# CHAPTER III

# MATERIALS AND METHODS

## Materials

### Equipments

1. Fluorescence spectrophotometer, Perkin Elmer MPF-3 and thin-layer chromotography accessories.
2. Recorder, Hitachi QPD 33.
3. Spectrophotometer, Hitachi 220.
4. Hot air oven, Lab-line Instruments.
5. Vortex-genie, Scientific Industries, Inc. K-550-Ge.
6. Centrifuge, Precision Scientific Co..
7. Ultraviolet lamp, long-wavelength, 366nm.
8. Thin-layer chromatographic chamber, Desaga.
9. Template, Desaga.
10. Microcaps, 1 microliter, Drummond.
11. TLC plate aluminum oxide 150 F254 (type T) pre-coated, Merck Art. 5727.

### Chemicals

All chemical listd below were reference standards.

1. Methyltestosterone reference standard, International chemical reference substance from WHO center, control no. 167023.
2. Vitamin A (Retinol acetate) USP reference standard in cottonseed oil, containing 34.4 mg/g.

All chemicals listed below were pharmaceutical grade.

1. Ethinyl estradiol
2. Vitamin A (Retinol palmitate), oily concentrate, containing 1,500,000 i.u./g.
3. Vitamin $D_3$ crystal (Cholecalciferol) An. 365,987, Roche.
4. Vitamin E (Tocopheryl acetate), Art. 8284, Merck.
5. Vitamin $K_1$
6. Vitamin $B_1$ (Thiamine hydrochloride)
7. Vitamin $B_2$ (Riboflavine)
8. Vitamin $B_6$ (Pyridoxine hydrochloride)
9. Vitamin $B_{12}$ (Cyanocobalamin)
10. Vitamin C (Ascorbic acid)
11. Niacinamide
12. Calcium pantothenate
13. Folic acid
14. Rutin
15. Strychnine sulfate
16. Yohimbine hydrochloride
17. Inositol

All chemicals listed below were analytical grade.

1. Dicalcium phosphate (B.D.H.Chemicals)
2. Cupric sulfate, anhydrous (B.D.H. Chemicals)
3. Ferrous sulfate, anhydrous (J.T. Baker Chemicals)
4. Magnesium sulfate, anhydrous (Merck)
5. Manganese sulfate (Merck)
6. Potassium iodide (Ajax Chemicals)
7. Potassium sulfate, anhydrous (Merck)

8. Sodium sulfate, anhydrous (Carlo Erba)
9. Zine sulfate (Merck)
10. Benzene (J.T. Baker Chemicals)
11. Diethyl ether (May & Baker)
12. Chloroform (J.T. Baker Chemicals)
13. Ethanol, absolute (Merck)

Reagent

Mixed solvent

Equal volume of chloroform and ethanol were mixed.

Standard solutions

1. Standard methyltestosterone stock solution (0.5 mg/ml)

   A 25 mg methyltestosterone reference standard was dissolved in 50 ml of the mixed solvent. This solution should be stored in a refrigerator.

2. Standard methyltestosterone solution

   The standard solution was prepared by diluting the stock solution with the mixed solvent to final concentration of 0.025 - 0.25 mg/ml.

## Methods

### General procedure

One microliter of standard and sample solution were applied separately with the microcaps on a TLC plate aluminum oxide $F_{254}$ (type T) previously activated by heating at 110° for 10 minutes and cooled in a desiccator. The application of the spots should be at points about 1.5 cm apart and at about 2.5 cm from the lower edge of the plate, and allowed to dry. The plate was developed in the chromatographic chamber containing mobile phase, until the solvent front reached about 15 cm above the line of appication. The plate was removed, allowed the solvent dry throughly with the current air and heated at 150° for 20 minutes. The plate was kept in a desiccator about 1 hour before measuring the fluorescence intensity by using densitometer.

The general procedure was used throughout the experiments.

1. Determination of the Mobile Phase

Standard solution

Methyltestosterone 0.1 mg/ml in mixed solvent.

Sample solution

One commercial vitamin-hormone preparation was selected as an example.

Each capsule contains:-

| | | |
|---|---|---|
| Ethinyl Estradiol | 0.01 | mg |
| Methyltestosterone | 2.0 | mg |
| Vitamin A (Palmitate) | 5,000 | I.U. |
| Vitamin $D_3$ (Calciferol) | 1,000 | I.U. |
| Vitamin $B_1$ (Thiamine mononitrate) | 3.0 | mg |
| Vitamin $B_2$ (Riboflavin) | 2.5 | mg |
| Vitamin $B_6$ (Pyridoxine HCl) | 0.75 | mg |
| Vitamin $B_{12}$ (Cyanocobalamin) | 1.5 | mcg |
| Nicotinamide | 20.0 | mg |
| Vitamin C (Ascorbic acid) | 50.0 | mg |
| Calcium pantothenate | 5.0 | mg |
| Folic acid | 0.34 | mg |
| Vitamin E (dl-Tocopheryl acetate) | 3.0 | mg |
| Calcium (as Dicalcium phosphate) | 215.0 | mg |
| Phosphorus (as Dicalcium phosphate) | 166.0 | mg |
| Iron (as Ferrous sulfate) | 13.4 | mg |
| Copper (as Cupric sulfate) | 1.0 | mg |
| Zinc (as Zinc sulfate) | 1.4 | mg |
| Magnesium (as Magnesium sulfate) | 7.5 | mg |
| Manganese (as Manganese sulfate) | 1.5 | mg |

| | |
|---|---|
| Sodium (as Sodium sulfate) | 0.4 mg |
| Potassium (as Potassium sulfate) | 5.0 mg |
| Choline bitartrate | 100.0 mg |

A portion of the sample mixture was dissolved in the mixed solvent to obtain a solution of 0.1 mg/ml of methyltestosterone.

Mobile phase

1. Dichloromethane
2. Dichloromethane-hexane 80:20, 90:10, 95:5
3. Benzene-absolute ethanol 95:5, 99:1
4. Benzene-diethyl ether 40:60, 50:50, 60:40, 70:30, 90:10

Procedure

The general procedure was performed.

The chromatograms were examined under the long-wavelength ultraviolet light (366 nm).

The $R_f$ value of the spots was calculated from the following formula:

$$R_f \text{ value} = \frac{\text{migration distance of the substance}}{\text{migration distance of the solvent}}$$

The $R_f$ values, the shape of spots and the results obtained were shown in Table 1.

2. Determination of the Maximum Excitation Wavelength and the Maximum Emission Wavelength

Standard solution

Methyltestosterone 0.1 mg/ml in mixed solvent.

Mobile phase

Benzene-diethyl ether 60:40

This mobile phase was used throughout the experiments.

Densitometric condition

The fluorescence spectrophotometer in connection with the thin-layer chromatography accessories was operated in a condition:

| | | |
|---|---|---|
| Measurement | : | fluorescence |
| Excitation wavelength | : | 366 nm, slit 10; scan |
| Emission wavelength | : | 454 nm, slit 10; scan |
| Reference sensitivity | : | 3 (Reference mode) |
| Sample sensitivity | : | 1 |
| Slit | : | 2 x 6 mm |
| Secondary filter | : | no. 43 |
| Scan speed | : | 150 nm/minute |
| Chart speed | : | 120 nm/minute |

Procedure

The general procedure was performed. The excitation spectrum with emission at 454 nm and the emission spectrum with excitation at 366 nm were obtained by densitometer which was operated in a condition described above.

The results obtained were shown in Figure 1.

3. Determination of the Effect of Time on Stability of Fluorescence Intensity

Standard solution

Methyltestosterone 0.1 mg/ml in mixed solvent.

Densitometric condition

The fluorescence spectrophotometer in connection with the thin-layer chromatography accessories was operated in condition:

| | | |
|---|---|---|
| Measurement | : | fluorescence |
| Excitation wavelength | : | 366 nm, slit 10 |
| Emission wavelength | : | 454 nm, slit 10 |
| Reference sensitivity | : | 3 (Reference mode) |
| Sample sensitivity | : | 1 |
| Slit | : | 2 x 6 mm |
| Secondary filter | : | no. 43 |
| Scan speed | : | 25 nm/minute |
| Chart speed | : | 30 nm/minute |

The condition was used throughout the experiments.

Procedure

The general procedure was performed and the TLC plate was kept in a desiccator for 1 hour after heating at 150° for 20 minutes. Then the chromatogram fluorescence intensity was measured by using densitometer at the time interval of 0, 30, 60, 90, 240, 270 and 300 minutes, and 24 hours.

The results obtained were shown in Table 2.

4. Reproducibility of Fluorescence Intensity between Spots within One Plate

Standard solution

Methyltestosterone 0.1 mg/ml in mixed solvent.

Procedure

The general procedure was performed in which the standard solution was applied separately ten spots on one plate.

The chromatogram was obtained by using densitometer which was operated in a condition described under "Determination of the Effect of Time on Stability of Fluorescence Intensity".

The results obtained were shown in Table 3 and Figure 2 and 3.

5. Determination of Adherence to Beer's Law

Standard solutions

Methyltestosterone 0.025, 0.075, 0.100, 0.125, 0.150, 0.200, 0.225 and 0.250 mg/ml in mixed solvent.

Procedure

The general procedure was performed and the chromatogram was obtained by using the densitometer. The calibration curve was obtained by plotting between the peak heights against the concentrations of methyltestosterone.

The results obtained were shown in Table 4 and Figure 4.

6. Interference of Other Substances in Vitamin-Hormone Preparations

6.1 Other Hormone (Ethinyl Estradiol)

Standard solutions

1. Methyltestosterone, 0.1 mg/ml
2. Ethinyl estradiol, 0.5 µg/ml

in mixed solvent.

Procedure

The general procedure was performed in which each of the standard solutions was applied separately. At the third spot, both solutions were applied together.

The chromatogram was examined under the long-wavelength ultraviolet light (366 nm) and the fluorescence intensities were measured by densitometer.

The results obtained were shown in Table 5 and Figure 5.

6.2 Fat-Soluble Vitamins

Standard solutions

1. Methyltestosterone, 0.1 mg/ml
2. Retinol acetate, 2.0 mg/ml
3. Retinol palmitate, 0.8 mg/ml
4. Tocopheryl acetate, 0.6 mg/ml
5. Cholecalciferol, 2.5 µg/ml
6. Vitamin $K_1$, 10.0 µg/ml

in mixed solvent.

Procedure

The general procedure was performed in which each of the standard solutions was applied separately. At the last two spots, all of the solutions were applied with and without methyltestosterone solution.

The chromatogram was examined under the long-wavelength ultraviolet light (366 nm) and the fluorescence intensities were measured by densitometer.

The results obtained were shown in Table 6 and Figure 6.

6.3 Water-Soluble Vitamins

Standard solutions

1. Methyltestosterone, 0.1 mg/ml
2. Thiamine hydrochloride, 0.5 mg/ml
3. Riboflavine, 0.5 mg/ml
4. Pyridoxine hydrochloride, 0.3 mg/ml
5. Cyanocobalamin , 0.15 µg/ml
6. Nicotinamide, 2.5 mg/ml
7. Ascorbic acid, 5.0 mg/ml
8. Calcium pantothenate, 0.5 mg/ml
9. Folic acid, 0.04 mg/ml

   in mixed solvent.

Procedure

The general procedure was performed in which each of the standard solutions was applied separately. At the last two spots, all of the solutions were applied with and

without methyltestosterone solution.

The chromatogram was examined under the long-wavelength ultraviolet light (366 nm) and the fluorescence intensities were measured by densitometer.

The results obtained were shown in Table 7 and Figure 7.

6.4 Minerals

Standard solutions

1. Methyltestosterone, 0.1 mg/ml
2. Dicalcium phosphate, 91.4 mg/ml
3. Potassium iodide, 0.04 mg/ml
4. Cupric sulfate, 0.24 mg/ml
5. Potassium sulfate, 1.1 mg/ml
6. Manganese sulfate, 0.48 mg/ml
7. Magnesium sulfate, 14.2 mg/ml
8. Ferrous sulfate, 3.6 mg/ml
9. Sodium sulfate, 0.28 mg/ml
10. Zinc sulfate, 0.5 mg/ml

in mixed solvent.

Procedure

The general procedure was performed in which each of the standard solutions was applied separately. At the last two spots, all of the solutions were applied with and without methyltestosterone.

The chromatogram was examined under the long-wavelength ultraviolet light (366 nm) and the fluorescence

intensities were measured by densitometer.

The results obtained were shown in Table 8 and Figure 8.

6.5 Other Compounds

Standard solutions

1. Methyltestosterone, 0.1 mg/ml
2. Yohimbine, 0.3 mg/ml
3. Strychnine sulfate, 0.05 mg/ml
4. Inositol, 1.0 mg/ml
5. Rutin, 0.5 mg/ml

in mixed solvent.

Procedure

The general procedure was performed in which each of the standard solutions was applied separately. At the last two spots, all of the solutions were applied with and without methyltestosterone solution.

The chromatogram was examined under the long-wavelength ultraviolet light (366 nm) and the fluorescence intensities were measured by densitometer.

The results obtained were shown in Table 9 and Figure 9.

6.6 Vegetable Oil

Standard solutions

1. Methyltestosterone, 0.1 mg/ml
2. Cottonseed oil, 20.0 mg/ml

in mixed solvent.

Procedure

The general procedure was performed in which each of the standard solutions was applied separately. At the third spot,both solutions were applied together.

The chromatogram was examined under the long-wavelength ultraviolet light (366 nm) and the fluorescence intensities were measured by densitometer.

The results obtained were shown in Table 10 and Figure 10.

7. Determination of the Percent Labelled Amount of Methyltestosterone in Methyltestosterone Tablet Using Spectrofluorodensitometric Method and USP Method

Spectrofluorodensitometric method

Standard solution

Methyltestosterone 0.1 mg/ml in mixed solvent.

Sample solution

Twenty methyltestosterone tablets (25 mg/tablet) were weighed and finely powdered. A portion of the powdered sample equivalent to about 1 mg of methyltestosterone was accurately weighed and transfered into a 10-ml volumetric flask. Then 8 ml of the mixed solvent was added, and the solution was shaken about 10 minutes. The solution was diluted to volume with the mixed solvent. The resulting solution was centrifuged for 10 minutes and a few milliliters was pipetted into the small stoppered-tube. Five replicates were determined by this procedure.

Procedure

The general procedure was performed in which the standard and sample solutions were applied separately two spots for each solution.

The chromatograms were obtained by using densitometer.

The amount of methyltestosterone in tablet was calculated from the following formula:

$$\text{Percent of labelled amount} = \frac{P_u}{P_s} \times \frac{C_s}{C_u} \times 100$$

Where, $P_u$ = peak height of the sample in mm.

$P_s$ = peak height of the standard in mm.

$C_u$ = concentration of methyltestosterone in mg/ml of assay preparation.

$C_s$ = concentration of methyltestosterone in mg/ml of standard solution.

USP method

Procedure

A portion of the finely pulverized tablet equivalent to about 10 mg of methyltestosterone was accurately weighed, and suspended in 5 ml of water. The suspension was extracted with four 25 ml volumes of chloroform and filtered each portion through chloroform-washed cotton-wool. The combined extracts were evaporated to dryness in a stream of air on a boiling water-bath. The residue was dissolved in ethanol and diluted to 50.0 ml. Then 5.0 ml of this solution was diluted to 100.0 ml with ethanol. The absorbance of this solution and the solution of standard methyltestosterone in the same medium having a final concentration of about 10 µg/ml, were determined in 1-cm cells at the wavelength of maximum at about 241 nm, using ethanol as the blank. Five replicates were determined by this procedure.

The amount of methyltestosterone in tablet was calculated from the following formula:

$$\text{Percent of labelled amount} = \frac{A_u}{A_s} \times \frac{C_s}{C_u} \times 100$$

Where, $A_u$ = absorbance of the sample.

$A_s$ = absorbance of the standard.

$C_u$ = concentration of methyltestosterone in mg/ml of assay preparation.

$C_s$ = concentration of methyltestosterone in mg/ml of standard solution.

The results obtained were shown in Table 11.

8. Determination of the Reproducibility of Spectrofluorodensitometric Determination of Methyltestosterone in Vitamin-Hormone Preparations

Standard solution

Methyltestosterone 0.1 mg/ml in mixed solvent.

Sample solutions

Five commercially available formulations of vitamin-hormone preparations were taken.

The sample preparations were carried out as described under the determination of percent labelled amount of methyltestosterone in methyltestosterone tablet using spectrofluorodensitometric method. Triplicate were determined for each preparation by using the same procedure.

Procedure

The general procedure was performed. The standard and sample solutions were applied separately two spots for each solution.

The chromatograms were obtained by using densitometer. The amount of methyltestosterone was calculated as described under the determination of the percent labelled amount of methyltestosterone in methyltestosterone tablet.

The results obtained were shown in Table 12.

9 Determination of the Percent Recovery of Methyltestosterone in Vitamin-Hormone Preparation

Standard solutions

Methyltestosterone 0.10, 0.15 and 0.20 mg/ml in mixed solvent.

Sample solutions

Each portion of the sample mixture equivalent to about 0.5 mg of methyltestosterone was accurately weighed and transfered to three 10-ml volumetric flasks. Then 1.0, 2.0 and 3.0 ml of the standard methyltestosterone stock solution (0.5 mg/ml) were added into each flask, respectively. The mixed solvent was added to each flask to about 8 ml, and the solutions were shaken for about 10 minutes. The solutions were diluted to volume with the mixed solvent. The resulting solutions were centrifuged for 10 minutes and a few milliliters of each clear solution was pipetted into three small stoppered-tubes.

Procedure

The general procedure was performed. The standard and sample solutions were applied separately two spots for each solution.

The chromatograms were obtained by using densitometer.

The percent recovery was calculated from the following formula:

$$\% \text{ recovery of methyltestosterone} = \frac{(W_f - W_s)}{W_a} \times 100$$

where, $W_f$ = total amount of methyltestosterone found in mg.

$W_s$ = amount of methyltestosterone from the sample in mg.

$W_a$ = amount of methyltestosterone added in mg.

Triplicate were determined by using the same procedure and the results obtained were shown in Table 13.

10. Analysis of Vitamin-Hormone Preparations Containing Methyltestosterone

To test the validity of the method, twelve commercially available formulations with various amounts of methyltestosterone were analyzed.

Standard solution

Methyltestosterone 0.1 mg/ml in mixed solvent.

Sample solutions

The sample preparations were carried out as described under the determination of percent labelled amount of methyltestosterone in methyltestosterone tablet using spectrofluorodensitometric method. Triplicate were determined for each preparation by using the same procedure.

Procedure

The general procedure was performed. The standard and sample solutions were applied separately two spots for each solution.

The chromatograms were obtained by using densitometer. The amount of methyltestosterone was calculated as described under the determination of the percent labelled amount of methyltestosterone in methyltestosterone tablet using spectrofluorodensitometric method.

The results obtained were shown in Table 15.