# CHAPTER III

# EXPERIMENTAL

## Apparatus

An HPLC System :

Isocatic elutions were performed with a high-performance liquid chromatographic system (Model SP 8810 Isocratic pump) (Spectra Physics, CA , U.S.A.) ; Chrom-A-Scope Model 1970 variable-wavelength detector (BarSpec System Inc., Israel). Chromatograms were recorded and processed with a Barspec Data system (Barspec). Injections were made using a 20 µL sample loop (Rheodyne valve 7125 injector, Altech, U.S.A.) and controlled by HP Vectra VL2 computer.

A phenylpropanolamine bonded silica column, 5 µm (80 Å), 150 x 4.6 mm I.D.

A Millipore Alpha-Q water purification system (Millipore, U.S.A. ).

A Eldex Heater Column CH-150 (Eldex Laboratories Inc., U.S.A.).

A titrator DL-21 combined with pH electrode DG 111-Sc (Mettler, Switzerland).

A pH - meter (Schott, Germany).

A magnetic stirrer (Thermolyne, U.S.A.).

A vortex mixture (Scientific industries Inc., U.S.A.).

Micropipettes 10-100, 20-200 and 100-1000 µL (Socorex, SWISS).

Cellulose nitrate filter 0.45 µm (Whatman Limited, Japan).

Cellulose acetate filter 0.45 µm (Sartorius, Japan).

Helium gas (Thai Industrail Gas Co.,Ltd., Thailand).

## Chemicals

Analytical grade phenol was from E. Merck (Darmstadt, Germany). L-ascorbic acid of laboratory grade was purchased from Sigma (St. Louis, U.S.A.). Laboratory grade acetylsalicylic acid was purchased from Rhone-Poulenc Thai Industries LTD., (Thailand). Laboratory grade of benzoic acid was from Unilab (Arburn, Australia). Analytical grade salicylic acid was from Univar (Arburn, Australia). All standards were greater than 97 percent purity. HPLC grade acetonitrile was form May & Baker (Dagenham, England). Analytical grade orthophosphoric acid was from BDH Limited (Poole, England). Water was purified on a Millipore Alpha-Q water purification system and filtered through a 0.45 µm cellulose nitrate filter.

## Chromatographic Prodedures

### *1. Mobile Phases*

30 mM orthophosphoric acid in water was prepared and adjusted with 10 M , 2 M and 0.1 M sodium hydroxide, respectively to pH 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5 ± 0.02. Subsequently, individual buffer was mixed with acetonitrile to make mobile phase that contained acetonitrile in concentration of 10, 20, 30, 40 and 50% (v/v). The mobile phases were degassed with helium gas 15 minutes prior to use.

### *2. Preparation of Standard Solutions*

Standard solutions of L-ascorbic acid, phenol, benzoic acid, acetylsalicylic acid and salicylic acid of which concentrations of each are 0.06, 0.2, 0.2, 0.4, 0.5 g / 10 ml in acetonitrile except L-ascorbic acid was in 30 mM phosphate buffer were prepared and labeled as a stock #1 solution.

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*3. Effect of solvent strength on chromatographic behavior of analytes on phenylpropanolamine column.*

Solution of stock #1 was diluted to stock #2 which have 0.3, 1.0, 1.0, 2.0 and 2.5 mg / mL of L-ascorbic acid, phenol, benzoic acid, acetylsalicylic acid and salicylic acid respectively. Subsequently, 20 μL each of these stock #2 solutions was mixed with various amount of acetonitrile (50 to 900 μL) and diluted to 1000 μL with 30 mM phosphate buffer. These freshly prepared solutions were injected onto the column under the chromatographic conditions as shown in Table 3.1.

. The column was equilibrated with at least 30 mL new mobile phase before injecting a sample solution and was thermostated precisely to ± 0.1 °C by thermostat oven to 30 °C.

**Table 3.1** Chromatographic conditions for the study of effect of solvent strength on chromatographic behavior of analytes on phenylpropanolamine column.

| HPLC parameter | HPLC conditions |
|---|---|
| Analytical Column | Phenylpropanolamine column, 5 μm, 150 x 4.6 mm I.D. |
| Mobile Phase | Acetonitrile : 30 mM phosphate buffer solution were 10:90, 20:80, 30:70, 40:60 and 50:50 (v/v) |
| Solvent Strength for sample | 5% to 90% (v/v) acetonitrile |
| Flow Rate | 1.0 mL / min |
| Column Temperature | 30 °C |
| Detector | UV 254 nm |

**Construction of species distribution of organic acids.**

Species distribution of any observed compounds, *i.e.* L-ascorbic acid, benzoic acid, acetylsalicylic acid and salicylic acid have been studied by potentiometric titration technique. The species of ionized and unionized forms were constructed from calculated concentration of corresponding acidic constants obtained from the experiment.

Acidity constants of studied compounds were derived from the potentiometric titration by Superquad program [34]. All potentiometric titrations were carried out at $25 \pm 0.1$ $^{o}$C and ionic strength of 0.1 M $KNO_3$ in 10% acetonitrile solution. Solution of 0.094 M NaOH (in same electrolyte) was used as a titrant. The initial volume of each titration was 30 ml of each studied compound in 10% acetonitrile solution. An autometric titrator of Mettler DL 21 including combination pH electrode (DG 111-Sc) was calibrated by two standard pH buffers. pH 4.00 / 7.00 buffers were used for the calibration of electrode throughout the experiment.