Stability of Retinol Analogs. IX.1) Stability of Vitamin A Acetate in Aqueous Ethanolic Solutions and Quantitative Analysis of Its Decomposition Products by High Performance Liquid Chromatography

YASUJI TAKASHIMA, TOSHIKI NAKAJIMA, SHIGEO TANAKA, MITSUNORI WASHITAKE, TOSHI ANNO,2) and HIDEO MATSUMARU3)

Research Laboratory, Taisho Pharmaceutical Co., Ltd.2) and Tokyo College of Pharmacy2)

(Received December 18, 1978)

The stability of vitamin A (VA) acetate in ethanolic solutions with various water contents and the formation and disappearance of its major decomposition products were studied quantitatively by high performance liquid chromatography (HPLC) using Li-Chrosorb Alox T (basic alumina). Three major decomposition products were fractionated by HPLC and identified as anhydride VA, 4-ethoxy anhydride VA, and VA ethyl ether. VA ethyl ether has an ultraviolet (UV) spectrum similar to that of VA acetate and caused interference with the estimation of VA acetate in these test solutions by UV spectrophotometry. The structure of 4-ethoxy anhydride VA, which has a UV spectrum similar to that of the retro VA analog, was assigned by mass spectroscopy, nuclear magnetic resonance, UV, and infrared analyses. This substance was largely converted into anhydride VA in aqueous ethanolic solution and is thus considered to be a precursor of anhydride VA.

Keywords—VA acetate; stability; aqueous ethanolic solution; anhydride VA; VA ethyl ether; 4-ethoxy anhydride VA; high performance liquid chromatography

Vitamin A (VA) is often used in aqueous pharmaceutical preparations such as creams and syrups, in which it is generally decomposed rapidly because of the water content. In these aqueous preparations, VA is usually solubilized with surfactants, and the stability of VA is affected by the nature of the surfactants and the state of solubilization.3)

In the previous paper of this series,4) the effect of water content on the stability of VA acetate and its decomposition mechanism was investigated in ethanolic solutions with various water contents without surfactants by alumina column chromatography. In this paper, VA acetate and its decomposition products in these solutions were investigated by high performance liquid chromatography (HPLC) which has better resolution.

Applications of HPLC have been reported for the determination of VA in pharmaceutical preparations such as creams and tablets,5) in food,6) liver,7) and serum,8) and the separation of VA isomers.9) However, the separation of the decomposition products of VA acetate by HPLC has not been reported. In this study, the HPLC conditions for estimation of VA acetate and its decomposition products were established, and the stability of VA acetate and its decomposition mechanism were investigated.

2) Location: a) 1-403 Yoshino-cho, Omiya-shi, Saitama-ken, 330, Japan; b) 1432-1, Horinouchi, Hachioji, Tokyo, 192-03, Japan.
Experimental

Preparation of Test Solutions—All-trans-VA acetate pure crystals (VA acetate) were obtained from Hoffmann-La Roche Co., Ltd., Basel. VA acetate was dissolved in absolute EtOH, 90%, and 60% (v/v) aqueous EtOH at a concentration of 20 mg/100 ml. These solutions were poured into amber ampules and stored at 35°, 40°, or 50° for 7 days.

Determination Methods—HPLC Method: VA acetate and its major decomposition products in test solutions were determined quantitatively by HPLC. One ml aliquots of the absolute ethanolic test solutions were evaporated to dryness under reduced pressure and dissolved in 1 ml n-hexane. Aqueous ethanolic test solutions were extracted with n-hexane as follows: 30 ml of n-hexane was added to 1 ml of the test solutions and the mixture was shaken for 5 min then centrifuged for 3 min, and 25 ml of the upper layer was taken and dried over 10 g of anhydrous Na₂SO₄. This solution was evaporated to dryness under reduced pressure and dissolved in 1 ml of n-hexane. Thirty μl of each prepared sample was subjected to HPLC. The concentrations of VA acetate and its major decomposition products in the test solutions were calculated by means of calibration curves as described later. Volumetric corrections were made, especially for the aqueous ethanolic test solutions. The instrument used was a Hitachi 633 liquid chromatograph with a 50 cm × 4.0 mm i.d. stainless steel column packed with LiChrosorb Alox T (10 μm) (E. Merck, Darmstadt, Germany) containing 3% H₂O by the dry packing technique. Other conditions were as follows. Eluent, 1% ether in n-hexane; flow rate, 1.0 ml/min (80 kg/cm²); temperature, ambient; detector, ultraviolet (UV) absorption at 350 nm (VA acetate and its major decomposition products are detectable); sensitivity, 0.2 AUFS.

UV Method: The determination of VA acetate in the test solutions was also carried out by UV spectrophotometry as follows, 1 ml of test solution was diluted with isopropanol to 50 ml and the absorbance at 326 nm was measured by a Shimadzu UV-200 double beam spectrophotometer.

Fractionation of Decomposition Products—Two hundred mg of VA acetate was dissolved in 1000 ml of absolute EtOH, and this solution was poured into amber ampules. After storage at 50° for 2 days, it was evaporated to dryness under reduced pressure and dissolved in 10 ml of n-hexane. About 1000 μl of this solution was subjected to preparative HPLC and the main decomposition products of VA acetate were collected after passage through a detector cell, removing small amounts of the initial and final regions of peaks in the chromatogram. The column for this preparative HPLC was a 50 cm × 8.0 mm i.d. stainless steel column packed with LiChrosorb Alox T (10 μm) containing 2% H₂O by the dry packing technique. Other conditions for preparative HPLC were as follows. Eluent, 2% ether in n-hexane; flow rate, 2.0 ml/min (20 kg/cm²); temperature, ambient; sensitivity, 10 AUFS. The same Hitachi 633 instrument was used.

Procedure for Thin-Layer Chromatography (TLC)—The decomposition products were examined by TLC with 250 μm Silica Gel 60 precoated plates without fluorescence indicator (Merck, Darmstadt, Germany). The coloration reagent was conc. H₂SO₄, and the developing solvent was ether: n-hexane (15:85). Fifty μl of solution extracted with n-hexane by the above-mentioned method from each test solution was applied to the plate.

Identification of Decomposition Products of VA Acetate—UV spectra were obtained with a Shimadzu UV-200 double beam spectrophotometer. Infrared (IR) spectra were obtained with a JASCO IRA-2 diffraction grating infrared spectrophotometer using the liquid film method. Nuclear magnetic resonance (NMR) spectra were obtained in a JNM-FX 100 MHz spectrometer using tetramethylsilane as an internal standard. Mass (MS) spectra were obtained using a Shimadzu LKB GC–MS, model 9000, by the direct injection method.

Results and Discussion

TLC Analysis

TLC results for VA acetate stored in 90% aqueous EtOH at 35° are shown in Fig. 1. In the initial sample, VA acetate was detected at Rf 0.31, and a trace of isomer was noted at Rf 0.37. In the stored samples, besides these two spots, several spots such as Rf 0.68 (anhydro VA), Rf 0.60 (substance I), Rf 0.55 (substance II), Rf 0.45 (VA ethyl ether), Rf 0.10 (VA alcohol) and some trace spots tailing from the application point to Rf 0.10 were noted.
However, the latter spots were scarcely observable at 7 days. Standard anhydro VA, VA ethyl ether, and VA alcohol were also developed (e, f, and g, respectively, in Fig. 1). These standard samples were prepared and identified by the methods described later.

**Separation of Decomposition Products by HPLC**

VA acetate and VA ethyl ether could not be separated by the alumina column chromatography described in the previous report, and substances I and II could not be recognized in absolute EtOH in 60% EtOH

![Chromatograms](image)

**Fig. 2. Chromatograms of Vitamin A Acetate and Its Decomposition Products on Storage in Ethanolic Solutions at 35°**

- initial, ————: 1 day, ————: 4 days.
- a, anhydro vitamin A; b, substance II; c, vitamin A ethyl ether; d, vitamin A acetate isomer; e, vitamin A acetate.
- Conditions: column, LiChrosorb Alox T 10 µm (50 cm×4.0 mm i.d.); eluent, 1% ether in n-hexane; flow rate, 1.0 ml/min; detector, UV at 350 nm; temperature, ambient; sensitivity, 0.2 AUFS.

![Calibration Curves](image)

**Fig. 3. Calibration Curves for Vitamin A Acetate and Its Decomposition Products in HPLC**

- ■-■: vitamin A acetate,
- ○-○: anhydro vitamin A,
- △-△: substance II,
- □-□: vitamin A ethyl ether.
clearly. Thus, HPLC conditions which could separate these decomposition products were established.

Chromatograms of VA acetate stored in absolute EtOH and 60% aqueous EtOH at 35° are shown in Fig. 2. The retention times of VA acetate and its major decomposition products were as follows: anhydro VA (5 min), substance II (8 min), VA ethyl ether (11 min), isomer of VA acetate (20 min), and VA acetate (26 min). These peaks were identified by TLC, UV, and MS after fractionation. Substance I, which was recognized as a faint spot in TLC, could not be seen in these HPLC chromatograms, because its amount was small and it had little UV absorption at 350 nm. To check the retention time of substance I, the eluate from anhydro VA to substance II was collected in large amounts by preparative HPLC. This eluate was condensed and subjected to quantitative HPLC. By detection of UV absorption at 272 nm, a peak of substance I could be seen between anhydro VA and substance II. This absorption is the UV maximum of substance I. However, this substance did not interfere with the determination of anhydro VA and substance II by HPLC detected by means of the UV absorption at 350 nm.

**HPLC Calibration Curves for VA Acetate and Its Major Decomposition Products**

VA acetate purified by preparative HPLC was used as the standard sample. Substance

![Graph showing rates of decomposition](image-url)

**Fig. 4. Rates of Decomposition of Vitamin A Acetate and Formation of Its Products in Absolute Ethanolic Solutions at 35°, 40°, or 50°**

- ■: vitamin A acetate,
- ○: anhydro vitamin A,
- △: substance II,
- □: vitamin A ethyl ether.

*a) The molecular weight of substance II was obtained from its mass spectrum (M*, 514).*

![Graph showing rates of decomposition](image-url)

**Fig. 5. Rates of Decomposition of Vitamin A Acetate and Formation of Its Products in Aqueous Ethanolic Solutions at 35°**

- ■: vitamin A acetate,
- ○: anhydro vitamin A,
- △: substance II,
- □: vitamin A ethyl ether.
II and VA ethyl ether were also prepared from stored test solutions in which VA acetate and its decomposition products were contained by preparative HPLC. Anhydro VA was prepared by the method described in the previous report. These purified substances were used as standard samples. Each standard sample was dissolved in n-hexane at various concentrations and subjected to HPLC. The calibration curves are shown in Fig. 3. The curves for the decomposition products were based on peak height, but the curve for VA acetate was based on peak area, because the peak shape was broad.

**Stability of VA Acetate and Formation of Its Major Decomposition Products**

The results of quantitative analysis of VA acetate stored in absolute EtOH at 35°, 40°, or 50° are shown in Fig. 4. At 35°, VA acetate was decomposed slowly and the amounts of its major decomposition products increased gradually up to 7 days; substance II was the major product, followed by VA ethyl ether, and anhydro VA in that order. The decomposition rate of VA acetate and the formation rates of its decomposition products at 40° were greater than at 35°. The three decomposition products were formed in almost equal amounts after 6 days at 40°. At 50°, the decomposition of VA acetate and the formation of its decomposition products were most rapid. Substance II increased for 2 days and then decreased, while VA ethyl ether increased for 6 days and then decreased, but anhydro VA continued to increase up to 7 days. At 6 days, VA ethyl ether was the major product, followed by anhydro VA and substance II in that order.

VA acetate was stored in 90% and 60% aqueous EtOH at 35° to examine the effects of water on its decomposition and on the formation and disappearance of its decomposition products. The results are shown in Fig. 5. In 90% aqueous EtOH, VA acetate decomposed more rapidly than in absolute EtOH, and the formation rates and amounts of anhydro VA and VA ethyl ether were greater than in absolute EtOH, but substance II was formed more slowly and its amount was less.

In 60% aqueous EtOH, VA acetate decomposed rapidly; the residue at 1 day was less than about 10%. Anhydro VA was formed rapidly as VA acetate was decomposed; it increased in amount for 1 day and then decreased. VA ethyl ether was also formed to a marked extent at 1 day and its amount subsequently remained almost unchanged. Under the conditions used, substance II decomposed so fast that it was scarcely present at 1 day and could not be detected after 3 days.

As the relationship between storage time and the logarithm of the residual VA acetate as determined by HPLC showed good linearity for given storage conditions, this reaction was regarded as an apparent first-order reaction. The rate constants \((k)\) were calculated by the least-squares method, and from these data the half-lives were obtained. These results are shown in Table I. It can be seen that the decomposition rate increased with increasing temperature. A comparison of the stability in the presence of various water contents showed that the \(k\) value increased with increasing water content.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Temp. (°C)</th>
<th>(k(\text{hr}^{-1}))</th>
<th>(r(\text{hr}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute EtOH</td>
<td>35</td>
<td>3.21 \times 10^{-3}</td>
<td>2.15 \times 10^2</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>6.50 \times 10^{-3}</td>
<td>1.07 \times 10^2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.17 \times 10^{-2}</td>
<td>3.19 \times 10</td>
</tr>
<tr>
<td>90% EtOH</td>
<td>35</td>
<td>1.53 \times 10^{-2}</td>
<td>4.53 \times 10</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>2.97 \times 10^{-2}</td>
<td>2.33 \times 10</td>
</tr>
<tr>
<td>60% EtOH</td>
<td>35</td>
<td>1.42 \times 10^{-1}</td>
<td>4.88</td>
</tr>
</tbody>
</table>

Comparison of the Residual VA Acetate as determined by the HPLC and UV Methods

The residual VA acetate after storage in 90% aqueous EtOH at 40° and in 60% aqueous EtOH at 35° was determined by the UV method and compared with that determined by HPLC. The results are shown in Fig. 6. The residual amounts determined by the UV method were greater than those found by HPLC under all conditions. This indicates that VA acetate was separated from its decomposition products by HPLC, whereas decomposition products such as VA ethyl ether, which have the same absorption spectrum as VA acetate, were determined in addition to VA acetate by the UV method. When decomposition products such as VA ethyl ether were formed, they could not be checked by the \( f \) value (JP-IX), so the UV method should not be used in this case.

![Graph showing comparison of residual vitamin A acetate by HPLC and UV](image)

**Fig. 6. Comparison of Residual Vitamin A Acetate determined by the HPLC and UV Methods**

- •: in 60% EtOH at 35°
- ••: in 90% EtOH at 40°

<table>
<thead>
<tr>
<th>Substance I</th>
<th>Substance II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TLC (Rf)</strong></td>
<td>0.60 (15% ether in n-hexane)</td>
</tr>
<tr>
<td><strong>MS m/e</strong></td>
<td>314 (M⁺), 299 (M−CH₃)</td>
</tr>
<tr>
<td>285 (M−CH₂CH₃), 269 (M−OCH₃CH₂)</td>
<td>285 (M−CH₂CH₃), 269 (M−OCH₃CH₂)</td>
</tr>
<tr>
<td>268 (M−OCH₂CH₃, H)</td>
<td>268 (M−OCH₂CH₃, H)</td>
</tr>
<tr>
<td>253 (M−OCH₂CH₃, CH₃, H)</td>
<td>253 (M−OCH₂CH₃, CH₃, H)</td>
</tr>
<tr>
<td><strong>NMR δ (CDCl₃)</strong></td>
<td>—</td>
</tr>
<tr>
<td>1.30, 1.35 (s, each, C₆H−(CH₃)₂)</td>
<td>1.30, 1.35 (s, each, C₆H−(CH₃)₂)</td>
</tr>
<tr>
<td>1.58 (s, C₆H−CH₃, C₂H₅−CH₃)</td>
<td>1.58 (s, C₆H−CH₃, C₂H₅−CH₃)</td>
</tr>
<tr>
<td>1.92 (s, C₂−CH₃)</td>
<td>1.92 (s, C₂−CH₃)</td>
</tr>
<tr>
<td>1.15 (t, ( J = 6.9 ) Hz, OCH₃CH₃)</td>
<td>1.15 (t, ( J = 6.9 ) Hz, OCH₃CH₃)</td>
</tr>
<tr>
<td>3.40 (q, ( J = 6.9 ) Hz, OCH₃CH₃)</td>
<td>3.40 (q, ( J = 6.9 ) Hz, OCH₃CH₃)</td>
</tr>
<tr>
<td>( \lambda_{max} ) nm (( E_{1%} ))</td>
<td>( \lambda_{max} ) nm (( E_{1%} ))</td>
</tr>
<tr>
<td>272, 283</td>
<td>330 (830), 345 (1260), 363 (1160)</td>
</tr>
<tr>
<td>UV</td>
<td>2920: −OCH₂CH₃</td>
</tr>
<tr>
<td>IR ν_{max} cm⁻¹</td>
<td>1570, 1610: polyene (C=C)</td>
</tr>
</tbody>
</table>
Investigation of Decomposition Products

Anhydro VA and VA ethyl ether were identified by comparison with authentic samples as described in the previous reports.\textsuperscript{10,11} Substances I and II were investigated to determine their structures and physical properties. These results are shown in Table II.

Substance I—Substance I appeared at $R_f$ 0.60 on TLC and was eluted between anhydro VA and substance II on quantitative HPLC when detected by means of the UV absorption at 272 nm. The MS spectrum of substance I is shown in Fig. 7. The main MS peaks were as follows, 314 ($M^+$), 299 ($M^+\text{–}CH_3$), 285 ($M^+\text{–}CH_3CH_3$), 269 ($M^+\text{–}OCH_3CH_2$), 268 ($M^+\text{–}OCH_3CH_2\text{–}H$), 253 ($M^+\text{–}OCH_3CH_2\text{–}CH_2\text{–}H$). The UV spectral changes of substance I stored in 60\% aqueous EtOH at 50° are shown in Fig. 8. Its absorption maxima appear at 272 and 283 nm, and no absorption at 330–350 nm was noted immediately after it had been fractionated. The results indicate that substance I is converted into substance II and then into anhydro VA, because the UV spectrum of substance I changed into one similar to that of substance II and the absorbance at 390 nm (one of the absorption maxima of anhydro VA)

increased. The NMR and IR spectra of substance I could not be obtained because it was formed in small amounts and decomposed rapidly.

The MS spectrum of substance I suggested that it was formed by loss of an acetyl group from VA acetate and introduction of an ethoxy group. Therefore substance I was regarded as a precursor of substance II and was thought to have a similar structure.

**Substance II**—Substance II appeared at \( Rf \) 0.55 on TLC and the retention time was 11 min in quantitative HPLC. As it was relatively stable and formed in considerable amounts, the physical properties could be determined after purification.

The MS peaks \((m/e)\) were as follows: 314 (M⁺), 299 (M⁺—CH₃), 285 (M⁺—CH₂CH₃), 269 (M⁺—OCH₂CH₃), 268 (M⁺—OCH₂CH₃, H), 253 (M⁺—OCH₂CH₃, CH₃, H). These peaks were similar to those of substance I, as shown in Fig. 7. The parent peak of substance II was higher than that of substance I. A peak at \( m/e \) 269 in both substances (due to removal of an ethoxy group) was recognized. In substance I, the parent peak was low and the peak at \( m/e \) 299 derived by loss of a methyl group was high. Thus, substance II seems to be more stable than substance I, and both substances readily release an ethoxy group, yielding anhydro VA.

![UV Spectral Changes of Substance II on Storage in Ethanolic Solutions at 50°](image)

Fig. 9. UV Spectral Changes of Substance II on Storage in Ethanolic Solutions at 50°

- **in 60% EtOH**
  - : initial
  - ---: 1 hr,
  - ---: 2 days.
- **in 80% EtOH**
  - : initial
  - ---: 3 days,
  - ---: 4 days.
- **in absolute EtOH**
  - : initial
  - ---: 7 days,
  - ---: 14 days.

The initial spectrum of substance II is shown in Fig. 9. Its absorption maxima appear at 330, 345, and 363 nm, suggesting that it has an \( \alpha \)-ionone ring and a vinlylog structure, such as retro VA or anhydro VA.

The NMR spectra of substance II and a partial spectrum of anhydro VA are shown in Fig. 10. In the NMR spectrum of substance II, signals at 1.15 ppm (\( t, J=6.9 \) Hz, -OCH₂CH₃) and 3.40 ppm (q, \( J=6.9 \) Hz, -OCH₂CH₃) were recognized and these signals indicate that an ethoxy group is present. As no signals due to a \( C_4 \)-proton (5.7 ppm) in anhydro VA could be recognized in this spectrum of substance II, this may be the position of the ethoxy group. In addition to these signals, a signal at 0.88 ppm (t, \( J=5.7 \) Hz) was seen, so substance II had a methyl group in the terminal region of the polyene. The structure of substance II thus appears to be 4-ethoxy anhydro VA, as shown in Fig. 10.

Fig. 10. NMR Spectra of Substance II and Partial NMR Spectrum of Anhydro Vitamin A (in CDCl₃, 100 MHz, TMS)

However, retro VA ethyl ether and isoanhydro VA were considered as analog substances which have an α-ionone ring, an ethoxy group, and a molecular weight of 314. These structures are shown in Chart 1.

Chart 1

The formation of retro VA ether has been reported in pharmaceutical preparations, but its structure has not really been investigated especially in relation to the introduced alkoxy group and its position. Γ Retro VA ethyl ether must have C₄-H (5.7 ppm, t) and C₁₄-CH₂O- (4.0 ppm, t) groups. Substance II lacks these signals, so it is not retro VA ethyl ether.

Isoanhydro VA has been reported by Oroshnik¹³ and Tsukida,¹² so this substance was prepared by their methods and compared with substance II. The Rf value of isoanhydro VA (Rf 0.50) in the TLC differed from that of substance II (Rf 0.55). In the NMR, isoanhydro VA shows C₄-H (5.7 ppm, t) and C₁₄-CH₃ (1.24 ppm, d) signals. Substance II lacks these signals, so it is not isoanhydro VA either. Thus, substance II was regarded as 4-ethoxy anhydro VA.

The IR bands of substance II were as follows: IR cm⁻¹, 2920 (C₄-OCH₂CH₃), 1450 (–CH₃), 1385 (C₃-C(CH₃)₂), 1570 (polyene, C=C). These bands are consistent with the proposed structure.

**Conversion of 4-Ethoxy Anhydro VA to Anhydro VA**

Purified 4-ethoxy anhydro VA was dissolved in absolute, 80%, and 60% aqueous EtOH at a concentration of about 20 µg/ml. These solutions were poured into amber ampules and stored at 50°. The UV spectral changes are shown in Fig. 9. With increasing storage time, an increase of UV absorption at 390 nm owing to the formation of anhydro VA was recognized. To examine the formation of anhydro VA and other decomposition products, these samples were subjected to HPLC. The chromatograms are shown in Fig. 11. Two peaks were noted.

![Chromatograms of Substance II and Its Decomposition Products on Storage in Ethanolic Solutions at 50°](image)

**Fig. 11.** Chromatograms of Substance II and Its Decomposition Products on Storage in Ethanolic Solutions at 50°

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60% EtOH</td>
<td>0</td>
</tr>
<tr>
<td>80% EtOH</td>
<td>0</td>
</tr>
<tr>
<td>Absolute EtOH</td>
<td>0</td>
</tr>
</tbody>
</table>

In 60% EtOH: initial, 1 hr, 2 days. In 80% EtOH: initial, 3 days, 5 days. In absolute EtOH: initial, 7 days, 14 days.

HPLC conditions were the same as in Fig. 2.

![Rates of Decomposition of Substance II and Formation of Its Decomposition Products in Ethanolic Solutions at 50°](image)

**Fig. 12.** Rates of Decomposition of Substance II and Formation of Its Decomposition Products in Ethanolic Solutions at 50°

at 5 and 11 min. The former and latter peaks were identified as anhydro VA and VA ethyl ether, respectively, by UV, MS, and TLC. The rates of decomposition of 4-ethoxy anhydro VA and formation of anhydro VA and VA ethyl ether are shown in Fig. 12. The decomposition of 4-ethoxy anhydro VA was accelerated with increasing water content. In 80% aqueous EtOH at 50°, the amount of anhydro VA increased for 7 days and then decreased. In absolute EtOH, the formation of anhydro VA was small but continued up to 14 days.

As shown in Fig. 5, anhydro VA was formed in large amounts, but 4-ethoxy anhydro VA was formed only to a small extent from VA acetate in 60% aqueous EtOH at 35°. It was considered that 4-ethoxy anhydro VA was not accumulated under these conditions, since it was converted into anhydro VA quite rapidly in aqueous ethanolic solutions, as shown in Fig. 12. Once formed, 4-ethoxy anhydro VA was mostly converted into anhydro VA in aqueous and absolute EtOH, so it was considered to be an important precursor of anhydro VA.

The amount of VA ethyl ether formed from 4-ethoxy anhydro VA was less than that of anhydro VA. Comparing the amounts of VA ethyl ether formed from 4-ethoxy anhydro VA (Fig. 12) and from VA acetate (Figs. 4 and 5), the latter was large, especially during later periods of storage. Thus, VA ethyl ether was mainly formed directly from VA acetate, and a smaller amount was also formed from 4-ethoxy anhydro VA.

Acknowledgement The author thanks Prof. T. Tabata, Showa College of Pharmacy, for advice and constructive criticism, and Dr. I. Tanaka, Director of this Research Laboratory, for his encouragement.