination of pyridoxine and congeners in biological fluids of man after high-dose therapy

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Pyridoxine, a vitamer of vitamin B_6, is used alone and in combination with other agents such as tryptophan in the treatment of depressive illness [1, 2]. For this purpose the usual dose ranges from 0.1 to 1 g a day and greatly exceeds the recommended daily intake of the compound as a vitamin (2 mg [3]). As part of research into the clinical applications of pyridoxine in psychiatry, it was necessary to investigate the pharmacokinetics of the drug and metabolites at the dose levels used. To obtain this information a simple direct assay for pyridoxine and several of its metabolites has been developed using high-performance liquid chromatography (HPLC).

Assays available for the compound in biological material involve enzymatic, microbiological and chemical techniques [4—6]. The enzymatic procedures usually make use of the co-enzyme function of pyridoxine and some congeners,

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particularly pyridoxal [7]. These methods are adapted to the determination of sub-microgram quantities of material and are not readily applicable to routine pharmacokinetic studies.

The microbiological assays are tedious and somewhat inaccurate for pharmacokinetic studies and are generally non-specific for pyridoxine metabolites [8]. Many of the chemical methods involve lengthy separation procedures [9—12] or electrophoresis [13, 14]. Other chemical procedures require selective oxidation of the vitamers to 4-pyridoxic acid lactone and determination of this compound fluorimetrically [15—17]. Some assays have been developed for analysis of pyridoxine and some vitamers in food and drug analysis [9, 18, 19]. The gas chromatographic separation of the non-phosphorylated vitamers has been published [20], but the technique requires derivatization.

Stewart et al. [18] have proposed an HPLC method for pyridoxine in pharmaceutical products using an ion-pair development method. Other HPLC methods allow the determination of vitamin B_6 components in food products and biological fluids of man using fluoromonitor detectors [19, 21], but column separation may take up to 2 h and therefore limits the number of samples assayed per day.

The assay described here permits the rapid and specific determination of pyridoxine and some of its metabolites, pyridoxal and 4-pyridoxic acid, in human plasma and urine after oral administration of the drug at psychiatric dose levels.

MATERIALS AND METHODS

Apparatus

A Spectra Physics 3500B high-performance liquid chromatograph was used, equipped with a variable-wavelength spectrophotometric detector (Model 700). The detector was connected to a 1-mV recorder (BD 7; Kipp and Zonen, Emmen, The Netherlands). The stainless-steel column, 25 cm × 4.6 mm I.D., was commercially packed with Partisil 10-ODS, particle size 10 μm (Chrompack, Middelburg, The Netherlands). The injection loop was 100 μl size. Detection of pyridoxine and metabolites was effected at 291 nm.

Solvent

The solvent used was a 0.067 *M* potassium dihydrogen phosphate solution in double-distilled water, to which 10 ml of a phosphoric acid solution (40%) were added per liter. The final pH of this mixture was 2.6 and the flow-rate used was 1.6 ml/min at a pressure of 155 atm.

Drugs

Pyridoxine hydrochloride, pyridoxal hydrochloride, 4-pyridoxic acid and pyridoxamine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.) and were chromatographically pure in all systems used. Pyridoxal-5-phosphate and pyridoxamine-5-phosphate (Sigma) exhibited minor contaminating peaks (< 0.5%) due to parent compounds in the system described. To prepare standard solutions the compounds were dissolved in water (1 mg/ml). In the case of 4-pyridoxic acid a small amount of 1 *N* sodium hydroxide solution was

added to the acid solution. Standard solutions were kept in a refrigerator. All were stable except pyridoxal, which was renewed within three weeks, and the phosphate derivative solutions, which were freshly prepared.

Subjects and clinical sampling

Pyridoxine was administered to Caucasian volunteers from the Department of Clinical Pharmacy and to patients in the Departments of Psychiatry of the St. Radboud Hospital (Nijmegen, The Netherlands) and of the Bethesda Hospital (Tiel, The Netherlands). The drug was administered orally as tablets of 250 mg.

From volunteers, blood samples of 0.5 ml were collected at scheduled time intervals by fingertip puncture (Microlance No. 433, Becton and Dickinson) in 2-ml Eppendorf vials, in which 0.1 mg of solid calcium heparin (Organon, Oss, The Netherlands) was used as anticoagulant (strength ±150.000 I.E./g). Spontaneously voided urine samples were collected over 24 h. In patients blood samples were obtained by venapuncture (5 ml) at scheduled time intervals, while urine collection was made over 24 h. Blood samples were centrifuged soon after collection (2500 *g* for 10 min). All samples (plasma and urine) were frozen after collection and assayed within 72 h.

Sample preparation

Plasma. A 0.2-ml sample of plasma was added to 0.3 ml of perchloric acid (0.33 *M*) and mixed by inversion. The mixture was allowed to stand for 5 min and then centrifuged for 5 min at 2500 *g*. A 100-µl aliquot of the clear supernatant was injected onto the column.

Urine. Ten microliters of urine were mixed with 0.3 ml of perchloric acid (0.33 *M*) on a vortex mixer. A 100-µl aliquot of the clear mixture was injected onto the column.

Calibration

A calibration curve was made by adding known concentrations of pyridoxine, pyridoxal, 4-pyridoxic acid and pyridoxamine to blank human plasma and urine. A calibration curve was constructed by plotting peak height versus concentration. Three to five standards were included as a control with each series of determinations.

RESULTS

Pyridoxine and its vitamers were well separated from endogenous compounds in plasma and urine by this HPLC technique (Fig. 1). The relative retention times of the vitamers and the major endogenous peaks, which were the same in plasma and urine, are shown in Table I. In Plasma, a linear response was obtained over a range of 0.3—25 µg/ml and in urine the curve was linear from 0.5 to 125 µg/ml (Table II). The correlation coefficients indicate the excellent linearity of the response. Recovery of pyridoxine added to human plasma in the concentration range of 1—30 µg/ml was found to be 100 ± 5%. The lowest concentration which can be measured accurately is 0.3 µg/ml for pyridoxine, pyridoxal and 4-pyridoxic acid, and 1.0 µg/ml for pyridoxamine. The total

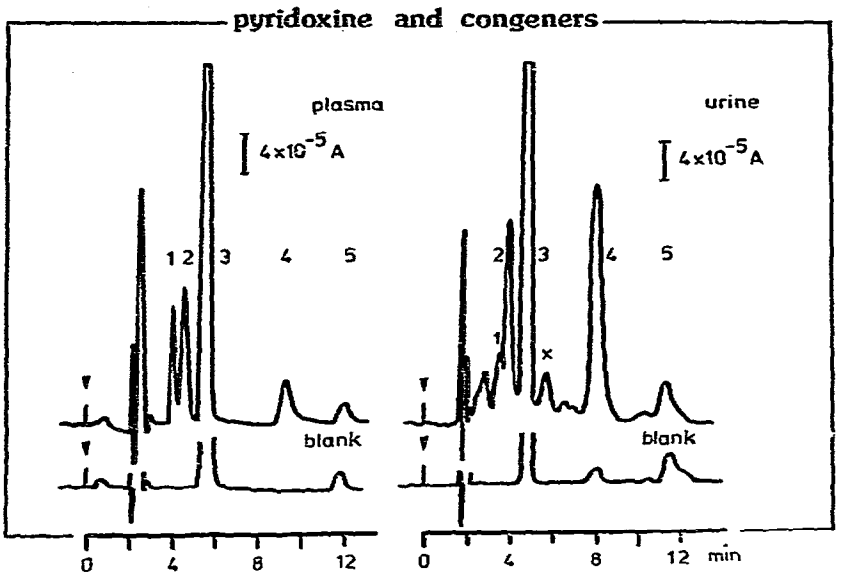

Fig. 1. HPLC chromatograms of pyridoxine and congeners in plasma and urine samples obtained from volunteers after the intake of 750 mg of pyridoxine and their respective blanks, sampled prior to drug intake. Peaks: 1 = pyridoxal; 2 = pyridoxine; 4 = 4-pyridoxic acid; 3 and 5 are unknown plasma and urine compounds; x represents an unknown metabolite in urine.

TABLE I

RELATIVE RETENTION TIME OF PYRIDOXINE AND CONGENERS

Compound	Relative retention time
Pyridoxamine	0.30
Pyridoxamine-5-phosphate	0.46
Pyridoxal	0.82
Pyridoxal-5-phosphate	1.25
Pyridoxine	1.46
Unknown plasma and urine peak	1.80
Unknown metabolite	2.36
4-Pyridoxic acid	3.75

time required for an analysis is 15 min. A series of assays was carried out to determine the reproducibility of the assay. In plasma it appeared to be 100 ± 2%; in urine at high levels (575 μg/ml) it was 100 ± 1% and at low levels (7.5 μg/ml) 100 ± 3.5% (n = 10).

When the method was applied in preliminary pharmacokinetic studies of pyridoxine, a number of clear-cut results were obtained. The measurements showed that this drug was rapidly absorbed with a peak plasma level appearing in less than an hour after oral administration (Fig. 2), and indicated an elimination half-life for the parent compound as well as for the metabolites of 1—1.6 h.

In urine, high levels of the vitamers, particularly 4-pyridoxic acid, were observed, up to 500 μg/ml in some samples. The limit of sensitivity in urine was about 0.5 μg/ml.

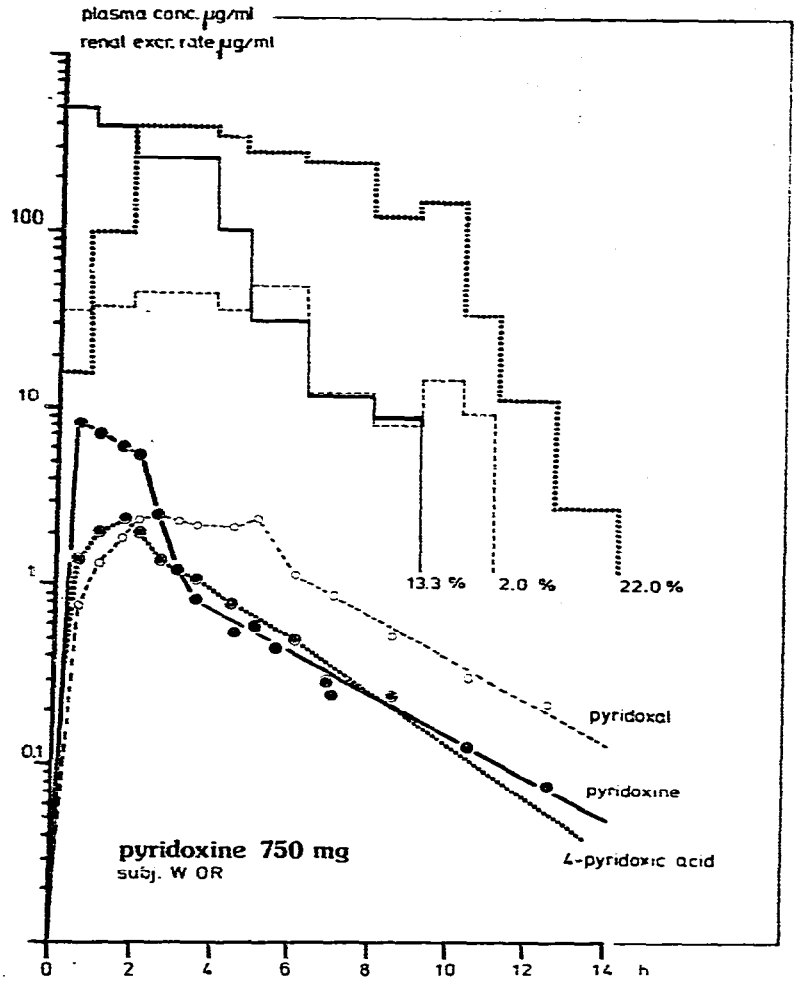

Fig. 2. Pharmacokinetics of pyridoxine and congeners (pyridoxal and 4-pyridoxic acid) in man after the intake of 750 mg of pyridoxine.

TABLE II

STANDARD CURVE DATA FOR PYRIDOXINE AND CONGENERS

The standard curve was constructed from peak height versus plasma or urine concentration (µg/ml).

Compound	Plasma*			Urine**		
	Slope	Intercept with y-axis	Corr. coeff.	Slope	Intercept with y-axis	Corr. coeff.
Pyridoxine	6.78	1.12	0.99	0.57	0.08	0.98
Pyridoxal	5.49	0.40	0.99	0.41	−0.50	0.99
4-Pyridoxic acid	1.85	0.10	0.99	0.17	0.80	0.98

*Concentration range 0—25 µg/ml.
**Concentration range 0—125 µg/ml.

The major metabolic products of pyridoxine were pyridoxal and 4-pyridoxic acid (Fig. 2) the latter being the predominant urinary product (Table III). The total amount of recovered pyridoxine, as free drug and metabolites, represents approximately 35% of the administered dose, so there must be other unidentified metabolites or pathways yet to be elucidated (Table III). One unidentified metabolite was observed in all subjects (Table I) and appeared to exhibit a similar pharmacokinetic profile to pyridoxine and 4-pyridoxic acid. More extensive studies in pyridoxine pharmacokinetics will be carried out in order to elucidate the structure of this unknown metabolite.

TABLE III

PHARMACOKINETIC PARAMETERS OF PYRIDOXINE AND CONGENERS AFTER THE ORAL ADMINISTRATION OF 750 mg OF PYRIDOXINE TO FOUR VOLUNTEERS

Values are expressed as mean ± S.D.

	Pyridoxine	Pyridoxal	4-Pyridoxic acid	Total
Half-life (h)	1.7 ±0.4	1.8 ±0.3	1.8 ±0.4	—
Percentage of the dose excreted in urine over 24 h calculated as percentage of free pyridoxine	13.61±2.56	1.92±0.62	20.05±2.32	35.58±2.65

DISCUSSION

In these studies, the only vitamers identified in plasma and urine after high-dose therapy were pyridoxine, pyridoxal and 4-pyridoxic acid. Therefore discussion of the assay will be limited to these compounds. The results indicate that pyridoxamine and probably the phosphorylated congeners could be quantitated by a modification of the method described, but since these compounds were not observed, their estimation was not further investigated.

The method described permits the rapid assay of pyridoxine, pyridoxal and 4-pyridoxic acid in plasma and urine in the concentration achieved by high-dose therapy. The day-to-day reproducibility of the assay was constantly checked by the inclusion of standards in all series and was excellent. In contrast to the available enzymatic and microbiological methods [4—8], the technique is sufficiently sensitive and highly specific for the non-phosphorylated vitamers. An assay requires only 15 min per sample compared to lengthy ion-exchange procedures [10], gel filtration [9] or thin-layer chromatographic methods [13]. No complicated oxidative procedures are required as in the technique of Fujita and co-workers [15, 16]. The HPLC assay developed by Stewart et al. [18] was designed to assay simultaneously pyridoxine and isoniazid in pharmaceutical products. This technique involves the use of an ion-pair reagent (sodium dioctyl sulphosuccinate) on a reversed-phase column. In our hands this method gave inadequate separation of the three vitamers, pyridoxine, pyridoxal and 4-pyridoxic acid. The gas—liquid chromatographic method of Williams [20] gives a slightly lower sensitivity than the present assay but has the disadvantage of requiring derivatization of the polar and non-volatile vitamers. Moreover, it requires considerably more time.

Greater sensitivity, however, would permit the assay of normal plasma levels of the compounds (normal vitamin B_6 activity is 15—35 ng/ml [3]). The endogenous concentrations obtained in urine by back projection of the standard curve in one subject gave a total daily urinary output of the vitamers as 2.6 mg. This is close to the recommended daily intake [3], which probably reflects the vitamin B_6 content of an average diet. If lower levels of the compound were to be quantitated, extraction or concentration procedures may be required. Because the compounds are extremely polar, attempts at solvent extraction from plasma and urine were unsuccessful. The use of other detection methods and suitable pretreatment could increase the sensitivity of the present assay and might permit the direct determination of clinically normal and deficient concentrations of vitamin B_6.

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