# CHAPTER II

# EXPERIMENTAL

## I Instruments and Materials

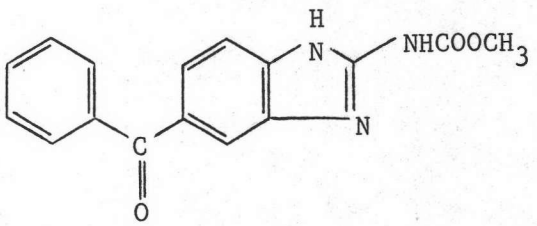
### 1. Instruments

1. Shimadzu UV 180 Spectrometer
2. Shimadzu Infrared Spectrometer-440
3. Jeol FX 90 Q Nuclear Magnetic Spectrometer
4. Jeol DX 300 double focusing Mass Spectrometer

### 2. Materials

1. Mebendazole (18,31) USP

The chemical name of mebendazole is carbamic acid, (5-benzoyl-1H-benzimidazol-2-yl), methyl ester, and its structure is shown below



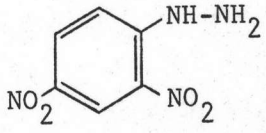

$C_{16}H_{13}N_3O_3$

MW. 295.30

Mebendazole is a white to slighly yellow powder, melts at about 280℃ with decomposition. It is almost insoluble in water,

ethanol, ether and chloroform but readily soluble in formic acid.

2. 2,4-Dinitrophenylhydrazine (32) - Merck




$C_6H_6N_4O_4$

MW. 198.14

2,4-Dinitrophenylhydrazine is a red crystalline powder, melts at about 200°C. It is slightly soluble in water or alcohol, but soluble in moderately dilute inorganic acids.

3. Methanol - Merck
4. Chloroform - Merck
5. Sulphuric acid - Merck
6. Isopropyl alcohol - Merck
7. Formic acid - Merck

All of chemicals used were analytical grade except mebendazole was pharmaceutical grade.

## II Mebendazole Solution

Mebendazole was weighed accurately about 0.1 g, transferred to a 100-ml volumetric flask with the aid of 20 ml methanol. A 0.8 ml of concentrated sulphuric acid was added and swirled until it was dissolved. And then followed by adding about 60 ml of water, mixed, cooled with tap water and added up to volume with water. The final concentration of the solution was about 1 mg/ml.

## III Chemical Reaction of 2,4-Dinitrophenylhydrazine and Mebendazole

### 1. 2,4-Dinitrophenylhydrazine Solution

The method of preparation of 2,4-DNPH solution was followed to A.I. Vogel's method (33) with a slightly modification. Sulphuric acid was used instead of hydrochloric acid.

A 0.335 g of 2,4-DNPH was dissolved in 100 ml of 2 N sulphuric acid and heated on water bath until a clear solution was obtained, cooled and filtered if necessary. The solution of 2,4-DNPH should be freshly prepared.

### 2. Preparation of 2,4-Dinitrophenylhydrazone Derivative

A 200 ml of 2,4-DNPH solution was added to 50 ml of mebendazole solution in a 500-ml conical flask. After heating for 1 hour at 70°C, the mixture was allowed to stand at room temperature for 1 hour. The orange precipitate was filtered off, washed with 2 N sulphuric acid, then with water to remove the excess reagent and the acid respectively.

### 3. Identification of 2,4-Dinitrophenylhydrazone Derivative

The orange precipitate obtained was identified by thin layer chromatography, ultraviolet-visible spectrometry, infrared spectrometry, nuclear magnetic spectrometry and mass spectrometry without further purification.

#### 3.1 Thin Layer Chromatography (TLC)

Thin layer chromatography was used to show Rf values of mebendazole, 2,4-DNPH and 2,4-DNPH' zone derivative. The solutions of the freshly prepared 2,4-DNPH' zone derivative, 2,4-DNPH and mebendazole were prepared in chloroform-methanol (2:1) mixture.

Each of 5 µl of these three solutions were chromatographed on a silica gel 60 GF 254 glass plate, and developed in chloroform tank until solvent front was about 10 cm from started line. 2,4-DNPH and 2,4-DNPH' zone derivative could be located visually but mebendazole had to be located under short-wavelength ultraviolet light (254 nm).

3.2 Ultraviolet-Visible Spectrometry (UV-V Spectrometry)

All absorption spectra were scanned on Shimadzu UV-180 Spectrometer using 1-cm quartz cells. Mebendazole and 2,4-DNPH were dissolved in chloroform-methanol (2:1) mixture to obtain the concentration of about 5 mcg/ml. 2,4-DNPH' zone derivative solution was prepared by dissolving the freshly prepared 2,4-DNPH' zone derivative in chloroform-methanol (2:1) mixture to obtain the concentration which was equivalent to about 5 mcg/ml of mebendazole. These three solutions were scanned for UV-V absorption from 200 to 500 nm against chloroform-methanol (2:1) mixture as blank.

3.3 Infrared Spectrometry (IR Spectrometry)

Infrared Spectrometry of mebendazole, 2,4-DNPH and its derivative were taken in KBr pellets on a Shimadzu IR 440 Spectrometer at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

3.4 Proton Nuclear Magnetic Resonance Spectrometry ($H^1$-NMR Spectrometry)

The $H^1$-NMR spectra of mebendazole and freshly prepared 2,4-DNPH' zone derivative were recorded on a Jeol FX 90 Q NMR spectrometer with a frequency of 90 MHz at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

DMSO-$d_6$ was used as a solvent. In addition, the $H^1$-NMR spectrum of mebendazole in acidified DMSO-$d_6$, prepared by adding a drop of concentrated hydrochloric acid to mebendazole-DMSO-$d_6$ solution, was investigated.

### 3.5 Mass Spectrometry

Mass spectrum of freshly prepared 2,4-DNPH' zone was obtained on a Jeol DX 300 double focusing mass spectrometer, electron impact (EI) type, at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

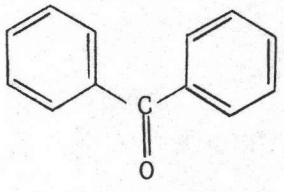
## IV Chemical Reactions of 2,4-Dinitrophenylhydrazine and Other Carbonyl Compounds

The chemical reactions of 2,4-DNPH and other carbonyl compounds were studied in order to concluded whether the reaction was at carbonyl group of ketone or carbamate.

### 1. Carbonyl Compounds

#### 1.1 Benzophenone (34)

Chemical name of benzophenone is diphenylketone and its structure is shown below.



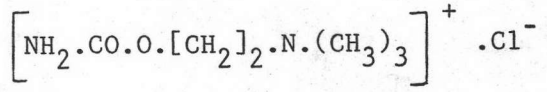
$C_{13}H_{10}O$

MW. 182.21

One gram of benzophenone dissolves in 7.5 ml alcohol.

1.2 Carbachol (35)

The chemical name of carbachol is (2-Carbamoyloxyethyl) trimethylammonium chloride and its structure is shown below.



$C_6H_{15}ClN_2O_2$

MW. 182.7

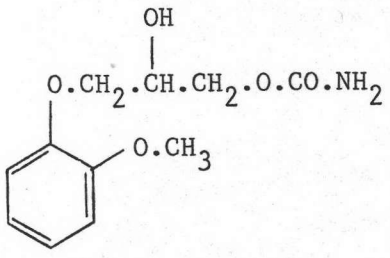
Carbachol is a white hygroscopic crystalline powder, melts at about 210℃ with decomposition. It is soluble in water and ethanol, very slightly soluble in dehydrated ethanol but more readily so on boiling, almost insoluble in ether and chloroform.

1.3 Mebendazole

Mebendazole was mentioned in the instruments and material.

1.4 Methocarbamol (36)

The chemical name of methocarbamol is 2-Hydroxy-3-O-methoxyphenoxypropyl carbamate and its structure is shown below



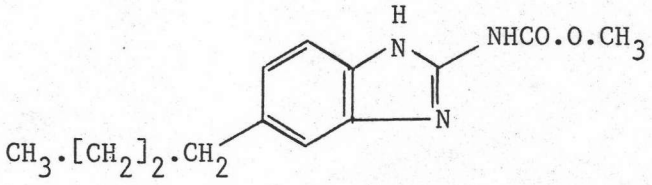
$C_{11}H_{15}NO_5$

MW. 241.2

Methocarbamol is a white crystalline powder, melts at about 92° to 94℃. It is soluble in water and ethanol.

1.5 Parbendazole (37,38)

The chemical name of parbendazole is methyl N-(5-butyl-benzimidazol-2-yl) carbamate, and its structure is shown below.



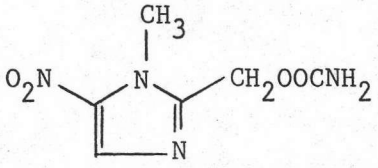
$C_{13}H_{17}N_3O_2$

MW. 247.29

Parbendazole is a white to yellowish-white crystalline powder, melts at about 224° to 227℃ with decomposition. It is practically insoluble in water.

1.6 Ronidazole (39)

The chemical name of ronidazole is 1-methyl-5-nitro-imidazole-2-methanol carbamate and its structure is shown below.



$C_6H_8N_4O_4$

MW. 200.16

Ronidazole is pale yellow crystals, melts at about 167° to 169℃. It is soluble in water (at pH 6.5) about 2.9 mg/ml at room temperature; more soluble in acid solutions; and soluble in methanol, ethanol, chloroform and ethyl acetate.

All of carbonyl compounds used were pharmaceutical grade, except benzophenone was analytical grade.

2. 2,4-Dinitrophenylhydrazine Solution

Two solutions of 2,4-DNPH were prepared as followed :

Solutions A is the solution of 3.35 mg/ml 2,4-DNPH in 2 N $H_2SO_4$. The preparation method was metioned under the chemical reaction of 2,4-dinitrophenylhydrazine and mebendazole.

Solution B is the solution of 20 mg/ml 2,4-DNPH in acidified methanol. The preparation method was done by suspending 2.0 g of 2,4-DNPH in 100 ml of methanol. A 4.0 ml of concentrated sulphuric acid was added slowly and cautiously to the mixture, and filtered if necessary.

3. Method of Study

3.1 Each carbonyl compound was weighed accurately about 5 mg and transferred to each 15-ml tube, and dissolved by adding of 3.0 ml of methanol and 0.1 ml of concentrated sulphuric acid. Then 5.0 ml of Solution A was added, warmed at 70°C for 1 hour, cooled and allowed to stand at room temperature for 1 hour. The chemical reactions were observed and recorded.

3.2 Each carbonyl compound was weighed accurately about 5 mg and transferred to each 15-ml tube, and dissolved by adding of 1.0 ml of methanol and 0.1 ml of concentrated sulphuric acid. Then 5.0 ml of Solution B was added, mixed, warmed at 70°C for 1 hour, cooled, and allowed to stand at room temperature for 1 hour. After chemical reactions were observed and recorded, 5.0 ml of 2 N sulphuric acid was added to each tube, mixed and allowed them to stand at room

temperature for 30-60 minutes. Again, chemical reactions were observed and recorded.

V Affecting Factors on Assay of Mebendazole by Using 2,4-Dinitro phenylhydrazine (2,4-DNPH) Method

Mebendazole and 2,4-Dinitrophenylhydrazine Solutions have been previously mentioned under the chemical reaction of 2,4-dinitrophenylhydrazine and mebendazole.

1. Determination of Maximum Absorption Wavelength

A 1.0 ml of mebendazole solution and a 5.0 ml of 2,4-DNPH solution were pipetted into a 15-ml tube, mixed and warmed at 70°C for 1 hour. After cooling to room temperature, this mixture was allowed to stand at room temperature for 1 hour. The orange precipitate obtained was filltered quantitatively through sintered glass crucible No. 4, washed with 2 N sulphuric acid to remove excess 2,4-DNPH solution and followed by washing with water until it was free from acid. The precipitate was dissolved in chloroform-methanol (2:1) mixture, transferred quantitatively to a 10-ml volumetric flask, and added up to the volume with the same solvent. A 0.50 ml of this solution was pipetted into another 10-ml volumetric flask and added up to volume with the same solvent. The resulting solution was scanned from 200 to 500 nm against solvent blank on Shimadzu UV-180 Spectrometer in 1-cm cells, and the maximum absorption wavelength was determined.

2. Determination of Effect of Temperature

1.0 ml of mebendazole solution and 5.0 ml of 2,4-DNPH

solution were pipetted into each of six 15-ml tubes. Each of five tubes was warmed at different temperature : 50°, 60°, 70°, 80° and 100°C respectively for 1 hour, cooled and allowed to stand at room temperature for 1 hour. The sixth tube was allowed to stand at room temperature for 2 hours. The 2,4-DNPH' zone derivative obtained from each tube was treated in the same procedure mentioned under the determination of maximum absorption wavelength. The absorbance of each of the resulting solutions was measured in a 1-cm cell at 393 nm against chloroform-methanol (2:1) mixture.

3. Determination of Effect of Time at 70°C

1.0 ml of mebendazole solution and 5.0 ml of 2,4-DNPH solution were pipetted into each of six 15-ml tubes. Each of five tubes was warmed at 70°C for 15,30,45,60 and 90 minutes, cooled, and allowed to stand at room temperature for 105,90,75,60 and 30 minutes respectively. The sixth tube was allowed to stand at room temperature for 2 hours. The reaction time for all tubes were 2 hours. The 2,4-DNPH' zone derivative obtained from each tube was treated in the same procedure mentioned under the determination of maximum absorption wavelength. The absorbance of each of the resulting solutions was measured in a 1-cm cell at 393 nm against chloroform-methanol (2:1) mixture.

4. Determination of Effect of Time for Completed Reaction at Room Temperature

1.0 ml of mebendazole solution and 5.0 ml of 2,4-DNPH solution were pipetted into each of five 15-ml tubes. All five tubes were warmed at 70°C for 1 hour, cooled, and allowed each of them to stand at room temperature for different time : 30,60,90,120 minutes

and 1 day respectively. Each 2,4-DNPH' zone derivative obtained was treated in the same procedure mentioned under the determination of maximum absorption wavelength. The absorbance of each of the resulting solutions was measured in a 1-cm cell at 393 nm against chloroform-methanol (2:1) mixture.

5. Determination of Effect of Acidity

The acidity of the reaction medium was studied at various acidic strength by using 2,4-DNPH solution repersented as reaction medium. Each of the 2,4-DNPH solution was prepared to have its acidic strength 1.5,2.0,2.5,3.0 and 4.0 N sulphuric acid while its concentration was maintained at 3.35 mg/ml. The preparation of 2,4-DNPH solutions at various acidic strength were done as follows : each 0.335 g of 2,4-DNPH was suspended in 100 ml of 1.5,2.0,2.5,3.0 and 4.0 N sulphuric acid respectively. The mixtures were warmed on water bath to dissolve 2,4-DNPH, cooled and filtered if necessary.

A 1.0 ml of mebendazole solution and a 5.0 ml of 2,4-DNPH in 1.5 N sulphuric acid were pipetted into a 15-ml tube and mixed. This mixture was warmed at 70°C for 1 hour, cooled, and allowed to stand at room temperature for 1 hour. The 2,4-DNPH' zone derivative obtained was treated in the same procedure mentioned under the determination of maximum absorption wavelength. The absorbance of the resulting solution was measured in a 1-cm cell at 393 nm against chloroform-methanol (2:1) mixture. The other four 1.0 ml mebendazole solutions were proceeded in the same manner but 2,4-DNPH in 2.0,2.5,3.0 and 4.0 N sulphuric acid were used instead of 2,4-DNPH in 1.5 N sulphuric acid.

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

1.0 ml of mebendazole solution was pipetted into each of seven 15-ml tubes, and different volumes of 2,4-DNPH solution : 1.0,2.0,3.0, 4.0,5.0,6.0 and 7.0 ml were added into these seven tubes respectively. The mixture were mixed briefly, warmed at 70°C for 1 hour, cooled and allowed to stand at room temperature for 1 hour. Each 2,4-DNPH' zone derivative obtained was examined in the same procedure mentioned under the determination of maximum absorption wavelength. The absorbance of each of the resulting solutions was measured in a 1-cm cell at 393 nm against chloroform-methanol (2:1) mixture.

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

A 1.0 ml of mebendazole solution and a 5.0 ml of 2,4-DNPH solution were pipetted into a 15-ml tube, mixed it briefly. This mixture was warmed at 70°C for 1 hour, cooled, and allowed to stand at room temperature for 1 hour. The 2,4-DNPH' zone derivative was filtered off, washed with 2 N sulphuric acid and with water to remove the excess of the reagent and the acid respectively. The derivative was transferred quantitatively with the aid of chloroform-methanol (2:1) mixture to a 10-ml volumetric flask. An aliquot of this solution (3.0 ml) was diluted to 50.0 ml with the same solvent. The absorbance of the resulting solution was measured in a 1-cm cell at 393 nm at different time : 5,15,30,45,60,90,120,150,180 minutes, 18 and 24 hours.

8. Determination of the Linearity of Absorbance against Concentration of Mebendazole

Four determinations were performed for each concentration. 0.2,0.4,0.6,0.8,1.0,1.2 and 1.4 ml of mebendazole solution were pipetted separately into each 15-ml tube, and added 1.0,2.0,3.0,4.0, 5.0,6.0 and 7.0 ml of 2,4-DNPH solution to each tube respectively. These mixtures were warmed at 70°C for 1 hour, cooled and allowed to stand at room temperature for 1 hour. The derivative obtained from each tube was treated in the same procedure mentioned under the determination of maximum absorption wavelenght. The absorbance of each of the resulting solutions was measured in a 1-cm cell at 393 nm against chloroform-methanol (2:1) mixture.

VI Determination of The Percent Labelled Amount of Mebendazole Tablets

1. 2,4-Dinitrophenylhydrazine Method

Standard Solution - An accurately weighed about 100 mg of mebendazole standard was transferred into a 100-ml volumetric flask with the aid of 20 ml methanol. A 0.8 ml of concentrated sulphuric acid was added and swirled until it was dissolved, and then followed by adding about 60 ml of water, shook, cooled with tap water and added up to volume with water.

Assay Solution - Twenty mebendazole tablets were weighed and ground to fine powder. An accurate portion of the powder, equivalent to about 50 mg of mebendazole, was transferred with the aid of 10 ml of methanol and 0.4 ml of conc. sulphuric acid to a 50-ml

volumetric flask. The mixture was sonicated or shaken for about 15 minutes, cooled and diluted with water to volume. After mixing it briefly, it was filtered through filter paper, rejected the first 15 ml and collected the filtrate.

Procedure - Each of 1.0 ml of standard solution and of assay solution was pipetted into each 15-ml tube. Added 5.0 ml of 2,4-DNPH solution into standard tube and assay tube, warmed at 70°C for 1 hour, cooled, and allowed to stand at room temperature for 1 hour. The derivatives obtained were filtered quantitatively through sintered glass crucible No 4. The derivatives and their tubes were washed with 2 N sulphuric acid until they were free from excess reagent, then washed with water until they were free from acid. The derivatives on sintered glass crucibles and the remaining in their tubes were dissolved with chloroform-methanol (2:1) mixture, transferred quantitatively to each 10-ml volumetric flask, and added up to volume with the same solvent. Each 0.5 ml of these two solutions obtained was pipetted into the other two 10-ml volumetric flasks, added up to volume with the same solvent, and mixed. The absorbance of the resulting standard and assay preparations were measured in a 1-cm cell at 393 nm against chloroform-methanol (2:1) mixture. The amount of mebendazole in tablets was calculated from the following formula

$$\% \text{ Labelled Amount} = \frac{Au \times Cs \times Av.Wt. \times 50}{As \times Wt. \times L} \times 100$$

As = Absorbance of standard preparation

Au = Absorbance of assay preparation

Cs = Concentration of standard solution in mg per ml

L = Labelled amount of tablet in mg

Av.Wt. = Average weight per tablet in mg

Wt. = Weight of sample powder taken in mg

2. USP XXI Method*

Standard preparation with some modification-Transferred about 20 mg of USP Mebendazole RS, accurately weighed, to a 200-ml volumetric flask, and added 90 ml of chloroform, 7 ml of isopropyl alcohol, and 2 ml of a 1 in 10 solution of formic acid in water. Agitated until the solid has dissolved, added isopropyl alcohol to volume, and mixed. Pipetted 5.0 ml of this solution into a 100-ml volumetric flask, diluted with isopropyl alcohol to volume, and mixed to obtain a solution having a known concentration of about 5 mcg/ml.

Assay preparation - Weighed and finely powder not less than 20 mebendazole tablets. Transferred an accurately weighed portion of the powder, equivalent to about 100 mg of mebendazole, to a 100-ml volumetric flask with the aid of 50 ml of formic acid, and heated in water bath at a temperature of 50°C for 15 minutes. Cooled, added water to volume, mixed, and filtered through a medium-porosity sintered glass filter. Pipetted 10.0 ml of the filtrate into a

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*USP XXI pp. 622-623

250-ml separator, and added 50 ml of water and 50 ml of chloroform. Shook for about 2 minutes, allowed the phases to separate, and transferred the chloroform layer to a second 250-ml separator. Washed the aqueous layer with two 10-ml portions of chloroform, added the chloroform washings to the second separator, and discarded the aqueous layer. Washed the combined chloroform solutions with a mixture of 4 ml of 1 N hydrochloric acid and 50 ml of a 1 in 10 solution of formic acid in water, and transferred the chloroform layer to a 100-ml volumetric flask. Extracted the aqueous washing with two 10-ml portions of chloroform, added these chloroform extracts to the chloroform solution in the volumetric flask, diluted with isopropyl alcohol to volume, and mixed. Pipetted 5.0 ml of this solution into another 100-ml volumetric flask, diluted with isopropyl alcohol to volume, and mixed.

Procedure - Mixed 45.0 ml of chloroform with 1 ml of a 1 in 10 solution of formic acid in water in a 100-ml volumetric flask, added isopropyl alcohol to volume, and mixed. Pipetted 5.0 ml of this solution into a second 100-ml volumetric flask, diluted with isopropyl alcohol to volume, and mixed to obtain a reagent blank. Concomitantly determined the absorbance of the Assay preparation and the Standard preparation in 1-cm cells at the wavelength of maximum absorbance at about 247 nm, with a suitable UV-spectrometer, using the reagent blank to set the instrument. Calculated the quantity, in mg, of $C_{16}H_{13}N_3O_3$ in the portion of tablets taken by the formula 20 C (Au/As).

$$\% \text{ Labelled amount} = \frac{20C \times Au \times Av.Wt.}{As \times Wt. \times L} \times 100$$

C = the concentration, in mcg/ml, of USP Mebendazole RS in the Standard preparation

Au = Absorbance of Assay preparation

As = Absorbance of Standard preparation

Av.Wt. = Average weight per tablet in mg

Wt. = Weight of the portion of tablets taken in mg

L = Labelled amount of tablet in mg

VII Determination of the Percent Recovery of Mebendazole in Mebendazole Tablets

1. 2,4-Dinitrophenylhydrazine Method

Standard solution - The procedure was the same in the determination of percent labelled amount of mebendazole tablet.

Assay solution - Twenty mebendazole tablets were weighed and ground to fine powder. Three accurate portions of the powder, equivalent to about 30 mg of mebendazole, were transferred with the aid of 10 ml of methanol and 0.4 ml of conc. sulphuric acid to each three 50-ml volumetric flasks. The mixture was sonicated or shaken for about 15 minutes, added 5.0, 10.0 and 15.0 ml of mebendazole standard solution (2 mg per ml of solvent as standard preparation) respectively, swirled and diluted to volume with water. After mixing, they were filtered through filter paper, rejected the first 15 ml, and collected the filtrates.

Procedure - Proceeded as described in the determination of percent labelled amount of mebendazole tablet.

2. USP XXI Method

Standard preparation - The procedure was the same as directed in determination of percent labelled amount of mebendazole tablet.

Assay preparation - Weighed and finely powdered not less than 20 mebendazole tablets. Three portions of the powdered tablets equivalent to 30 mg of mebendazole were weighed accurately and transferred with the aid of 20, 15 and 10 ml of formic acid to each three 50-ml volumetric flasks respectively. These three flasks were warmed at 50°C for 15 minutes and cooled. Then 5.0, 10.0 and 15.0 ml of mebendazole standard solution (2 mg per ml of formic acid) were added respectively, swirled, added water to volume, mixed and filtered through a medium-porosity sintered glass filter and each 10.0 ml of these three filtrate was extracted in the same procedure as directed in the determination of percent labelled amount of mebendazole tablet.

Procedure - Proceeded as directed in the determination of percent labelled amount of mebendazole tablet.

The quantity, in mg, of mebendazole was calculated by the formula 10 C (Au/As) instead of 20 C (Au/As) because the dilution factor was changed. Assay preparation were prepared in a 50-ml volumetric flask instead of a 100-ml volumetric flask.

Percent recovery of both 2,4-DNPH method and USP XXI method were calculated from the following formula

$$\% \text{ Recovery} = \frac{(Wf - Ws)}{Wa} \times 100$$

Wf = Weight of mebendazole found in mg

Ws = Weight of mebendazole from tablets in mg

Wa = Weight of mebendazole added in mg