CHAPTER III

RESULTS AND DISCUSSION

Mebendazole, an anthelmintic drug, reacted with 2,4-dinitrophenylhydrazine in acidic medium, at high temperature to yield an orange precipitate of 2,4-dinitrophenylhydrazone derivative. This derivative was identified by chromatographic and spectrometric methods, the results obtained were the following.

I Identification of 2,4-Dinitrophenylhydrazone Derivative

1. Thin Layer Chromatography

Mebendazole, 2,4-DNPH and freshly prepared 2,4-DNPH' zone derivative were chromatographed on a silica gel 60 GF 254 glass plate by using chloroform as developing solvent. The Rf value results of the three compounds were shown in Table 1.

The Rf value of 2,4-DNPH' zone derivative differed from those of mebendazole and 2,4-DNPH indicating that the hydrazone compound was produced by the reaction. The compound was mobilized by chloroform better than mebendazole, perhaps it contained functional group of 2,4-DNPH and mebendazole in its complex structure which might be solubilized in chloroform.

2. UV-V Spectrometry

Absorption spectra of mebendazole, 2,4-DNPH and 2,4-DNPH' zone derivative in chloroform-methanol (2:1) mixture were shown in

Figure 2. 2,4-DNPH showed a maximum absorption wavelength at about 350 nm, while mebendazole showed an absorption wavelength at about 313 nm and 249 nm. 2,4-DNPH' zone derivative showed maximum absorption wavelength, separated from those two compounds, at 393 ± 2 nm due to auxochromes and chromophores of 2,4-DNPH. These gave an advantage in the determination of mebendazole by forming 2,4-DNPH' zone. The bathochromic shift was the resulting from its more conjugation.

In addition, the spectrum of the derivative showed combined absorbance of both mebendazole and 2,4-DNPH at various wavelengths indicating that the derivative consisted of functional groups of the two compounds.

3. Infrared Spectrometry (IR Spectrometry)

The IR spectra of mebendazole, 2,4-DNPH and 2,4-DNPH' zone derivative in KBr were shown in Figures 3-5. The analysis of the absorption peaks of each compounds were demonstrated below (40,41).

Mebendazole

3420 cm^{-1}	N-H stretching
1720 cm^{-1}	C=O stretching of carbamate group
1650 cm^{-1}	C=O stretching of aromatic ketone
	N-H bending of carbamate group
	C=N stretching of imidazole ring

2,4-DNPH

3340, 3120 cm^{-1}	N-H stretching
1570-1650 cm^{-1}	N-H bending
1330, 1520 cm^{-1}	NO_2 stretching
1220-1330 cm^{-1}	C-N bending of aromatic amine.

2,4-DNPH' zone derivative

3300, 3350 cm^{-1}	N-H stretching
1740 cm^{-1}	C=O stretching of carbamate group
1650 cm^{-1}	C=N of hydrazone and imidazole ring
1330, 1520 cm^{-1}	NO_2 stretching
1220-1340 cm^{-1}	C-N bending of aromatic amine

2,4-DNPH' zone derivative showed N-H stretching at 3300 and 3350 cm^{-1} which was closed to the N-H stretching of 2,4-DNPH. The sharp N-H stretching at 3420 cm^{-1}, which represented a N-H stretching in imidazole ring, was broad in spectrum of 2,4-DNPH' zone derivative because the protonation occured.

C=O stretching of aromatic ketone of mebendazole at 1650 cm^{-1} was difficult to predict whether it was present or absent in 2,4-DNPH' zone because the spectrum of 2,4-DNPH' zone at this frequency was composed of C=N stretching of hydrazone and C=N stretching of imidazole. But C=O stretching of carbamate at 1740 cm^{-1} still remained, indicating that 2,4-DNPH did not react at carbonyl carbon of carbamate. Therefore it should react at carbonyl carbon of aromatic ketone.

In addition, the IR spectra of this derivative also showed peaks composed of both mebendazole and 2,4-DNPH, such as C=O stretching and C=N stretching of mebendazole, NO_2 stretching and $C_{aromatic}$-N bending of 2,4-DNPH.

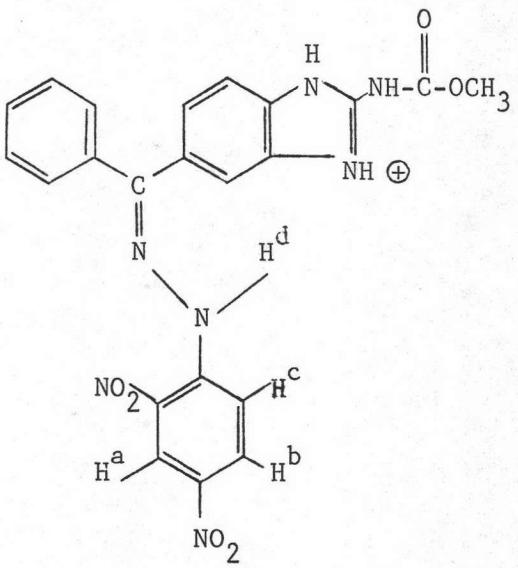
4. H^1-Nuclear Magnetic Resonance Spectrometry

H^1-NMR spectra of mebendazole and 2,4-DNPH' zone derivative were obtained by using DMSO-d_6 as solvent and TMS as reference

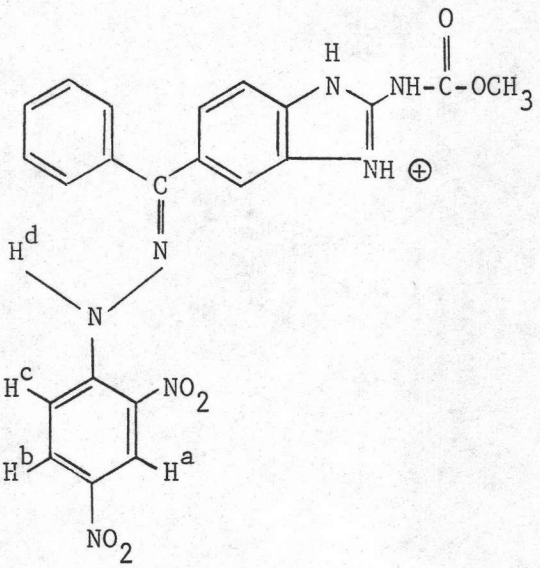
standard. H^1-NMR spectrum of mebendazole was shown in Figure 6 and analysed as follows : (42).

δ 3.78	singlet	3H of $-OCH_3$
δ 7.55-8.28	multiplet	protons of heteroaromatic ring
δ 11.94	singlet (broad)	N-H of imidazole ring

H^1-NMR spectrum of 2,4-DNPH' zone derivative in DMSO-d_6 was shown in Figure 7 and was analysed as follows : (42,43).



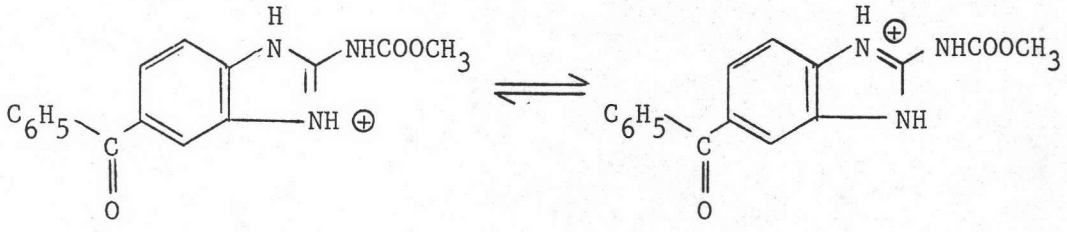
Syn-isomer



Anti-isomer

δ 3.80	singlet	3H of $-OCH_3$
δ 7.50-7.70	multiplet	protons of heteroaromatic ring
δ 8.83	singlet	H^a
δ 8.36	doublet (J = 9 Hz)	H^b
δ 7.15	doublet (J = 9 Hz)	H^c
δ 11.05, 11.25	singlet, singlet (syn and anti)	H^d

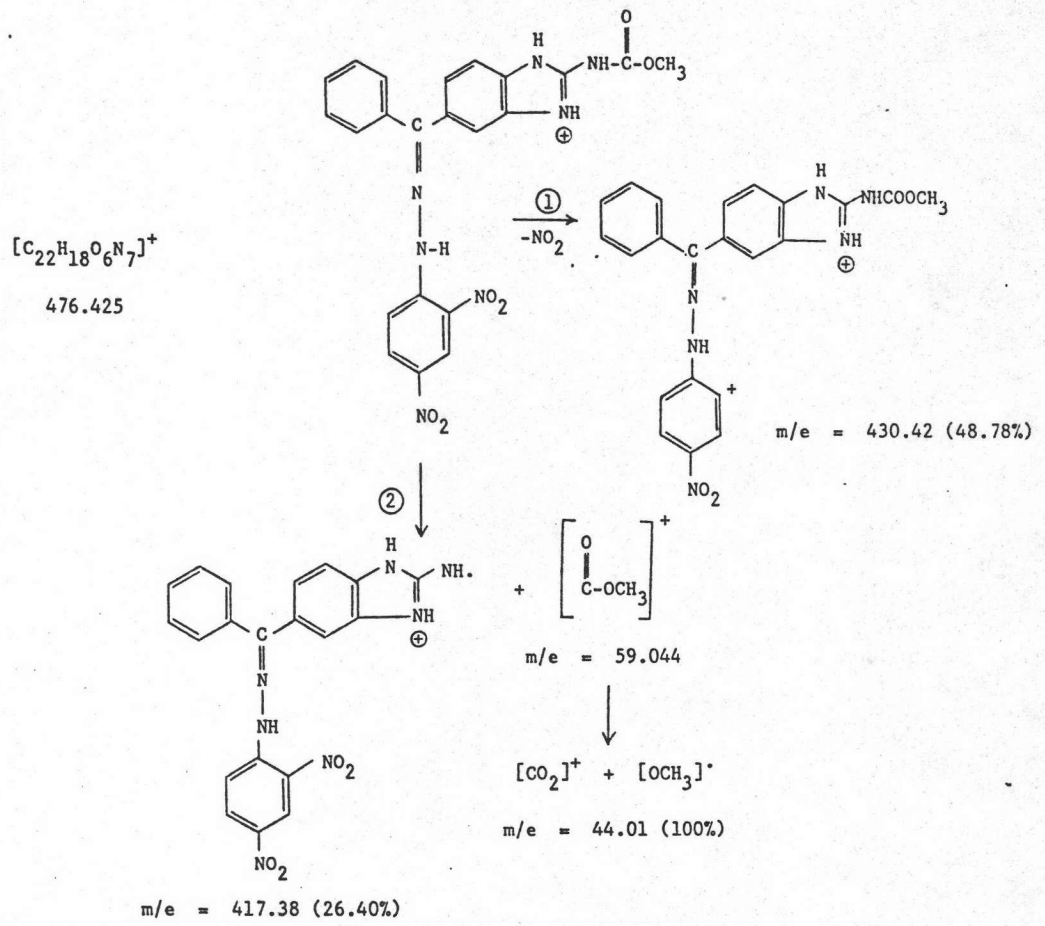
Proton of N-H of imidazole did not show signal in the spectrum of 2,4-DNPH' zone because it was protonated by acid, and the resonance was occured. This reason was confirmed by examining H^1-NMR of mebendazole in acidified DMSO-d_6. The spectrum of H^1-NMR of mebendazole in acidified DMSO-d_6 was shown in Figure 8. There was no signal of proton of N-H.



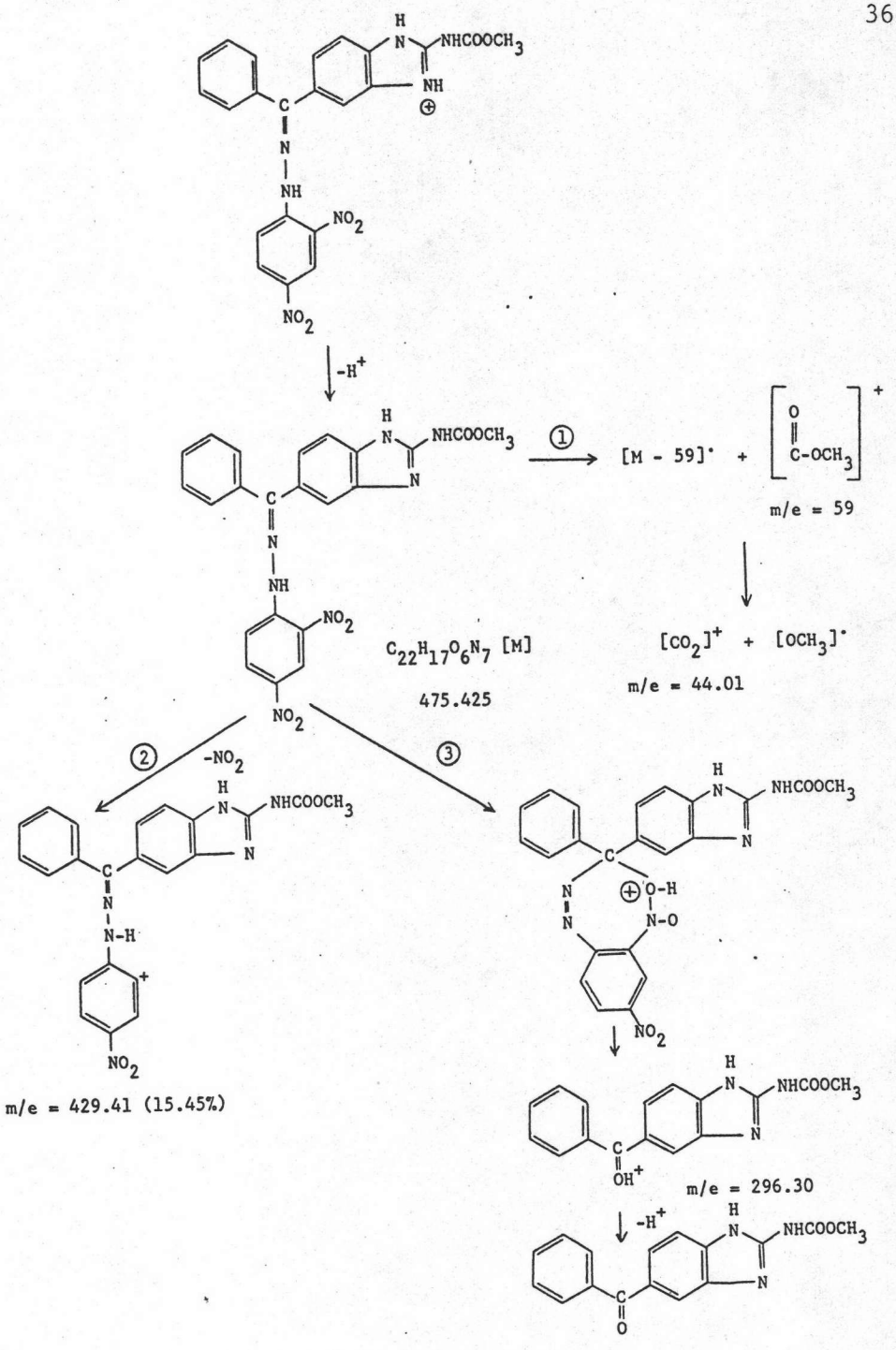
The information from H^1-NMR spectra indicated that an orange precipitate obtained was 2,4-DNPH'zone derivative and C=O of aromatic ketone was the only site which 2,4-DNPH attacked.

5. Mass Spectrometry

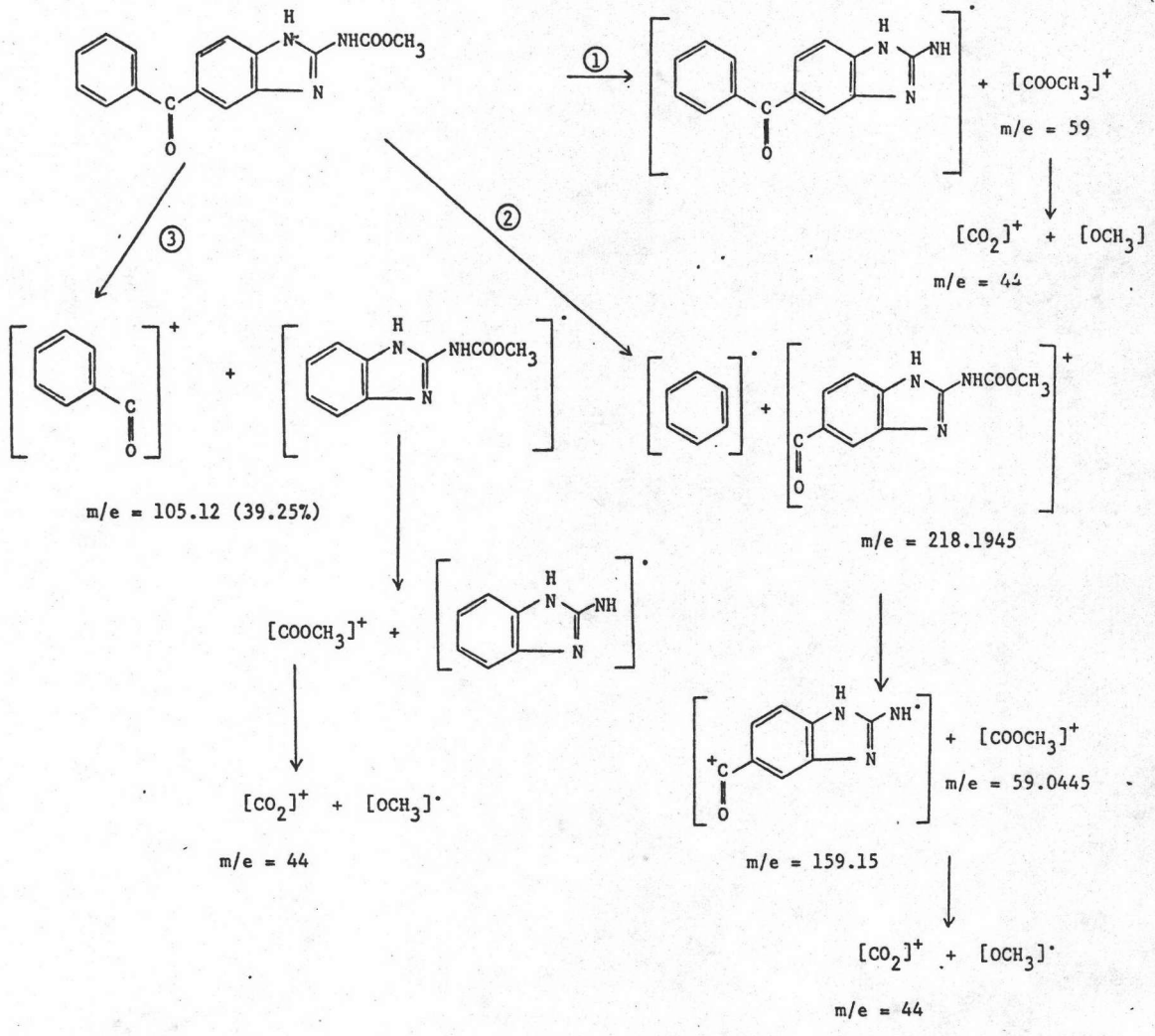
Mass spectrometric identification of freshly prepared 2,4-DNPH' zone derivative was done on electron impact mass spectrometer (EIMS). Mass spectrum was shown in Figure 9. The most mass/charge (m/e) was 458.1, so it indicated that the mole ratio of mebendazole to 2,4-DNPH was one to one (1:1). Interesting fragmentation (44-46) and m/e (% relative intensity) were shown on Scheme A and B.


[C22H18O6N7]+
476.425
-NO2
m/e = 430.42 (48.78%)
m/e = 59.044
[CO2]+ + [OCH3]·
m/e = 44.01 (100%)
m/e = 417.38 (26.40%)

Scheme A


NHCOOCH3
NH
NO2
-H+
①
[M - 59]· + [C-OCH3]+
m/e = 59
[CO2]+ + [OCH3]·
m/e = 44.01
C22H17O6N7 [M]
475.425
②
-NO2
③
N-H
O-H
N-O
m/e = 429.41 (15.45%)
OH+
m/e = 296.30
-H+
O

Scheme B


①
②
③
+ [COOCH3]+
m/e = 59
[CO2]+ + [OCH3]
m/e = 44
m/e = 105.12 (39.25%)
m/e = 218.1945
[COOCH3]+ +
[CO2]+ + [OCH3]·
m/e = 44
+ [COOCH3]+
m/e = 59.0445
m/e = 159.15
[CO2]+ + [OCH3]·
m/e = 44

II Chemical Reactions of 2,4-Dinitrophenylhydrazine with Other Carbonyl Compounds

Results of chemical reactions of 2,4-DNPH with carbonyl compounds; benzophenone, carbachol, mebendazole, methocarbamol, parbendazole and ronidazole; were shown in Table 2 and 3. Positive result was judged by forming yellow to orange precipitate. In acidified methanol medium, if no solid separated within 1 hour, the solution was carefully diluted with 2 N sulphuric acid (47) in order to decrease its solubility and bring it to separate from solvent while 2,4-DNPH did not separate.

The carbonyl compounds which gave positive results from both two medium, 2 N sulphuric acid and acidified methanol after diluting with 2 N sulphuric acid, were benzophenone and mebendazole. The carbonyl compounds which gave no change were carbachol, methocarbamol, parbendazole and ronidazole. Therefore, it indicated that only carbonyl carbon of ketone reacted with 2,4-DNPH but carbonyl carbon of carbamate did not react with 2,4-DNPH. Mebendazole had carbonyl carbon of ketone and of carbamate, so only carbonyl carbon of ketone reacted with 2,4-DNPH to form 2,4-DNPH' zone derivative.

III Affecting Factors on Assay of Mebendazole by Using 2,4-Dinitrophenylhydrazine Method

The optimum conditions for reaction of mebendazole and 2,4-DNPH were examined in order to perform the assay of mebendazole tablets. To obtain good results, four determinations were repeated for each condition, then mean values and percentage of coefficient of variation

(% CV) were calculated. The selected optimum conditions were depended on those values and also on the time consumed.

1. Maximum Absorption Wavelength

The absorption spectrum of 2,4-DNPH' zone derivative in chloroform-methanol (2:1) mixture, scanned from 200 to 500 nm was performed and the maximum absorption was determined. The absorbance maximum at 393 ± 2 nm was used as a wavelength in the determination of affecting factors on the assay of mebendazole. Although 2,4-DNPH showed an absorption band at 354 nm (Figure 2), which is closed to the absorption band at 393 nm, the assay can be performed by washing off the excess of 2,4-DNPH from precipitate before measuring the absorbance.

2. Effect of Temperature

The effect of temperature on chemical reaction of mebendazole and 2,4-DNPH was investigated at temperature of 50°, 60°, 70°, 80°, 100°C and room temperature for 1 hour. The results shown in Table 4 and Figure 11 indicated that absorbance, due to the formation of derivative, increased when the temperature increased from room temperature to 60-80°C. The optimum temperatures were in the range of 60-80°C, so the temperature at 70°C was selected. At 100°C there was a great change in reaction medium due to the loss of water by evaporation, resulted in a large standard deviation. Thus for a long time about 1 hour, this temperature was not suitable unless it was refluxing which was not convenient to do.

3. Effect of Time at 70°C

Several mixtures of mebendazole and 2,4-DNPH were warmed at 70°C, the optimum temperature, for different periods of time 0, 15, 30, 45, 60, and 90 minutes and allowed to set at room temperature for 120, 105, 90, 75, 60 and 30 minutes, respectively, to have equal net reaction time 2 hours of all mixtures. The results shown in Table 5 and Figure 12 indicated that the optimum warming time at 70°C was 45-90 minutes, and 60 minutes was selected.

4. Effect of Time at Room Temperature

After the reaction mixtures were warmed at 70°C for 1 hour, the reaction time for setting at room temperature were determined at 30, 60, 90, 120 minutes and 1 day, respectively. The results shown in Table 6 and Figure 13 indicated that precipitation depended on time. Precipitation was increased when time was increased. Two hours and 1 day did not give much difference in absorbance. The time for setting at room temperature was selected at 1 hour although it did not give maximum absorption, it gave acceptable standard deviation and was not too long for the determination of mebendozole tablets. To obtain a good result for the determination of mebendozole tablets, the reaction time used for standard and sample should be the same.

5. Effect of Acidity

In this work the suitable acidity of reaction medium was examined by varying the normality of acid solution. The experiment data in Table 7 and Figure 14 indicated that optimum acidity was between 1.5 N and 2.5 N. The acidity selected was 2 N sulphuric acid.

6. Optimum Ratio of Mebendazole to 2,4-Dinitrophenylhydrazine

To determine amount of 2,4-DNPH required for maximum formation of 2,4-DNPH' zone, various amount of 2,4-DNPH solution 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 ml were added to 1.0 ml of mebendazole solution. The results were shown in Table 8 and Figure 15 and ratios of mebendazole to 2,4-DNPH were shown in volume by volume (ml), weight by weight (mg) and mole by mole. Suitable ratios were 1:3 to 1:6 (by volume) or 1:15 to 1:30 (by mole). In order to ensure that an excess of reagent was used in quantitative analysis, 5.0 ml of 2,4-DNPH solution was selected for 1.0 ml of mebendazole solution (1 mg/ml).

7. Effect of Time on Stability of 2,4-Dinitrophenylhydrazone Derivative

Stability of 2,4-DNPH' zone derivative at room temperature was examined at selected intervals of time within 3 hours and at 18 and 24 hours. The results shown in Table 9 and Figure 16. 2,4-DNPH' zone derivative in chloroform-methanol (2:1) mixture was very stable at room temperature, so it could be measured at any time intervals which gave good results for quantitative analysis.

8. Linearity of Absorbance against Concentration of Mebendazole

For quantitative analysis, linearity of absorbance with concentration of drug should be investigated whether it obeyed Lambert-Beer's law or not. Results were shown in Table 10 and Figure 17, mebendazole showed linear absorbance-concentration relationship from 1 to 7 mcg/ml with correlation coefficient 0.9999

and their calibration curve passed through the origin.

IV Determination of Percent Labelled Amount of Mebendazole Tablet

The content of mebendazole in mebendazole tablets from five manufacturers were determined by 2,4-DNPH method and USP XXI method. The results were shown in Table 11, indicating an acceptable precision and a close relationship between the two methods. 2,4-DNPH method gave reproducible results and was comparable to USP XXI method.

The tablets from manufacturer A gave high percent coefficient of variation by using USP XXI method because the emulsion formed during extraction.

V Determination of Percent Recovery of Mebendazole in Mebendazole Tablets

The accuracy of 2,4-DNPH method was checked by using the percent recovery. In order to be sure that the excipients in dosage form did not interfere the determination of sample, the different accurate amounts of standard mebendazole about 10, 20 and 30 mg were added to mebendazole tablets. The determination of mebendazole by 2,4-DNPH method and USP XXI method were performed and the results were shown in Table 12, indicated that both methods gave acceptable recoveries for different weights of standard mebendazole added.