Differential Spectrophotometric Method for the Determination of Vitamin A (Retinol) by Using Trifluoroacetic Acid, and Its Application to Related Compounds

Samir A. Gharbo and Leo A. Gosser

Warren-Teed Research Center, Warren-Teed Pharmaceuticals Inc., 582 West Goodale Street, Columbus, Ohio 43215, U.S.A.

A differential spectrophotometric procedure for the determination of vitamin A (retinol) based on the formation of a pink colour in a trifluoroacetic acid - perchloric acid medium has been developed. Maximum absorbance of the pink colour with $\lambda_{\text{max}}$, at 502 nm was attained within 7-12 min and remained stable for at least 30 s; the intensity of this colour was equivalent to 45 per cent. of the intensity of the blue colour produced by the well known antimony(III) chloride procedure. The pink colour could be destroyed within 2-4 min by the addition of pentane-2,4-dione followed by hydrogen peroxide. A rectilinear graph of absorbance $versus$ amount of vitamin A was obtained for the 0-20-µg range, optimum results being achieved in the 6-10-µg range. The relative standard deviation of the method for 8-3 µg was ±0-7 per cent. The reactions of vitamin A acetate, retinal, retinoic acid, β-carotene, ergocalciferol, cholecalciferol, cholesterol, ergosterol, phytonadione and α-tocopherol were also investigated employing the same reagent additions.

Several methods are available for the determination of vitamin A (retinol)\textsuperscript{1} in foods,\textsuperscript{2} pharmaceutical preparations\textsuperscript{3} and blood, but of these the colorimetric method with antimony(III) chloride\textsuperscript{4} is the most widely used. The blue colour ($\lambda_{\text{max}}$, 616-620 nm) that develops in the reaction with antimony(III) chloride, trichloroacetic acid\textsuperscript{5} and other strong Lewis acids\textsuperscript{6} is very sensitive but is subject to rapid fading within several seconds. Vitamin A gives the same colour reaction with trifluoroacetic acid (TFA)\textsuperscript{6,7} with much less sensitivity to moisture\textsuperscript{8} and this reagent is preferred, especially in micro-determinations.\textsuperscript{9}

The blue colour produced with TFA decays to a more stable secondary pink colour ($\lambda_{\text{max}}$, 520-550 nm) within 1.5-2 h, but the intensity of this pink colour is less than one third of that of the blue colour. Thus, the difficulties with the colorimetric determination of vitamin A are that the blue colour, although very sensitive, is unstable while the pink colour, although more stable, is less sensitive and develops slowly.

During the investigation of chemical methods for the determination of vitamin A in vitamin mixtures, it was noted that temperature, solvents and oxidising agents all have an effect on the speed of development and the sensitivity of the pink colour. It was found that a mixture of trifluoroacetic acid and 0.1 N perchloric acid ($5 + 1$) added to a dichloromethane solution of vitamin A gave the best sensitivity within 7-12 min and the absorbance at 502 nm was stable for at least 30 s. This absorbance could be destroyed rapidly within 2-4 min by the addition of pentane-2,4-dione followed by hydrogen peroxide. In this paper, the optimum conditions for a differential spectrophotometric procedure for determining vitamin A are described. In addition, the reactions of related compounds that may be present with vitamin A in pharmaceutical preparations or in blood are reported.

Experimental

Reagents

\textit{Trifluoroacetic acid.} “OR” grade. (\textit{Caution}—Trifluoroacetic acid is an extremely corrosive acid and should be handled with care.)

\textit{Perchloric acid}, 0-1 N \textit{solution in glacial acetic acid}.

\textit{Dichloromethane}. Spectroscopic quality.

\textit{Pentane-2,4-dione (acetylacetone).}
Hydrogen peroxide solution, 30 per cent. Analytical-reagent grade.

All-trans-vitamin A.

Vitamin A acetate. USP reference standard in oil.

All-trans-retinal.

All-trans-retinoic acid.

β-Carotene, ergosterol, cholesterol and cholecalciferol, 100 per cent. pure, crystalline.

Ergocalciferol. USP reference standard.

Phytonadione. USP reference standard.

DL-α-Tocopherol.

Apparatus

Perkin-Elmer, Coleman Model 124, spectrophotometer.

Rotary evaporator.

Fast-delivery pipette. Capacity, 2.0 ml.

Procedure

Transfer an aliquot of a solution of the sample, preferably dry, in a volatile organic solvent, containing 5–10 μg of vitamin A, into a 10- or 25-ml flask and evaporate it to dryness under vacuum at 35–40 °C. Add 1 ml of dichloromethane to the flask and again evaporate to dryness. To the residue in the flask, add 0.20 ml of dichloromethane, mix well to effect dissolution, and quickly add 2.0 ml of the freshly mixed solvent trifluoroacetic acid - 0.1 N perchloric acid (5 + 1). Gently swirl the flask in order to mix the contents and immediately transfer the mixture into a 1-cm spectrophotometer cuvette. Monitor the absorbance at 502 ± 2 nm and record the maximum value attained (usually within 7–12 min of mixing), zeroing the instrument with the mixed solvent as blank. After the maximum absorbance has been recorded, add 1 drop of pentane-2,4-dione, with mixing, followed by 1 drop of hydrogen peroxide solution, with mixing, and measure the absorbance at the same wavelength 3 min ± 30 s after the addition of the hydrogen peroxide. Subtract the second absorbance reading from the maximum absorbance reading and calculate the amount of vitamin A by comparison with a similarly treated standard solution.

Results and Discussion

Efforts to utilise the pink colour developed in the reaction of vitamin A with trifluoroacetic acid, led to an investigation of this reaction and the parameters that affect it. Simply allowing vitamin A to react with TFA at room temperature gives an initial blue colour that decays to a pink colour. It was found that a fairly stable isosbestic point was reached within 5–10 min at 532 ± 2 nm, which was unsatisfactory as the basis of an analytical procedure, as the absorbance value obtained was relatively weak and easily affected by solvent and pH variations. Heating vitamin A in various mixtures of chloroform and TFA at 50 °C for time intervals of 1–4 min demonstrated that the pink colour could be developed more rapidly and completely, but again it was easily affected by slight variations in experimental conditions.

Encouraging results were obtained initially when it was found that the addition of hydrogen peroxide as an oxidant, in conjunction with TFA, gave increased sensitivity to the pink colour as the maximum absorbance at 502 nm developed within 10 min and remained stable for about 1 min. The effects of nitric acid, perchloric acid and mixtures of these acids, in lieu of hydrogen peroxide, were then investigated. The most satisfactory combination was found to be a 5 + 1 mixture of TFA with 0.1 N perchloric acid in glacial acetic acid. The presence of dichloromethane before the addition of TFA was found to be advantageous, but the sensitivity decreased somewhat with the use of 1,2-dichloroethane, chloroform, carbon tetrachloride and benzene, in that order, with absolute ethanol and acetone being definitely inferior.

During the initial work with hydrogen peroxide as an aid to TFA for the rapid development of the pink colour, it was found that the addition of a small amount of acetone could destroy the colour within 15 min. Then, when developing the colour with a trifluoroacetic acid - 0.1 N perchloric acid (5 + 1) mixture, it was found that the colour was only slightly affected by the addition of a small amount of either hydrogen peroxide or acetone alone; however, if
hydrogen peroxide and acetone were added successively or in a 1:1 mixture, the colour was removed within 15–20 min. On the assumption that the keto group of the acetone had a role in this reaction, pentane-2,4-dione was substituted for acetone with the result that the absorbance of the colour was reduced to less than 0.02 within 2–4 min. Based on these observations, the conditions described in the procedure were deemed to be the optimum for the determination of vitamin A.

The visible spectrum obtained in the reaction of trifluoroacetic and perchloric acid with vitamin A at maximum intensity (Fig. 1A) illustrates the absorbance band at 502 nm with only a slight shoulder at 528 nm. The absorbance at 502 nm increases rapidly in the first 5 min, attains maximum intensity within 12 min and remains stable for at least 30 s, before decreasing slowly. The primary blue colour, which appears at 595 nm and which decays rapidly within a few seconds of the addition of the reagent, has about twice the intensity of the secondary pink colour. On the addition of pentane-2,4-dione, with mixing, the absorbance falls only slightly, but on subsequent addition of hydrogen peroxide, with mixing, the absorbance falls rapidly in the first 1 min and then attains a stable intensity (less than 0.02 absorbance unit) within 2–4 min (Fig. 1B). Addition of the last two reagents in the reverse order gives essentially the same result. The spectrum shown in Fig. 1B is also given by a blank reaction without vitamin A. It is important that the sample and standard solution are treated in a similar way as the concentration of TFA may vary from batch to batch and degree of use. The perchloric acid should be mixed with the TFA shortly before use (preferably less than 1 h) as the mixture tends to lose potency with time.

Various dilutions of vitamin A [Fig. 2(a)] and of the USP reference standard vitamin A acetate [Fig. 2(b)] in chloroform were used to establish the linearity of response. The graphs of maximum absorbance at 502 nm versus the amount of vitamin A and that of the acetate was rectilinear in the range 0–20 μg, with optimum results being obtained in the 5–10-μg range. The relative standard deviations of the absorbance for six aliquots of vitamin A containing 8.3 μg and of the acetate containing 8.4 μg were found to be 0.66 and 1.07 per cent., respectively.
The $E_{1%}\text{cm}^\text{min}$ values calculated from the average of several determinations of vitamin A and its acetate (Table I) indicate that the value of the acetate is 2 per cent. higher than that of the alcohol, which is in contrast to a value expected to be 15 per cent. lower based on the difference in relative molecular masses and according to the comparable literature values for the blue colour with TFA,$^6,9$ Hydrolysis of vitamin A acetate by the USP XVIII$^{10}$ procedure and determination of the resulting alcohol by both the ultraviolet$^{10}$ and the colorimetric procedures gave $97-9\pm1-8$ and $96-9\pm2-4$ per cent. recoveries, respectively. Vitamin A acetate was adsorbed on deactivated neutral silica, chromatographed on neutral alumina, eluted with acetone and the acetate determined in the eluate by both the ultraviolet and the colorimetric procedures, which gave $99-9\pm0-7$ and $97-5\pm1-5$ per cent. recoveries, respectively. Apparently, the acetate does indeed produce a higher colour yield than does the alcohol on an equimolar basis.

The colorimetric procedure was applied to some related compounds that may be present with vitamin A in foods, pharmaceutical preparations and in blood, and the results are shown in Table I. The spectra of all of the compounds in the table showed single peaks except for $\beta$-carotene, which showed a secondary peak at 475 nm, and $\alpha$-tocopherol, which showed an additional shoulder at 430 nm. All the peaks disappeared on the addition of pentane-2,4-dione and hydrogen peroxide. However, it is evident that differential spectrophotometry could not be used for compounds that have their maxima at wavelengths below 460 nm as the blank solvents begin to make a significant contribution to the absorbance (Fig. 1B).

**TABLE 1**

**COMPARATIVE RESULTS OF THE APPLICATION OF THE DIFFERENTIAL SPECTROPHOTOMETRIC PROCEDURE FOR THE DETERMINATION OF VITAMIN A TO RELATED COMPOUNDS**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Wavelength ($\lambda_{\text{max.}}\pm2$)/nm</th>
<th>$E_{1%}\text{cm}^\text{min}$ at $\lambda_{\text{max.}}$</th>
<th>Time taken to reach maximum absorbance/min</th>
<th>Minimum time of stability of absorbance/s</th>
<th>$E_{1%}\text{cm}^\text{min}$ at 502 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Vitamin A acetate</td>
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<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Retinal</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>$\beta$-Carotene</td>
<td>$735$</td>
<td>$332$</td>
<td>$7 \pm 1$</td>
<td>$30$</td>
<td>$92$</td>
</tr>
<tr>
<td>Ergocalciferol</td>
<td>$475$</td>
<td>$108$</td>
<td>$17 \pm 2$</td>
<td>$60$</td>
<td>$102$</td>
</tr>
<tr>
<td>Cholecalciferol</td>
<td>$485$</td>
<td>$1151$</td>
<td>$3 \pm 1$</td>
<td>$60$</td>
<td>$641$</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>$412$</td>
<td>$163$</td>
<td>$2 \pm 1$</td>
<td>$30$</td>
<td>$517$</td>
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<tr>
<td>Ergosterol</td>
<td>$383$</td>
<td>$213$</td>
<td>$2.5 \pm 1$</td>
<td>$20$</td>
<td>$34$</td>
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<tr>
<td>Phytonadione</td>
<td>$400$</td>
<td>$125$</td>
<td>$60 \pm 5$</td>
<td>$120$</td>
<td>$0.8$</td>
</tr>
<tr>
<td>DL-$\alpha$-Tocopherol</td>
<td>$458$</td>
<td>$28^*$</td>
<td>$&gt;90$</td>
<td>—</td>
<td>$2.5^*$</td>
</tr>
</tbody>
</table>

* At 90 min.

Several advantages over other colorimetric methods that are used for the determination of vitamin A could be attributed to the method described in this paper. The developed
colour, in addition to giving an absorbance graph that is rectilinear with concentration, is relatively stable, sensitive and rapidly formed. The procedure is simple and provides a distinct advantage in the use of differential spectrophotometry in the presence of spectral interferences of a general background nature. There were no significant day-to-day variations in results noted under similar conditions.

References


Received *January 27th, 1975*
Accepted *May 6th, 1975*