MATERIALS AND METHODS

Instruments

1. UV-spectrophotometer (Shimadzu)

2. pH meter

3. Ostwald viscometer

4. picnometer

5. HPLC

   - Water Association chromatography pump

   - Water Model 510 pump

   - Water Association Model 440 absorbance detector

   - Water 740 Data Module printer

   - 15 cm x 4.6 mm i.d. Zobax C8 column

Chemicals

1. Paracetamol working standard 99.97% (GPO)

2. Sulpyrin working standard 99.01% (Department of Medical Science)

3. Ammonium sulfamate, analytical grade (Fluk AG, Buchssc)

4. Sodium nitrite, analytical grade (Riedel-De Haenag Seelze-Hannover)

5. Potassium dihydrogen phosphate, analytical grade (Riedel-De Haenag Seelze-Hannover)

6. Concentrated hydrochloric acid 37%, analytical grade (E-Merck)

7. Sodium hydroxide, analytical grade (E-Merck)

8. Methanol, HPLC grade (JT-Baker)

9. Commercially available liquid paracetamol preparations.

10. Commercially available paracetamol tablets.

Reagents

Preparation of Phosphate Buffer Solution

Dissolve 1.3609 gm of potassium dihydrogen phosphate in distilled water and make to 1,000 ml The solution was adjusted with concentrated phosphoric acid to make pH 4.0.

Preparation of Solvent Mixture

Mix 15 ml of methanol and 85 ml of 0.010 M. phosphate buffer pH 4.0 solution to be used as solvent for HPLC method.

Method

Official USP XXI Method*

USP method for determining of paracetamol in paracetamol suspension (23), official method, was used as reference method to compare with HPLC method (proposed method) in this study. The USP method is colourimetric method through pathway that paracetamol and nitrous acid reaction under mild condition to form 2-nitro-4-acetam idophenol, which can be measured in alkaline solution (Scheme 5).

(28)

λ max = 250,285 nm λ max = 370 nm λ max = 430 nm

(Scheme 5)
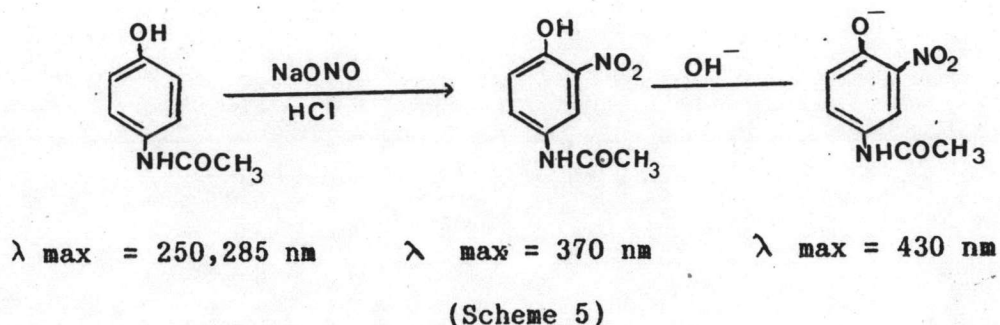
This method may be interfered by preparation's colour changing in brown colour in case of syrup.

Beer's Law Plot of paracetamol Solution

A 6.0, 5.0, 4.0, 3.0 and 2.0 ml of the 1.25 mg/ml paracetamol solution was transfered to 50-ml volumetric flasks, diluted with water to volume and mixed. A 10.0 ml each of the solution, and water to provide a blank was transfered to separate 50-ml volumetric flasks. Each solution was treated as follows.

1. A 2.0 ml of 6 N hydrochloric acid was added. The solution was mixed.

2. A 5.0 ml of 10% sodium nitrite solution was added. The solution was mixed and allowed to stand for 15 minutes.

3. A 5.0 ml of 15% ammonium sulfamate solution was added. The solution was swirled gently and allowed to cool to room temperature.

4. A 15.0 ml of 2.5 N sodium hydroxide was added and allowed to cool to room temperature.

5. The solution was diluted with water to volume and mixed.

Concomitantly determinated the absorbances of the solutions obtained from paracetamol in 1-cm cells relative to the blank at the wavelength of maximum absorbance at 430 nm.

Stability Determination of Commercially Available Liquid Paracetamol in Thailand.

A 5 ml each of commercially available liquid paracetamol preparation was pipetted to separate tight, light-resistant containers (vial). The prepared vials were kept in constant temperature incubators at 70°, 60°, 55°, 40°C and in a room which average temperature was 31°C (25° - 37°C). Each sample taken from different temperature were analysed at suitable time intervals. The first sample was assumed to contain 100% remaining paracetamol concentration and the subsequent samples were calculated as a relative percentage amount of the first sample paracetamol.

Effect of Colour of Incubated Syrup on Determining of Remaining Paracetamol Amount in Liquid Preparation by USP Method.

To detemine the effect of colour of incubated syrup on USP method. The experiment was divided into two parts.

Incubated syrup (Decomposed syrup)

1. 100 ml of USP syrup was kept in tight, light-resistant container with the level of syrup marked.

2. The container was incubated at 60°C until syrup was decomposed and colour changed to brown (about 4 monthes)

3. The container was removed from a incubator and adjusted to the marked volume.

4. 10 ml of paracetamol stock solution (120 mg/ml in 95% ethanol) was pipetted to a 50-ml volumetric flask.

5. The volumetric flask was adjusted to volume with incubated syrup and mixed

6. Paracetamol concentration in incubated syrup was determined by USP method and calculated by reference to standard curve of paracetamol.

Refrigerated syrup

1. 100 ml of USP syrup was kept in tight, light-resistant container with the level of syrup marked.

2. The container was kept in a frigerator as long as incubated syrup.

3. The container was removed from refrigerator and adjusted to the marked volume.

4. 10 ml of paracetamol stock solution (120 mg/ml in 95% ethanol) was pipetted to a 50-ml volumetric flask.

5. The volumetric flask was adjusted to volume with refrigerated syrup and mixed.

6. Paracetamol concentration in refrigirated syrup was determined by USP method and calculated by reference to standard curve of paracetmol.

The percent recovery of paracetamol in incubated syrup and refrigerated syrup were compared.

Determination of Remaining Paracetamol Amount in Commercially Available Liquid Paracetamol Preparations in Thailand.

Standard preparation - Dissolve an accurately weighed quantity of paracetamol reference standard in water. Dilute

quantitatively and stepwise if necessary with water to obtain a solution having a known paracetamol concentration of 100 mcg/ml.

Sample preparation - Accurately transfer each samples of paracetamol in vial to a separated 100-ml volumetric flask, add 60 ml of water, insert the stopper, and shake by mechanical means for 30 minutes. Dilute with water to volume and mix. Transfer 4.0 ml of this solution to a 50-ml volumetric flask. Adjust to volume with water.

Procedure - Transfer 10.0 ml of each standard, the sample preparation, and water to provide a blank, to a separated 50-ml volumetric flasks, and treat each as follows

1. Add 2.0 ml of 6 N hydrochloric acid, mix.

2. Add 5.0 ml of 10% sodium nitrite solution. Mix and allow to stand for 15 minutes.

3. Add 5.0 ml of 15% ammonium sulfamate solution. Swirl gently and allow to cool to room temperature.

4. Add 15.0 ml of 2.5 N sodium hydroxide allow to cool to room temperature.

5. Dilute with water to volume and mix.

Concomitantly determinate the absorbances of the solutions obtained from the standard preparation and the sample preparation in 1 cm cells relative to the blank at the wavelength of maximum absorbance at 430 nm. The results were calculated by reference to standard preparation.

HPLC Method (Proposed Method)

<u>Determination of the Linearity of the Ratio of the Peak
Height Paracetamol Divided Peak Height of Internal Standard
(Sulpyrin) with Concentration of Paracetamol Solution</u>.

Internal standard solution - Dissolve an accurately weighed
quantity of sulpyrin reference standard in the solvent mixture (15%
methanol: 85% phosphate buffer pH 4.0) and dilute quantitively to
make concentration about 3.4 mg/ml.

Paracetamol preparation - Weigh paracetamol reference
standard accurately 50 mg into a 50-ml volumetric flask and dissolve
with the solvent mixture then dilute quantitatively to volume and
mix. Transfer 2.0, 3.0, 4.0, 5.0 and 6.0 ml of the solutions to
separated 10-ml volumetric flasks, dilute with the solvent mixture.
Pipet 1.0 ml of each paracetamol solution to a separated 10-ml
volumetric flask. Add 1.0 ml of internal standard solution to every
volumetric flask then adjust to volume with the solvent mixture. Mix
the solutions.

<u>Stability Determination of Commercially Available Liquid
Paracetamol Preparations in Thailand By HPLC Method</u>

Each sample of commercially available liquid paracetamol
preparation was pipeted 1.0 ml to a separated tight, light-resistant
container (vial). The prepared vials were kept in constant
temparature incubators at 70°, 60°, 55°, 40°C and in room which
average temperature was 31°C (25°-37°C). The sample taken from each
incubator was analysed at suitable time interval. The first sample
was considered to contain 100% of remaining paracetamol

concentration. The subsequent samples were calculated as a relative percentage amount of the original paracetamol.

Determination Remaining Paracetamol Amount in Commercially Available Liquid Paracetamol Preparations in Thailand by HPLC Method (Proposed Method).

Internal standard solution - Dissolve an accurately weighed quantity of sulpyrin reference standard in the solvent mixture (15% methanol:85% phosphate buffer pH 4.0) and dilute quantitively to make concentration about 3.4 mg/ml.

Standard preparation - Accurately weigh paracetamol reference standard accurately 50 mg into a 10 ml volumetric flask and dissolve with the solvent mixture. Dilute to volume and mix. Transfer 1.0 ml of the solution to a 10-ml volumetric flask, then dilute with the solvent mixture to volume and mix.

Sample preparation - Transfer each sample of paracetamol in vial to a separated 50-ml volumetric flask and dilute with the solvent mixture to volume. Mix the solution.

Chromatographic condition - The mobile phase consists of 85% of phosphate buffer solution pH 4.0 which is filtered through a 0.45 mcm filter and 15% of methanol which is also filtered through a 0.45 mcm filter. A flow rate of 2.0 ml/minute is established.

Procedure - Pipet 1.0 ml each of standard preparation and sample preparation to a separated 10-ml volumetric flasks. Add 1.0 ml of internal standard solution to every volumetric flask then adjust to volume with the solvent mixture. Mix the solutions. Three replicate, a 20 mcl injection was made from each sample solution and the results were calculated by reference to a standard

preparation. The response of the standard was determined by making two replicate injection.

Calculation

USP Method (23)

The amount of paracetamol was calculated from following formula

$$\text{paracetamol (mg/ml)} = \frac{C \times A_u}{V \times A_s}$$

where

C = concentration, in mcg/ml, of paracetamol in standard preparation.

V = volume, in ml, of sample preparation taken.

A_u = the absorbance of the solution from sample preparation.

A_s = the absorbance of the solution from standard preparation.

HPLC Method

The amount of paracetamol was calculated from following formula

$$\text{paracetamol (mg/ml)} = \frac{U \times C \times D.F}{A}$$

C = weight, in mg, of standard.

A = ratio of peak height of standard divided peak height of internal standard.

U = ratio of peak height of sample divided peak height

of internal standard.

D.F. = dilution factor : 0.5

The first sample was considered to contain 100% of remaining paracetamol concentration. The subsequent samples were calculated as a relative amount of the first sample paracetamol.

USP Method and HPLC Method

Determination of Specific Rate Constant of Commercially Available Liquid Paracetamol Preparation Degradation and Activation Energy.

The specific rate constant (K) will be calculated from the slope of linear regression line of the plots of percentage amount of remaining intact drug versus time if specific rate constant is zero-order rate constant. Alternatively the specific rate constant (K) will be calculated from the slope of plot natural logarithm of percentage amount of remaining drug versus time if specific rate constant is first-order constant (68).

Stability of paracetamol solution was studied by Koshy and Lach. The hydrolysis of paracetamol was both acid and base catalysed. At a constant pH the reaction was apparent psuedo first-order (3). If drug concentration is fixed (e.g., by making a suspension), the reaction will be apparent psuedo zero-order (68). Thus, specific rate constant of paracetamol syrup and paracetamol elixir were psuedo first-order while paracetamol suspension was pseudo zero-order.

The activation energy of paracetamol calculated from

The activation energy of paracetamol calculated from Arrhenius equation

$$K = Ae^{-\frac{E_a}{RT}} \qquad \text{(Eq. 9)}$$

$$\ln K = \ln A - \frac{E_a}{R} \times \frac{1}{T} \qquad \text{(Eq. 10)}$$

where

K = specific rate constant of degradation

A = frequency factor as a constant

E_a = activation energy

R = gas constant (1.987 calories degree^{-1} mole^{-1})

T = absolute temperature ($^{\circ}$C + 273)

A plot of ln K versus 1/T yeilds a strainght line and slope is E_a/R. The activation energy was calculated from the product of slope and the gas constant. (68) Since the Arrhenius plot in linear, it is possible to predict the rate constant (K) at room temperature or at any lower temperature by extrapolation.

Determination of Shelf-lives of Commercially Available Liquid Paracetamol Preparations.

When the activation energy is known, it is possible to predict the specific rate constant of degradation at lower temperature from the rate obtained at one elevated temperature study by using equation

$$\ln \frac{K_2}{K_1} = \frac{E_a}{R} \times \frac{T_2 - T_1}{T_2 T_1} \qquad \text{(Eq. 11)}$$

where

K_1 = specific rate constant of degradation at T_1 temperature (Absolute temperature).

K_2 = specific rate constand of degradation at T_2 temperature (Absolute temperature).

Shelf-life (t_{90}) is defined as the time required for the product to decrease its concentration from 100% to 90% of labeled amount at normal storage temperature.

The shelf-life of a zero-order reaction can be calculated by equation

$$t_{90} = \frac{0.1 \, [D_0]}{K_0} \qquad \text{(Eq. 12)}$$

where

D_0 = drug concentration at $t = 0$ (solubility of drug)

K_0 = specific rate constant of degradation

The shelf-life of a first-order reaction can be obtain calculated by equation

$$t_{90} = \frac{0.1054}{K} \qquad \text{(Eq. 13)}$$

where

K = specific rate constant of degradation (67)

From the Arrhenius equation (Eq.10) may be modified as

follows :

$$\ln K = \ln A - \frac{E_a}{RT}$$

from Eq. 12, substituting into Eq. 10

for zero-order reaction

$$\ln t_{90} = \ln \frac{0.1 [D_o]}{A} + \frac{E_a}{RT} \qquad (Eq.\ 14)$$

from Eq. 13, substituting into Eq. 10

for first-order reaction

$$\ln t_{90} = \ln \frac{0.1054}{A} + \frac{E_a}{RT} \qquad (Eq.\ 15)$$

Determination Percent Labelled Amount of Remaining Paracetamol in Commercially Available Paracetamol Tablets in Thailand by HPLC Method.

Internal standard solution - Dissolve an accurately weighed quantity of sulpyrin reference standard in the solvent mixture (15% methanol: 85% phosphate buffer pH 4.0) and diluted quantitively to make concentration about 3.4 mg/ml.

Standard preparation - Weigh paracetamol reference standard accurately 50 mg into a 10 ml volumetric flask and dissolve with the solvent mixture then dilute quantitively to volume and mix. Transfer 1.0 ml of the solution to a 10 ml volumetric flask, dilute with the solvent mixture to volume and mix.

Sample preparation - Weigh and finely powder not less than 20 paracetamol tablets. Calculate average weight per tablet. Accurately weigh a portion of the powder, equivalent to about 50 mg of paracetamol and transfer with the aid of about 60 ml of solvent mixture to a 100 ml volumetric flask. Swirl by mechanical means for 30 minutes, dilute with solvent mixture to volume, mix.

Chromatographic condition - The mobile phase consisted of 85% of phosphate buffer solution pH 4.0 which was also filtered through a 0.45 mcm filter and 15% of methanol which was filtered through a 0.45 mcm filter. A flow rate of 2.0 ml/minute was established.

Procedure - Pipet 1.0 ml of each standard preparation and sample preparation to a separated 10-ml volumetric flask. Add 1.0 ml of the internal standard solution to every volumetric flasks then adjust to volume with the solvent mixture. Mix the solutions. Triplicate, 20 mcl injections were made from each sample solution and the results were calculated by reference to a standard preparation. The response of the standard preparation was determined by making duplicate.