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Note

New reagent for vitamin B_6 derivative formation in gas chromatography*

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Quantitative analysis of vitamin B_6 can be accomplished through bacterial[1], protazoan[2], and yeast[3] growth methods. These methods have several disadvantages, however, including (1) questionable accuracy in terms of selective specificity for vitamin B_6, (2) they measure only total vitamin B_6 levels without regard to the vitamer present, and (3) they are extremely time consuming.

The relatively ubiquitous presence of the gas chromatograph in even the most basic laboratory and the relative ease of operation makes this method of analysis extremely attractive. The direct analysis of vitamin B_6 is prohibited by its polar, non-volatile nature. Many volatile derivatives of this vitamin have been reported, Korytnyk[4] reviews several, and other, more recent methods are available[5–8]. These methods have various advantages and disadvantages which must be weighed by the analyst. The advent of the relatively new reagent, N-methyl-bis-trifluoroacetamide, provides yet another tool for the analyst and offers the advantage over previous methods of a rapid, clean, and simple analytical procedure.

EXPERIMENTAL

Aqueous solutions (5 m*M*) of pyridoxine hydrochloride (Nutritional Biochemicals, Cleveland, Ohio, U.S.A.), pyridoxamine dihydrochloride (Nutritional Biochemicals), desoxypyridoxine hydrochloride (Nutritional Biochemicals) and pyridoxal hydrochloride (ICN Pharmaceuticals, Cleveland, Ohio, U.S.A.) were prepared. Levels of each of these from 10–200 μl were added to microvials and dried under nitrogen at 70°. To each vial were added 40 μl of 100% ethanol to undergo hemiacetal formation[4] with the aldehyde vitamer to distinguish it from the alcohol after derivatization. The vials with closed tops were refluxed at 125° for 15 min. Evaporation to dryness at 70° under nitrogen removed the excess ethanol. Finally 30 μl of N-methyl-bis-trifluoroacetamide (Regis Chemical Co., Morton Grove, Ill., U.S.A.) was added to each vial, then with closed tops they were refluxed for 20 min at 125°. The samples were cooled and directly injected into the gas chromatograph.

Gas chromatography was carried out with a Tracor MT-220 gas chromato-

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graph fitted with a flame ionization detector (FID). For the FID, the air regulator was set at 20 p.s.i., and a flow-rate of 280 ml/min was used; the hydrogen regulator was set at 10 p.s.i., and a flow-rate of 50 ml/min was used. The detector temperature was 250°.

The column was 6 ft. × 2 mm I.D. glass, packed with 5% silicone oil DC-550 on Chromosorb P AW DMCS, 80–100 mesh (Chemical Research Services, Addison, Ill., U.S.A.). The injection port was operated at 220°, and the column temperature was 150°.

The carrier gas was argon, with a regulator pressure of 40 p.s.i. and a flow-rate of 40 ml/min. Resolution and retention time were dramatically effected by regulator pressure, as well as the flow-rate of the carrier gas.

RESULTS AND DISCUSSION

The DC-550 packing provided good peak resolution of all vitamers (Fig. 1). Alternatively, OV-17 may provide a more rapid analysis but the excess reagent peak may interfere slightly with the desoxypyridoxine and pyridoxine vitamers. Care must be taken in selecting other alternative columns based on the observations of Darbre and Blau[9] that some packings catalytically decompose trifluoroacetyl derivatives of hydroxyl groups. In preliminary column selection this effect seemed to occur.

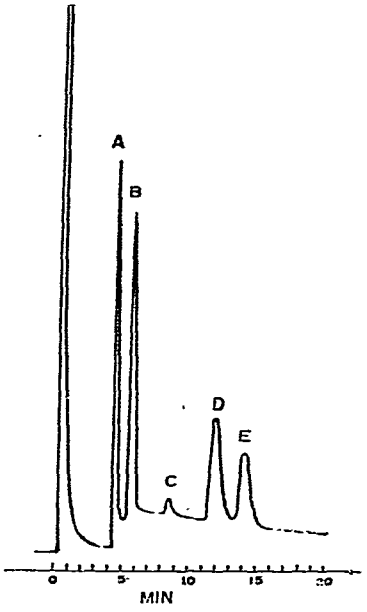

Fig. 1. Chromatogram of vitamin B_6 preparation. Peaks: A = desoxypyridoxine (474 ng); B = pyridoxine (514 ng); C = unidentified; D = pyridoxal (510 ng); E = pyridoxamine (504 ng).

Plots of peak areas, as determined by triangulation, *versus* the weight of the vitamer present in ng showed in all cases a linear response to at least about 250 ng (Figs. 2a–d). The slopes of these linear plots, however, were not the same and this non-proportionality between linearity curves must be recognized and dealt with

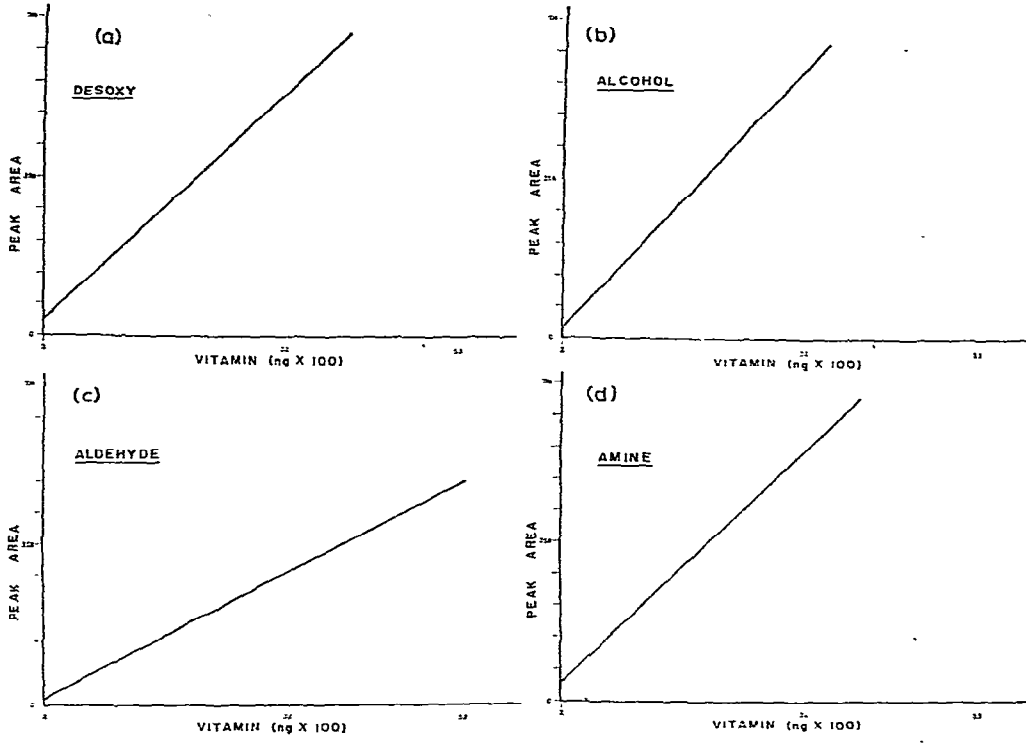

Fig. 2. Relative response in terms of peak area *versus* actual vitamin amounts (ng). (a) Desoxypyridoxine; (b) pyridoxine; (c) pyridoxal; (d) pyridoxamine.

particularly if the desoxypyridoxine is used as an internal standard compound. Ettre and Zlatkis[10] provide a detailed idscussion of the internal standard technique in relation to normalization of relative detector response.

Complete drying of the aqueous solutions is necessary. Incomplete drying of the excess ethanol will produce an extraneous peak.

Imanari and Tamura[7] reported the use of *n*-propylamine to undergo rapid, quantitative Schiff's base reaction with the aldehyde vitamer. It was found, however, that hemiacetal formation provides more consistent quantitative results. In all cases, reflux temperatures should be repeated as accurately as possible because they have dramatic effects on the products formed. A peak eluted immediately (approx. 1 min retention time) after the excess reagent peak, with extreme tailing, occurs when temperatures inside the vial are too low to reflux the N-methyl-bis-trifluoroacetamide reagent. Applied temperatures may have to be adjusted to give an inner vial reflux temperature of 125°.

Based on preliminary data this method may be applicable to quality control in the drug industry for both preliminary batch evaluation and final product quality control, and might also be used for *in vitro* metabolic studies where the vitamin is added to tissue homogenates. It is not, however, applicable to analysis of vitamin B_6 in biological samples since the detection minimum is around 250 ng with the FID. The extremely low levels of this vitamin in biological samples requires a more sensitive

detector. Preliminary work in this laboratory to adapt this method to use with an electron capture detector, or a Coulson electroconductivity detector operated in either the halogen or nitrogen modes, was not fruitful in that an extreme but low level tailing of the excess reagent peak could be seen with these more sensitive detectors.

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