KINETICS OF THE FORMATION OF PANTOLACTONE FROM PANTOTHENATES AND ITS QUANTITATIVE DETERMINATION

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The conditions of hydrolysis and lactonization of the calcium and sodium salts of pantothenic and pantoic acids have been studied. The selected conditions of hydrolysis (t = 80°C, C_{HCl} = 13%) led to a 100% yield of β-alanine and pantothenates after 60 min from the beginning of hydrolysis, while the formation of pantolactone was complete in 105 min for the calcium salt and in 75 min for the sodium salt. The hydrolysis and lactonization reactions have been performed under the conditions mentioned, and the GLC of the pantolactone formed has permitted the determination of various concentrations of calcium pantothenate. The results obtained from the GLC analysis of pantolactone indicate the possibility of a quantitative determination of salts of pantothenic and pantoic acids. The sensitivity of this method is 0.2-5 nmole.

In vitaminological investigations highly sensitive but insufficiently specific microbiological methods of analyzing pantothenic acid (I) have come into wide use [1]. Nevertheless, as early as the beginning of the seventies attempts were made to use gas chromatography for the quantitative determination of a cyclic derivative of pantoic acid — pantolactone — (II) or a volatile derivative of another component of (I) — β-alanine (III) [2-4]. An approach connected with the hydrolysis of (I) to (II) and the determination of (II) by GLC proved to be promising, the method being suitable both for the investigation of the level of (I) in human biological fluids [5, 6] and for the analysis of the amount of vitamin in food products [7]. A group of investigations performed in the laboratory of the Institute of Nutrition of the University of Bonn under the direction of Professor Hetzel [4, 7] permitted the GLC of pantothenate in biological materials to be carried out with a resolving capacity of the method of about 1 nmole of the vitamin. It has also been reported that quantitative GLC can be applied to the investigation of the coenzyme form of I — CoA [8]. However, in our preceding paper [9] attention was directed to certain difficulties in the hydrolysis of (I)-containing compounds. Furthermore, up to the present time there has been no adequate information in the literature on the kinetics of the hydrolysis of (I) and its salts, which gives rise to certain contradictions in the quantitative evaluation of the results of the determination of (I) by different methods.

The aim of the present investigation was, in the first place, to select the correct conditions for the hydrolysis of (I) for its quantitative determination and, in the second place, to select conditions for the chromatography of (II) ensuring its quantitative determination in the range of 0.2-1 μg, which will permit this method of analysis to be used for measuring amounts of the vitamin in biological fluids and tissues of small laboratory animals.

In the first stage of the investigations, in the light of literature information on the nature of the temperature factor and of the time of hydrolysis (t = 80°C, τ = 150 min) [3-6], we studied the influence of the acidity of the medium on the formation of (II) from calcium pantothenate (IV).

The complete hydrolysis of (IV) to (II) was achieved at an HCl concentration of 13%. In light of the different stabilities of the salts of (I), comparative investigations have been made of the hydrolysis of (IV) and of sodium pantothenate (V), the degrees of hydrolysis being determined simultaneously from the yields of (II) and (III). The first product was determined by GLC and the second spectrophotometrically from the colored complex with sodium 1,2-naphthoquinonesulfonate.

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In an investigation of the kinetics of the hydrolysis of (IV) and (V) under the conditions mentioned above, it was established that the formation of (III) took place identically from both compounds and reached a 100% yield within 60 minutes from the beginning of hydrolysis. The formation of (II) was completed (100% yield) for (IV) after 105 min, and for (V) after 75 min. Thus, the formation of (II) lagged behind that of (III). In this connection, it appeared necessary to investigate the lactonization reaction of pantoic acid.

The cyclization of the pantoic acid salts was completed 30 minutes earlier for (V) than for (IV). These results confirm those of our previous studies [9].

In the acid hydrolysis of amides, it is assumed [10] that the reaction takes place by an A_{AC2} mechanism and the rate of the hydrolysis reaction is proportional to the product of the concentration of the amide and the concentration of hydrogen ions, $V = K[A]^n[H^+]^n$ (the concentration of amide in any time interval was determined from the concentration of β-alanine). Since the concentration of H^+ is far greater than that of the pantothenate, we can write $V = K'[A]^n$, where K' is the observed rate constant of the reaction. To calculate the order and the rate constant of the reaction we used the graphical variant of Van't Hoff's differential method over the complete kinetic curve [11]. A graph in the coordinates $-\log V$ and $-\log C$ consisted of a straight line the tangent of the angle of slope of which gave the order of the reaction as n = 1.5, and the rate constant of the reaction was determined from the kinetic equation as $K = 0.569$ ($\text{liter}^{0.5} \cdot \text{mole}^{-0.5} \cdot \text{min}^{-1}$). The order and rate constant for the lactonization of salts of pantoic acid were found similarly. For (V) and (IV) the order of the reaction was 1.5 and the rate constants were, respectively, 0.222 and 0.123 ($\text{liter}^{0.5} \cdot \text{mole}^{-0.5} \cdot \text{min}^{-1}$).

Taking into account the conditions selected, the hydrolysis, lactonization, and the GLC of various concentrations of (IV) were performed. This showed a linear dependence of the ratios of the products of the retention time and the height of the pantolactone peak to the analogous product of the standard on the amount of pantolactone in the sample. The limit of detection or the sensitivity of the method, which permits (I) and its derivatives to be determined, was 0.2-1 μg in a sample. Since the amount of vitamin and its derivatives in the tissues of experimental animals varies between 100 and 500 nmole and in biological fluids from 5 to 20 nmole, the GLC method for the determination of (I) that is under consideration can be used successfully for investigating biological samples.

EXPERIMENTAL

The investigation was carried on a chromatograph of the Biokhrom 1 type with a flame ionization detector (FID). After preliminary testing, as the stationary phase we used Chromaton N-AW with a particle size of 0.200-0.250 mm impregnated with XE-60 silicone (Chemopol) in 3 m × 3 mm steel columns. The chromatography of the pantolactone and of γ-butyrolactone (internal standard) was carried out under the following conditions: temperature of the column thermostat 140°C and of the detector 250°C; linear rate of flow of hydrogen 35 ml/min, of gas 20 ml/min and of the carrier gas, helium, 40 ml/min.

A 250-mg sample of pantothenate (pantoate) was dissolved in water in a 50-ml measuring flask. Aliquots of 2.5 ml were taken and transferred quantitatively to 25-ml measuring flasks. To each of these was added 6.5 ml of 18% hydrochloric acid. The flasks were thermostated in a thermostat at 80°C for 105 min.

Determination of (II). After cooling, the volume of the reaction mixture in the flask was made up with water to 25 ml and when it had been stirred for 5 min 3 ml of the resulting mixture was extracted with chloroform (3 × 2 ml). The organic fractions were combined, and 1 ml of a 0.0116 M freshly prepared chloroform solution of γ-butyrolactone was added. The solution obtained was concentrated in the rotary evaporator to 1 ml, and 1 μl of the mixture obtained was injected into the evaporator. Two symmetrical peaks appeared on the chromatogram: of γ-butyrolactone and of (II), with retention times of 376 and 918 sec, respectively. To determine the amount of (II) in the sample, a calibration graph of the dependence of the ratio of the products of the retention time and the height of the peak of (II) to the analogous product for γ-butyrolactone on the amount of substance in the sample was plotted.

Determination of (III). To the flask containing the reaction mixture after hydrolysis and cooling was added two drops of 0.2% phenolphthalein solution, and the mixture was neutralized with 2 M caustic soda solution to a pink coloration, and then 0.1 M HCl was added dropwise until the coloration had disappeared. The volume of the solution was made up to

the mark (25 ml) with water and it was mixed. For the reaction, 2 ml of the solution prepared and 2 ml of pantothenate solution before hydrolysis (with the same concentration) — a control experiment for free (III) — were transferred to two 25-ml measuring flasks. Then to each were added 2 ml of 1.5% sodium tetraborate and 1 ml of 0.5% sodium naphthoquinonesulfonate, and the mixtures were kept in the boiling water bath for 10 min. After cooling, 2 ml of an acid solution of formaldehyde and 2 ml of 0.1 N solution of sodium thiosulfate were added to each and, after mixing, the volumes were made up to the marks with water. After 20 min, the optical densities of the solutions under investigation were measured on a spectrophotometer at a wavelength of 465 nm in a cell with a layer thickness of 1 cm against a control solution of the reagents. To determine the amount of (III) in the sample a calibration graph for the dependence of the optical density on the amount of (III) in the sample was plotted previously.

SUMMARY

1. The optimum conditions have been found for the formation of pantolactone from calcium and sodium pantothenates at $t = 80°C$, $C_{HCl} = 13\%$, $\tau = 105$ min.

2. The quantitative GLC of pantolactone has been performed, which ensures the determination of salts of pantothenic acid in amounts of from 0.2 to 5 nmole (minimum concentration).

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