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SHORT COMMUNICATION

A NEW PROCEDURE FOR THE ISOMERIZATION OF VITAMIN D AND ITS METABOLITES

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Summary—A new procedure for the isomerization of vitamin D and its metabolites is described. Vitamin D or its metabolites are dissolved in 100 μl methanol and 10 M HCl in 2-butanol is used as the reagent for isomerization. The isomerization reaction is carried out at 5°C for 2–3 min which gives quantitative yields of isotachysterols down to 10 ng level without use of any carrier.

INTRODUCTION

Determination of vitamin D and its metabolites in body fluids is important to evaluate vitamin D status in various clinical disorders [1]. Because of the thermal rearrangements of vitamin D and its metabolites at higher temperatures, multiple peaks from a single compound were observed in GC analysis [2]. Vitamin D and its metabolites, however, when isomerized to their isotachysterols, each give a single peak during GC analysis [3]. High performance liquid chromatography (HPLC) has also been used for the determination of vitamin D and its metabolites in human plasma after converting these to their corresponding isotachysterols [4]. Seamark *et al.* reported an isomerization technique using HCl to provide quantitative yield for isomerization down to 100 ng level [2]. Below this level it was necessary to add carrier vitamin D in order to avoid any loss by destruction.

In this report, a simple procedure has been developed for the quantitative conversion of vitamin D and its metabolites to their corresponding isotachysterols(s) down to a level of 10 ng without use of any carrier vitamin D. Isomerization was carried out with 10 M HCl in 2-butanol. The isotachysterols thus formed were then analysed by HPLC.

EXPERIMENTAL

Vitamin D2 (ergocalciferol), and vitamin D3 (cholecalciferol) were purchased from Sigma Chemical Co., St. Louis, Mo. 25-OH vitamin D3 (calcifediol) was purchased from United States Pharmacopeia, Rockville, Md. 2-Butanol was from Aldrich Chemical Co., Milwaukee, Wis. HPLC grade hexane, ethylacetate, and methanol were from Fisher Scientific Co., Springfield, N.J. Stoppered centrifuge tubes (40 ml) were used for isomerization.

Isomerization reagent

The reagent, 10 M HCl in 2-butanol (36.5 g HCl in 100 ml 2-butanol), was prepared by passing dry HCl gas into 2-butanol cooled to 0°C.

Aqueous sodium carbonate solution

Reagent grade sodium carbonate was dissolved in distilled water to prepare a saturated solution at room temperature.

Liquid chromatography apparatus

A Laboratory Data Control Constametric III pump equipped with a Rheodyne 7105 syringe injection valve with 50 μl sample loop, SpectroMonitor III variable wavelength detector (LDC, Riviera Beach, Fla) and Hewlett Packard 3390A integrator (Hewlett Packard, Palo Alto, Calif.). Chromatographic column; Spherisorb normal phase, stainless steel column, 25 cm × 2 mm, 3 μm (Phase Separation Inc., Norwalk, Conn.). Mobile phase; hexane:ethylacetate: methanol (97:2.5:0.05). Flow rate; 0.7 ml/min.

Isomerization

A solution of vitamin D or metabolite was placed in a 40 ml centrifuge tube and evaporated to dryness under a slow stream of nitrogen. The contents of the tube were redissolved in 100 μl methanol and cooled to 5°C. The isomerization reagent (0.6 ml), cooled to 5°C, was added to the centrifuge tube and the tube shaken well on vortex mixer for about 15–20 s. The centrifuge tube was kept at 5°C for 2 min while shaking on a vortex mixer 2–3 times in between. After 2 min, 5 ml aqueous sodium carbonate solution was added to the tube and shaken well on the vortex mixer to destroy excess reagent. The isomerized vitamin D or metabolite was extracted by adding 2 ml chloroform to the tube and shaking well on the vortex mixer. The chloroform layer was withdrawn and passed through a small column filled with anhydrous sodium sulfate. A disposable pipet was used for this purpose. The aqueous layer was extracted two more times with 2 ml chloroform each time and the chloroform extract was passed through the same sodium sulfate column. All the extracts were collected in a 15 ml centrifuge tube and evaporated to dryness under a slow stream of nitrogen. The sample was redissolved in 250 μl of hexane and analysed by HPLC.

RESULTS

Isomerization was carried out as described in the Experimental Section and a number of parameters were varied to optimize the reaction conditions. It has been shown in earlier reports that vitamin D and its metabolites isomerize similarly [2]. Therefore, detailed experiments were carried out only on 50 ng quantity of cholicalciferol unless otherwise specified.

A standard curve was plotted for the concentration of isotachysterol versus peak height on HPLC, and the yields of the reactions were calculated by comparing the peak heights obtained by HPLC analysis of the isotachysterol with standard curve.

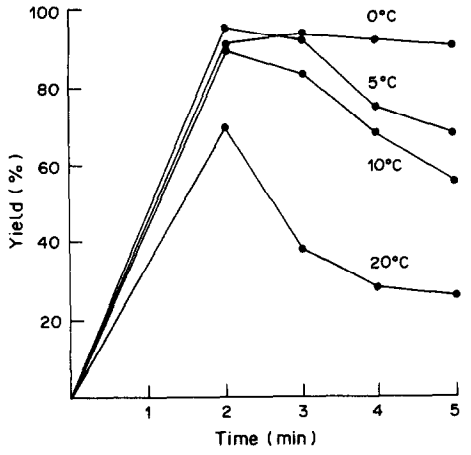

Fig. 1. Isomerization of vitamin D3 to isotachysterol at various temperatures.

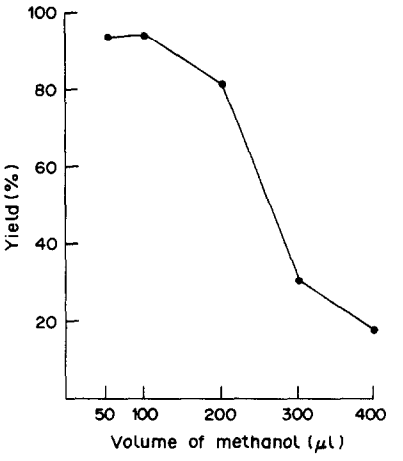

Fig. 2. Effect of volume of methanol on the isomerization of vitamin D3.

Suitability of the solvent

Seamark *et al.*[2] reported that the chloroform or dichloromethane was the best solvent for isomerization. No alcohols were tried as solvent in their study [2]. In this study, however, it was considered more appropriate to use alcohol as a solvent due to greater miscibility with the reagent which was prepared in 2-butanol. Both the solvents, chloroform and methanol, were tried and yields were higher when methanol was used.

Effect of time and temperature

Isomerization was carried out at various temperatures ranging from 0 to 20°C. The reaction time was varied from 2 to 5 min and it was observed that the yields were lower at 5 min at all the temperatures except at 0°C (Fig. 1). The maximum yield was obtained when the reaction was performed at 5°C for 2 min. No significant loss of the isomerized product was observed up to 3 min at 5°C. This suggested that the reaction can be carried out at 5°C for a period of 2–3 min to obtain the maximum yield.

Effect of volume

The volume of the reaction mixture played a significant role on isomerization and it was observed that 50 to 100 μl was the optimum reaction volume (Fig. 2). Any increase in the volume above 100 μl significantly reduced the isomerization yield, presumably due to the dilution of the derivatizing reagent. This also suggested that the concentration of the derivatizing reagent was also important.

Isomerization was carried out in the range of 10–1000 ng of vitamin D3 and the yields in all cases were above 93%. Two other compounds, vitamin D2 (ergocalciferol) and 25-OH vitamin D3 (calcifediol) were isomerized under similar conditions at 50 ng level and over 92% yield were obtained.

DISCUSSION

Isotachysterol has absorbtion maxima at 278, 288. and 301 nm and its molar absorption is more than double compared to that of vitamin D at 265 nm. Therefore, when monitored at 290 nm for HPLC analysis, it provides greater sensitivity and selectivity.

Isomerization of vitamin D and its metabolites is a very rapid reaction and a slight increase in reaction time may lead to the reduction in isomerization yield due to the destruction of isotachysterol, thus making quantitation difficult. For this reason it was found essential to destroy the excess reagent immediately after completion of the reaction. Initially, a similar approach as taken by Seamark *et al.*[2] was used which involved removing the excess reagent under a slow stream of nitrogen. It was however, observed that during the evaporating period some destruction of isotachysterol occurred, especially at a low level of vitamin D. In order to overcome this problem an aqueous sodium carbonate solution was used to destroy the excess reagent after the isomerization reaction was completed. This procedure provides a simple method for quantitative conversion of vitamin D and its metabolites to their corresponding isotachysterols down to 10 ng level.

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