Necessity of Monitoring HPLC by a $\bar{X}$-$R$ Control Chart on Measurement of Serum Fat-Soluble Vitamins

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Summary: In epidemiologic studies, many serum samples obtained from a large population have to be processed for measurement of fat-soluble vitamins. Quantification results by a high performance liquid chromatography (HPLC) should be stable in both intra- and inter-run, and a quality control may be performed by a $\bar{X}$-$R$ control chart. The present study shows that serum retinol, $\alpha$-tocopherol, $\alpha$-carotene, $\beta$-carotene, and lycopene could be measured simultaneously, by 1) use of photodiode-array (POD) detector, 2) selection of some optimal analytical conditions (e.g. column, mobile phase, column temperature), 3) applying 2 internal standards, and 4) monitoring by a $\bar{X}$-$R$ control chart. The present procedure yielded relatively low coefficients of variations (CVs) compared to some reported figures. However, determination of CVs only does not ensure the accuracy and precision of an assay system, and application of a $\bar{X}$-$R$ control chart may be necessary for a good quality control of measurement, such as serum fat-soluble vitamins.

Key words fat-soluble vitamins, $\beta$-carotene, stability, CV, $\bar{X}$-$R$ control chart, HPLC

INTRODUCTION

There is a great interest in the relationship between fat-soluble vitamins and diseases. Usually, many serum samples obtained from a large population have to be processed for measurement by a high performance liquid chromatography (HPLC) in epidemiologic studies. Quantification results should be stable in both intra- and inter-run, and the reproducibility of these measurements was usually ensured only by coefficient of variations (CVs) [1-5], but rarely by a $\bar{X}$-$R$ control chart [6] which has been widely used in the laboratory work for quality control [7].

The present paper reports an improved method for the simultaneous measurement of retinol, $\alpha$-tocopherol, $\alpha$-carotene, $\beta$-carotene and lycopene by HPLC with high resolution being monitored by a $\bar{X}$-$R$ control chart in order to ensure a satisfactory stability of these measurements.

MATERIALS AND METHODS

Reagents

All-trans retinol, DL-$\alpha$-tocopherol, DL-$\alpha$-tocopherol acetate, $\alpha$-carotene (Type V, from carrots), $\beta$-carotene (Type IV, from carrots) and Lycopene were purchased from Sigma Chemical Co., Ltd. (St. Louis, USA). Ethyl $\beta$-apo-8'-carotenoylacetate was purchased from Fluka Chemica (Buchs, Switzerland). Super special-grade ethanol, n-hexane, acetonitrile, methanol, dichloromethane and special-grade ammonium acetate were obtained from Wako Pure Chemical Co., Ltd. (Tokyo, Japan). They were used without further purification.

Human serum

The sera used were pooled sera which were residual sera after transfusion. Two kinds of sera were prepared; one was the working standards and the other was the dummy samples. It was subdivided into stock tubes, frozen at $-80^\circ$C, and subsequently

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used for the present experiments.

Preparation of the HPLC sample

Figure 1 shows an extraction procedure of a HPLC sample from serum. Two hundred microliters of ethanol containing 76 μmol/l of DL-α-tocopherol acetate and 8.7 μmol/l of ethyl β-apo-8'-carotenole as internal standards was added to 200 μl of serum in a 5 ml brown glass tube and then vortexed vigorously for 1 min. Subsequently, the mixture was extracted with 800 μl of n-hexane by vortexing vigorously for 5 min. After centrifuging at 375×g for 5 min at 20 °C, the clear supernatant solution was carefully drawn into a 5 ml brown glass tube and dried by a Speed Vac Concentrator (Savant Instruments, Inc., New York, USA). The residue was dissolved in 200 μl of a mobile phase. The extract was centrifuged at 3,373×g for 5 min at 20 °C and passed through a 0.5 μm membrane filter (Nippon Millipore, Tokyo, Japan). One hundred microliter of extract was injected into the HPLC system. The stock solution in ethanol with 760 μmol/l of DL-α-tocopherol acetate and 87 μmol/l of ethyl β-apo-8'-carotenole were stored at -4 °C and diluted ten times for each 10 runs. DL-α-tocopherol acetate or ethyl β-apo-8'-carotenole were used as the internal standards for retinol and α-tocopherol or for β-carotene, β-carotene and lycopene, respectively.

HPLC equipment and analytical condition

The HPLC system consisted of the following equipment: a SIL-10Axl automated sample injector (Shimadzu, Kyoto, Japan), a LC-10As pump (Shimadzu), a SPD-M10Avp photodiode-array (PDA) detector (Shimadzu), a CBM-10A communication base module (Shimadzu) and a L-7300 column oven (Hitachi, Tokyo, Japan). Data were stored and processed by using a CLASS-LC10 system (Shimadzu) by a Fujitsu FMV-SII165 (Fujitsu, Tokyo, Japan). The chromatographic analysis was performed on YMC-Pack ODS-AM column (250 cm×4.6 mm I.D., YMC, Kyoto, Japan), maintained at 30 °C. A precolumn filter (GL sciences, Tokyo, Japan) and a guard cartridge of ODS-AM (YMC) preceded the main column. The mobile phase consisted of acetonitrile-methanol-dichloromethane containing 0.01% ammonium acetate (70:16:14, v/v). Ammonium acetate was first dissolved in methanol, and then added to the rest of the solvent mixture. The flow rate was 1.5 ml/min. The chromatograms were plotted at 292 nm (for tocopherols), 325 nm (for retinol), 450 nm (for carotenoids) and 470 nm (for lycopene). One run consisted of 20 samples analysis. Three working standards were prepared and treated as if being the 1st, 11th and 20th sample of each run. Between each working standard, 17 dummy samples were injected in to the HPLC.

HPLC monitoring by X-R control chart

The whole data for the present study consisted of measurements by 20 runs. “Intra-run” variance indicates the mean variance due to the 3 working standards of each run, and “inter-run” variance indicates that due to these 20 runs. After the whole data were collected, X-R control charts were constructed. Values on X-R control chart were calculated from 3 working standards of 20 runs. Both the upper control limit (UCL) and the lower control limit (LCL) were set at the upper or lower limit of 3 σ of the total average (x) as usual for the X control chart. The UCL was also set at the upper limit of 3 σ of the total average (R) for the R control chart. Usual criteria were applied for out of control judgement, e.g. plot above UCL or below LCL, or upward or downward trend [7].

RESULTS

Typical HPLC chromatograms are shown in Fig.
2. Five vitamins and the 2 internal standards separated well from other peaks. The separation of \(\alpha\)- and \(\beta\)-carotene is at baseline (resolution: \(R_s=2.2\)). Micronutrients were quantified by determining peak high in the HPLC chromatograms calibrated against known amounts of standards.

A summary of intra- and inter-run CVs of the present study is presented in Table 1. Intra-run CVs were less than 3.1% and inter-run CVs were less than 3.7% for all constituents. The highest variations were seen for carotenes. Figures 3-7 show \(\bar{X}\)-R control charts obtained in the present study. All plots fall

TABLE 1.

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>Intra-run CVs (%)</th>
<th>Inter-run CVs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol</td>
<td>1.51</td>
<td>2.06</td>
</tr>
<tr>
<td>(\alpha)-tocopherol</td>
<td>1.12</td>
<td>1.26</td>
</tr>
<tr>
<td>(\alpha)-carotene</td>
<td>2.62</td>
<td>3.45</td>
</tr>
<tr>
<td>(\beta)-carotene</td>
<td>3.08</td>
<td>3.62</td>
</tr>
<tr>
<td>Lycopene</td>
<td>2.29</td>
<td>3.09</td>
</tr>
</tbody>
</table>

Fig. 2. Chromatograms of serum extract. Peak identification: a, DL-\(\alpha\)-tocopherol acetate; b, \(\alpha\)-tocopherol; c, Retinol; d, Ethyl \(\beta\)-apo-8'-carotenoate; e, \(\alpha\)-carotene; f, \(\beta\)-carotene; g, Lycopene

Fig. 3. \(\bar{X}\)-R control chart of serum Retinol concentration analysis. \(\bar{X}\), UCL and LCL on \(\bar{X}\) control chart; R, \(\overline{R}\) and UCL on R control chart were calculated from 3 working standards of 20 runs.

Fig. 4. \(\bar{X}\)-R control chart of serum \(\alpha\)-tocopherol concentration analysis. \(\bar{X}\), UCL and LCL on \(\bar{X}\) control chart; R, \(\overline{R}\) and UCL on R control chart were calculated from 3 working standards of 20 runs.
within the UCL of the R control chart, but some plots were above the UCL or below the LCL of X control chart. The number of plots outside the acceptable limits of the X control chart for retinol, tocopherol, \(\alpha\)-carotene, \(\beta\)-carotene, lycopene were 6, 1, 5, 4 and 2 runs, respectively.

**DISCUSSION**

Many samples of serum fat-soluble vitamins collected for epidemiologic studies are measured by separate runs in one laboratory. Therefore, it is required that measurement should be stable in both intra- and inter-run. A lot of previous studies ensure stability of the measurement in terms of CVs. The present study aimed at improving the measurement of serum fat-soluble vitamins with HPLC to yield satisfactory stability, and confirming this stability in intra- and inter-run. A PDA detector enabled measurements of each fat-soluble vitamin at more suitable wavelength than UV/VIS detector. Then \(\alpha\) and \(\beta\)-carotene could be separated well from each other with a high performance under the optimized conditions for the present HPLC. As shown in Table 1, the present condition yielded relatively low CVs [1-5] in both intra- and inter-run for all constituents. In spite of very low CVs and of all plots being within the control limit on R control charts for all constituents, some plots on X control charts were above or below the control limits and other plots did not fulfill the other out of control criteria on X-R control charts. This indicated that the present measurement procedure was of satisfactory stability at intra-run, but not at inter-run, and X-R control charts for the present study may not be used for quality control on the actual quantification. It is obvious that further improvement is required for the present assay system.

Because retinol, \(\alpha\)-tocopherol, \(\alpha\)-carotene and \(\beta\)-carotene in the serum are stable when stored at \(-80^\circ\)C for at least one year [8], satisfactory stable X-R control chart once obtained can be used for at least one year. It may be necessary, therefore, to monitor HPLC measurements with a X-R control chart for quality assessing control of measurement of fat-soluble vitamins.

**REFERENCES**


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