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Note

Pyridoxine O-methylether cyclic *n*-butane boronate: a new derivative for gas chromatography of pyridoxine

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Several chemical, microbiological and enzymatic methods are available for the assay of pyridoxal and its phosphorylated form. They have been listed in a recent review[1]. However, there is only one fluorometric method, *i.e.*, the "lactone method" available for the determination of pyridoxine[2]. This method involves oxidation of pyridoxine with potassium permanganate to 4-pyridoxic acid which subsequently is converted into its lactone with hydrochloric acid. This method is very unsatisfactory since the yields are low (30–32%) and inconsistent[3,4]. Furthermore, as the reagents are non-specific, pyridoxal also is oxidized and converted into the lactone under the reaction conditions[4]. Thus, prior separation of pyridoxine from pyridoxal is necessary for differential assay.

Several investigators have examined the gas chromatographic (GC) behaviour of acetyl, benzyl, isopropylidine, trimethylsilyl, trifluoroacetyl and heptafluorobutyryl derivatives of B_6 vitamers[5]. Some of these methods employ the more conventional flame ionization detector which has low sensitivity (*i.e.* in the μg range). Trifluoracetyl and heptafluorobutyryl derivatives, although more sensitive, require the use of a electron-capture detector instead of the more routinely available flame ionization detector. In fact, alkylating or silylating agents such as those mentioned above are single protecting group donors and they do not exploit the distinctive moieties of polyfunctional compounds which could allow the use of more specific reagents involving proximal groups[6]. The use of *n*-butylboronic acid can be very rewarding in this respect as this reagent can form a cyclic boronate with the 1,4-diol group of pyridoxine. In this communication, we have exploited this reaction to develop a new, simple, very specific and highly sensitive GC method for the determination of pyridoxine.

MATERIALS AND METHODS

Chemicals

2,2-Dimethoxypropane, *n*-butylboronic acid and pyridoxine were purchased from Sigma (St. Louis, MO, U.S.A.). Diethyl ether and methanol were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and were 99.9 mole% pure and used without further purification. Diazald, 99% (N-methyl-N-nitroso-*p*-toluenesulfonamide) was purchased from Aldrich (Milwaukee, WI, U.S.A.). 3% OV-101 on 100–200 mesh

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Gas-Chrom Q and methyl caprate were obtained from Applied Science Labs. (State College, PA, U.S.A.).

The infrared (IR) spectra were obtained on a Perkin-Elmer Model 337 spectrophotometer as Nujol mulls or neat film. Nuclear magnetic resonance (NMR) spectra were recorded with a Varian Model 56/60A spectrometer in C^2HCl_3 using tetramethylsilane as an internal standard. Mass spectra were obtained on a Finnegan 1015 quadruple mass spectrometer. Melting points were determined on a precalibrated "Thermopan" apparatus. Thin-layer chromatography (TLC) was performed on 0.1 mm thick silica gel plates purchased from Eastman-Kodak (Rochester, NY, U.S.A.).

Preparation of 4,5-dihydroxymethyl-3-methoxy-2-methylpyridine (A)

Pyridoxine (100 mg) was dissolved in anhydrous methanol (3 ml) and diazomethane in dry ether was added in small lots till no discoloration of diazomethane was observed. The solution was allowed to stand at room temperature for 1 h. Excess diazomethane and organic solvent were removed on a rotary evaporator under reduced pressure and the residue gave 104 mg of the desired ether in 96% yield. It was crystallized from a mixture of chloroform and ether, m.p. 89–90%. It gave a single spot on TLC plate in chloroform containing 5% methanol. It also gave a single peak in gas–liquid chromatography (GLC) when injected with *n*-butyl boronic acid in dimethoxy propane. The spectral properties of the methyl ether are as follows: IR, 3265 cm^{-1}, OH; 3360 cm^{-1}, OH; NMR, δ 3.8, OCH_3; δ 2.5, CH_3; δ 4.7, CH_2; δ 8.08, H (ring); δ 5.2, OH; the hydroxyl signal disappeared when the compound was shaken with ^2H_2O; mass spectrum, calculated for $C_9H_{13}NO_3$ 183; found M^+ 183.

Preparation of n-butylboronate of 4,5-dihydroxymethyl-3-methoxy-2-methylpyridine (B)

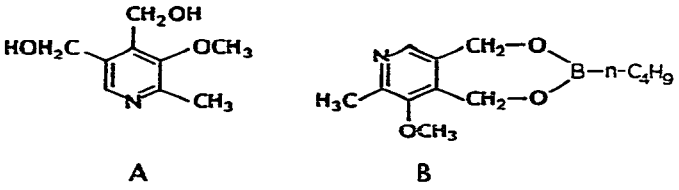
4,5-Dihydroxymethyl-3-methoxy-2-methylpyridine (36.4 mg) and chloroform (0.5 ml) were warmed in a 5-ml test tube provided with a rubber septum and a nitrogen in-let. *n*-Butane boronic acid (20.2 mg/molar proportion) was added and the mixture was warmed under nitrogen atmosphere for 30 min. The reaction solution was then transferred to a sublimation apparatus and the organic solvent removed by flushing nitrogen through the solution. Sublimation of the residue at 125–130°C/0.6 mm pressure yielded 47.0 mg of the pure cyclic boronate (94.4% yield) as a viscous liquid. NMR, δ 3.68, s, OCH_3; δ 2.57, s, CH_3; δ 0.4-1.5, m, C_4H_9; δ 5.0, s, CH_2; δ 5.1, s, CH_2; δ 8.2, s, H; mass spectrum, calculated for $C_{13}H_{20}BNO_3$ 249; found M^+ 249.

4,5-Dihydroxymethyl-3-methoxy-2-methylpyridine (1 mg) was also dissolved in dimethoxypropane (1 ml) and *n*-butylboronic acid (3 mg) was added. The reaction mixture was allowed to stand at room temperature for 30 min and then, 1 μl of the reaction mixture was injected into the gas chromatograph. A single peak of the cyclic boronate (B) was observed.

Similar results were observed when the methyl ether of pyridoxine and *n*-butylboronic acid were injected into the gas chromatograph directly.

Gas–liquid chromatography

This was accomplished with a Beckman GC-65 gas chromatograph provided with a flame-ionization detector. A U-shaped glass column (1.8 m × 2.0 mm I.D.) packed with 3% OV-101 on 100–200 mesh Gas-Chrom Q was used. The injector, inlet



A B

Fig. 1. Formulae of pyridoxine derivatives.

and detector temperatures were 200, 225 and 250°C, respectively. The column temperature was maintained at 160°C. The carrier gas (nitrogen), hydrogen and air flow-rates were 30, 45 and 280 ml/min, respectively. The attenuation range used was 100 × 2.

RESULTS

Diazomethane reacted with pyridoxine to give 4,5-dihydroxy-3-methoxy-2-methylpyridine (A) (Fig. 1). The reaction was complete in 1 h as followed by GLC of the reaction mixture. The presence of excess diazomethane did not affect the nature of the end product.

The reaction of 4,5-dihydroxymethyl-3-methoxy-2-methylpyridine with *n*-butylboronic acid was also quantitative. The mass spectrum of the cyclic boronate showed the molecular ion at 249. The peak at 149, which also is the base peak, represents loss of $C_4H_9BO_2$ from the molecular ion and thus is consistent with the cyclic ester structure (B). The other major peaks observed were at *m/e* 192, 178, 162, 119 and 83. The NMR spectrum lends further support to the structure assigned to the ester. In the ester the methylene protons are shifted δ 0.3 down field when compared to the methyl ether of pyridoxine. Furthermore, the IR and NMR spectra of the ester did not show any hydroxyl absorptions. Such addition of *n*-butane boronic acid to hydroxy and amino functions has been reported by other workers[7,8].

We have applied this procedure for the determination of pyridoxine. To ascertain the linearity of the reaction 10, 20, 30 and 40 μg, respectively of pyridoxine was treated separately with diazomethane as described under Materials and methods. The residue from each of these reactions was dissolved in 100 μl of dimethoxypropane containing 300 μg of methyl caprate as internal standard. An aliquot (1 μl) containing 100, 200, 300 or 400 ng of A from these reactions was injected into the gas chromatograph along with 3 μg of *n*-butylboronic acid in 0.5 μl of dimethoxypropane and the internal standard. The total volume of each injection was 1.5 μl. The reaction was linear and a plot of the peak areas *vs.* concentration is shown in Fig. 2. The retention time was 5.24 min measured from the appearance of the internal standard.

The sensitivity of this procedure was determined by running aliquots of standard containing 10–80 ng of B with 1 μg of *n*-butylboronic acid in a total volume of 1.0 μl of dimethoxypropane. A plot of the peak areas vs concentration is given in Fig. 2 (inset).

DISCUSSION

GC methods based on derivatization with alkylating or silylating agents lack

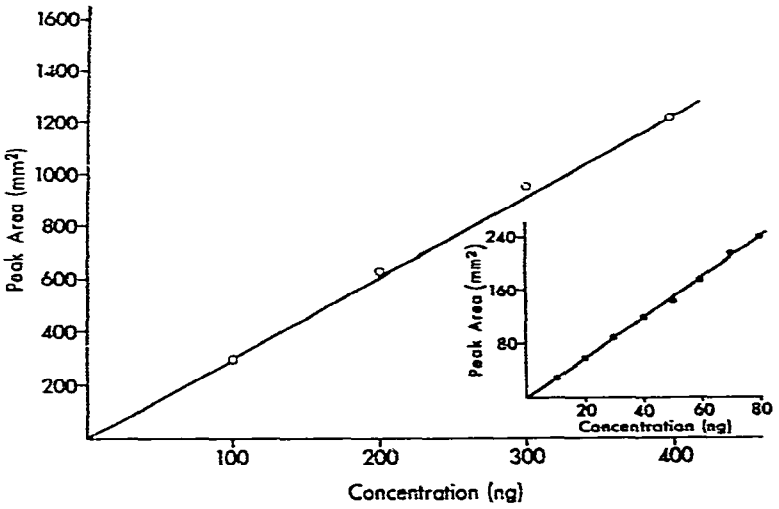


Fig. 2. Plot of peak areas (mm^2) *versus* concentration (ng/1.5 μl) to show linearity of reaction. Each dot represents the mean area from 10 experiments. Inset, plot of peak areas (mm^2) *versus* concentration (ng/1.5 μl) to show the lower response range of the pyridoxine boronate. Each dot represents mean area from 10 experiments.

specificity. The recovery of the target compound is low due to multiple product formation and consequent extraction procedures.

Brooks and Watson[9] introduced the use of boronic acids for the GC analysis of compounds bearing 1,2-diol group in the molecule to achieve specificity of the reaction[6,10-11]. These reagents form highly volatile cyclic boronates which have been used for the GC analysis of 3,4-dihydroxyphenylethyleneglycol, its methoxy derivative and 3,4-dihydroxyphenylacetic acid[6,10-13]. The presence of 1,4-diol group in pyridoxine makes it amenable to such derivitization. The phenolic group of pyridoxine was protected by ether formation with diazomethane prior to boronation.

The method reported here is free of complications since highly specific reagents such as *n*-butylboronic acid and diazomethane have been used for the preparation of the volatile derivative of pyridoxine. Diazomethane reacts only with the phenolic functionality while *n*-butylboronic acid forms cyclic boronates only in the presence of 1,4-diol group. No side products were observed in the presence of large excess of these reagents. Furthermore, organic solvent extraction operations, which reduce the yield of the target compounds, are not needed in this procedure. Therefore the yields are quantitative. Furthermore, the conventional flame ionization detectors are adequate to detect up to 10 ng of pyridoxine. This procedure, therefore has a great potential as a routine assay procedure for pyridoxine. Since pyridoxal and pyridoxamine can be converted into pyridoxine[2,13] these vitamers can also be determined with this method.

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