CHAPTER IV

RESULTS AND DISCUSSION

Determination of the Mobile Phase

The one- and two- component solvents were used as developing solvents for thin-layer chromatography as shown in Table 1. The chromatograms of methyltestosterone, in the standard and sample solutions, were obtained by using various solvent systems. The solvent, dichloromethane; the solvent mixtures of dichloromethane-hexane, 80:20, 90:10 and 95:5; and the mixtures of benzene-absolute ethanol, 95:5 and 99:1 showed poor separations but excellent separations could be achieved with the solvent mixtures of benzene-diethyl ether, 40:60, 50:50, 60:40, 70:30 and 90:10.

The solubility of the sample in the solvent system is an impotant criterion for selection of the solvent system for the good separation. Methyltestosterone is soluble in organic solvents whereas other ingredients such as fat-soluble vitamins, other hormone, ethinyl estradiol, are also soluble in organic solvents. The system of binary mixtures of organic solvents with different polarity gave more advantage than one component solvent for the good separation. The solvent strength should be considered in order to obtain a good chromatographic resolution. The mixture of benzene-diethyl ether showed better separation than other systems. As methyltestosterone spots were round and had higher R_f

values than the others, a 60:40 mixture of benzene and diethyl ether was selected as the mobile phase throughout the experiments.

Determination of the Maximum Excitation Wavelength and the Maximum Emission Wavelength

The spectrum of excitation and emission of the blue fluorescent spot of methyltestosterone were obtained by examining the chromatogram with a densitometer (a fluorescence spectrophotometer in connection with a thin-layer chromatography accessories), in the suitable condition. It was reported(27,28) that the fluorescence of testosterone and progesterone were detected at the excitation wavelength of 366 nm and at the emission wavelength of 440 nm. However, the wavelength of maximum excitation and maximum emission of methyltestosterone were determined for the best optimum condition in this experiment. The spectrum shown in Figure 1 indicated that the wavelength of maximum excitation was 366 nm and the wavelength of maximum emission was 454 nm.

Therefore, the excitation wavelength, 366 nm and the emission wavelength, 454 nm were fixed throughout the experiments.

Determination of the Effect of Time on Stability of Fluorescence Intensity

The stability of fluorescent spot intensity of methyltestosterone was studied by determining the effect of time on the fluorescence, using the densitometer. The flu-

orescence intensity of the spot was measured at selected intervals of time within 24 hours at room temperature. The experimental data in Table 2 indicated the slightly decrease of the fluorescence intensity of the spot. It was also reported(28) that the fluorescent spot intensity remained constant for a few days.

Reproducibility of Fluorescence Intensity between Spots within One Plate

The fluorescence intensity of ten spots of methyltestosterone in one plate was measured with the densitometer. The data evaluation obtained by using single value technique compared to the results using data-pair technique are shown in Table 3 and Figure 2 and 3. The coefficient of variation were 2.65 % and 1.46 % for single value technique and data-pair technique, respectively. The reproducibility of intensity between spots within one plate was good. The data-pair technique should be carried out in the experiments in order to minimize the systematic errors due to chromatographic parameters which tend to influence fluorescence values as a function of varying R_f values.

Determination of Adherence to Beer's Law

Calibration curve of methyltestosterone standard was determined in the optimum condition previously study. The relationship between peak height and concentration of methyltestosterone was adherence to Beer's law as shown in Table 4 and Figure 4. The calibration graph for methyltes-

tosterone standard was linear in the range 0.025-0.200 mg/ml for a 1 µl application volume with correlation coefficient of 0.9998. The non-linear curve at high concentration was observed because of concentration quenching effect.

Interference of Other Substances in Vitamin-Hormone Preparations

Many substances in vitamin-hormone preparations included other hormone, ethinyl estradiol; fat-soluble vitamins; water-soluble vitamins; minerals; other compounds such as yohimbine, strychnine,vegetable oils etc. were determined as interference in separation capability. The solvent system for completely separation was very important. The spot of methyltestosterone on the chromatographic plate should be completely separated enough to be interpreted the chromatogram fluorescence intensity by densitometer without interfering from other substances. The R_f values, peak heights of the spots and chromatogram fluorescence intensities of methyltestosterone and mixtures of methyltestosterone and other substances were determined as shown in Table 5-10 and Figure 5-10.

The results showed that the proposed method for determination of methyltestosterone were not interfered by other substances.

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

The single drug preparation of methyltestosterone,

in tablet form, was determined by the proposed method and by official spectrophotometric method(3). The results obtained were compared in Table 11. The mean percentage value for five determinations was 92.20 with 0.76 % of coefficient of variation by spectrofluorodensitometric method and 92.56 with 0.78 % of coefficient of variation by spectrophotometric method.

The data presented were shown a good precision and a close relationship between two methods. It was evident that the spectrofluorodensitometric method gave reproducible results which compared well with the official spectrophotometric method.

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

The five commercial finished products of vitamin-hormone preparations, were analyzed by using spectrofluorodensitometric method. The methyltestosterone contents in each sample were determined triplicate using the data-pair technique. As shown in Table 12, the coefficient of variations were between 1.89 and 3.81 %.

The results were such as to indicate that the three determinations in the same plate for each analysis were reproducible and satisfactory.

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

Standard methyltestosterone of various amounts added into vitamin-hormone preparations, containing the exact amount of methyltestosterone, the percent recoveries of methyltestosterone were determined by using spectrofluorodensitometric method. The mean percent recoveries were calculated from triplicate determinations as shown in Table 13. The percent recoveries were 99.73, 100.87 and 99.42 with 1.80, 2.71 and 1.67 % of coefficient of variation for the weight of methyltestosterone added: 0.50, 1.00 and 1.50 mg, respectively.

The results showed that the proposed method produced good recovery with high reproducibility. The other active ingredients and inactive excipients in the preparations showed no effect for the method.

Analysis of Vitamin-Hormone Preparations Containing Methyltestosterone

The twelve commercially available formulations with different amounts of methyltestosterone were analyzed by using spectrofluorodensitometric method. The results were the mean value of triplicate determinations of each sample expressed in percentage of the labelled amount as shown in Table 14. In formula A, which methyltestosterone combined with hormone and vitamins, the result was 1.98 % of coefficient of variation. In formula B, that combined with hormone, vitamins and minerals, the coefficient of variation was

1.23 - 3.84 %. In formula C, that combined with vitamins and alkaloids; strychnine and yohimbine, the result was 3.05 % of coefficient of variation.