CHAPTER II

EXPERIMENTATION

1. Instruments and Materials

1.1 Instruments

1.1.1 High Performance Liquid Chromatograph, consists of

- model 600 E Multisolvent delivery system, Waters, Milford; MA, U.S.A.
- model 484 Tunable Absorbance Detector, Waters, Milford; MA, U.S.A.
- model h746 Integrator, Waters, Milford; MA, U.S.A.
- Injection system, Rheodyne 7125 fitted with a 20 µl loop.
- Column, "Resolve" 5 µm Spherical C_{18}, (15 cm. x 3.9 mm.i.d.), Waters, Milford, MA, U.S.A.

1.1.2 syringe 100 µl, Hamilton Microliter Syringe, 710 Rheodyne.

1.1.3 Spectrophotometer, Hitachi 150-20 and recorder, Yamato Scientific Co., Ltd. Tokyo, Japan.

1.1.4 Analytical balance, Mettler AE 200, Instruments AG., Switzerland.

1.1.5 Water Bath, Refrigerated Bath RB-5, Techne with Tempette TE-80, England.

1.1.6 Vortex, Genie, Model K-550-GE, Scientific industries Inc. New York, U.S.A.

1.1.7 pH-Meter, model 605, Metrohm, Switzerland

1.2 Materials

1.2.1 Ephedrine hydrocloride, working standard

1.2.2 Codeine phosphate, hemihydrate. working standard

1.2.3 Promethazine hydrochloride, working standard

1.2.4 Promethazine hydrochloride, USP Reference standard

1.2.5 Ethylephedrine hydrochloride, working standard

1.2.6 Methylephedrine hydrochloride, working standard

1.2.7 Amberlite XAD-2 resin, BDH, England

1.2.8 Syrup USP.

1.2.9 Phensedyl cough linctus, May & Baker Co.

: Each 5 ml contains

Promethazine hydrochloride	3.6 mg.
Codeine phosphate	9.0 mg.
Ephedrine hydrochloride	7.2 mg.

1.2.10 Commercially available products

Five brands of cough syrup with the same active ingredients as Phensedyl cough linctus were studied. Phensedyl(R) represented as syrup No. 1.

All chemicals listed below were analytical grade, except 1.2.19

1.2.11 Ammonium acetate, Merck, Damstadt, F.R.

1.2.12 Sodium carbonate, Merck, Germany

1.2.13 Sodium carbonate, Marck Germany

1.2.14 Acetic acid, Merck, Germany

1.2.15 Ammonia solution, BDH, England

1.2.16 Methanol, Merck, Germany

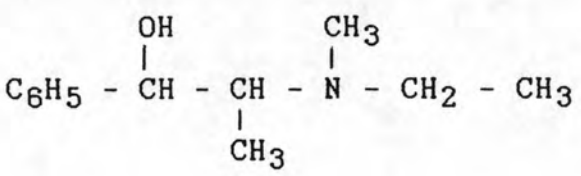
1.2.17 Dichloromethane, Merck, Germany

1.2.18 Chloroform, Merck, Germany

1.2.19 Methanol, HPLC grade, J.T. Baker, U.S.A.

1.3 Physical and Chemical properties of ethylephedrine and methylephedrine hydrochloride

Ethylephedrine (etafedrine) hydrochloride

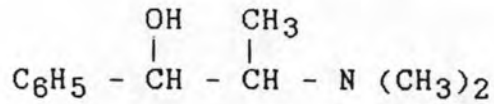


(-)-2-(Ethylmethylamino)-1-phenylpropan-1-ol

$C_{12}H_{19}NO \cdot HCl = 229.7$

It is soluble 1 in 1.5 of water and 1 in 8 of ethanol. The UV absorption with the maximum occur at 251, 217, 263 nm. (Clarke, ed., 1986)

Methylephedrine hydrochloride



(-)-2-(Dimethylamino)-1-phenylpropan-1-ol

$C_{11}H_{17}N.O.HCl = 215.7$

It is freely soluble in water, soluble in ethanol and practically insoluble in ether. The UV absorption with the maximum occur at 251, 257, 263 nm. (Clarke, ed., 1986)

2. Method

A Resolve C_{18}, 5 μm (Waters. Assoc., Milford, MA, USA.) column (150 x 3.9 mm. i.d) was used. The mobile phase as finally optimized was pumped at 2.0 ml per min. and consisted of methanol : 20 mM ammonium acetate solution (90 : 10, v/v, pH 7.0). Detection was effected at 265 nm, 0.05 AUFS, with chart speed of 0.25 cm/min. All experiments were carried out at ambient temperature.

2.1 Optimization of mobile phase

Initially, a search for the optimum condition to provide the best chromatographic resolution of ephedrine hydrochloride, codeine phosphate, promethazine hydrochloride and its photolytic degradation products; and the internal standard from each other was initiated.

The parameters investigated include :

a) The percentage of methanol in the mobile phase

Various composition of mobile phases (80-95% v/v of methanol) in 20 mM ammonium acetate at pH 7.0 (adjusting the pH of the final eluent) were prepared.

b) The pH of mobile phase used in the assay (after adding organic modifier)

Various pH of the mobile phase (pH 6.0-7.5) consisting of methanol : 20 mM. ammonium acetate solution (90 : 10, v/v) were prepared.

c) The molar concentration of the buffer salt (ammonium acetate, CH_3COONH_4).

Mobile phase consisting of methanol : ammonium acetate solution (90 : 10 v/v pH 7.0) were prepared by varying the concentration of ammonium acetate solution (10-30 mM.)

2.2 Preparation and optimization of Solid-Phase Extraction (SPE)

2.2.1 Preparation of Amberlite XAD-2 resin

The resin was first washed with distilled water until no chloride left. Then the resin was suspended in five bed-volumes of methanol for 2 hours,

allowed to settle and the methanol was discarded. Repeated the process once more. Allowed the resin to dry at room temperature and stored in a closed container.

2.2.2 Optimization of SPE procedure

A search for the optimum condition of the SPE procedure was performed by varying the following parameters : column size, polarity of sample solutions, polarity, type and volume of eluting solvent.

The XAD-2 resin columns were finally prepared according to the optimum conditions as follows. Inserted a cotton plug into a 20 x 1 cm.id. chromatographic tube with restricted end joined to a short 1-mm.id. capillary. Filled in the washed and dry XAD-2 resin until the height of the resin bed was about 7-8 cm (about 1 g.). Inserted a cotton wool pad on the top of the resin bed. The packed XAD-2 columns were then ready for sample preparation.

The SPE consists of four basic steps : conditioning, loading, rinsing and eluting. XAD-2 columns were conditioned sequently with 3 x 5 ml of dichloromethane, 3 x 5 ml of methanol, 3 x 5 ml of distilled water and 3 x 5 ml of carbonate buffer solution pH 11.

Without allowing the column to dryout, sample solution was applied to the top of the column, allowed it to flow through the sorbent bed and discarded

the waste. The sample was allowed to dry onto the column for 5-10 min.

The columns were rinsed with 3 x 5 ml of distilled water and allowed to dry for 10 min. The adsorbed drugs were then eluted with organic solvent for complete extraction.

Various types of eluting solvents including methanol, methanol acidified with acetic acid, chloroform and dichloromethane were investigated.

The fraction eluting from the XAD-2 column, 5 ml each, was examined for the presence of analytes by thin-layer chromatography (TLC) with the developing system of chloroform : methanol (9:1) and visualizing the plate with ultraviolet light at 254 nm.

2.3 Standard solution

Stock solutions of ephedrine hydrochloride, codeine phosphate, promethazine hydrochloride were prepared separately in distilled water with the concentration of 1.4, 1.8 and 0.7 mg/ml, respectively. From these solutions the appropriate dilutions were made whenever required.

Two internal standards, methylephedrine hydrochloride and ethylephedrine hydrochloride, were investigated. Stock solutions (9.0 mg/ml) of each standard were prepared separately in distilled water.

2.4 Buffer solution

A stock solution of ammonium acetate buffer was prepared by dissolving ammonium acetate to the concentration of 100 mM. in distilled water.

A carbonate buffer solution was prepared by dissolving 21.0 g of sodium carbonate and 420 mg of sodium bicarbonate in 500 ml of distilled water and adjusting the pH to 11.0,

2.5 Sample preparation

A sample solution was prepared by vortex mixing 1.0 ml of syrup, 1.0 ml of stock solution of finally optimized internal standard which was ethylephedrine hydrochloride and 0.5 ml of carbonate buffer solution. The solution was then passed through the SPE column which was already conditioned. The rinsing step was then carried out. Details of conditioning and rinsing steps were described under the topic "Optimization of SPE Procedure".

The eluting step was performed using 3 x 5 ml of dichloromethane. the eluent was collected in a 30-ml brown glass bottle and evaporated to dryness on water-bath. The residue was reconstituted in 25.0 ml of mobile phase. Three replicate 20-μl aliquots of the solution were injected onto the HPLC column.

3. Photolytic degradation study of promethazine hydrochloride

The solution of promethazine hydrochloride was prepared (0.7 mg/ml) in distilled water and placed on the table for exposure to neon light (60 watt, at distance 20 cm.) at room temperature for one week.

Following the exposure time, 1.0 ml of the resulting solution was diluted and adjusted to 25.0 ml with the mobile phase. Degradation study was conducted by triplicate injecting a 20-µl aliquot of the solution onto the HPLC column.

4. Method Validation

4.2 Linearity

Five samples of syrup USP spiked with known concentrations of standards and the internal standard were prepared and processed according to the SPE procedure.

The residue of each sample was reconstituted in 25.0 ml of mobile phase and triplicate injected onto the HPLC column. The injected concentration ranges of ephedrine hydrochloride, codeine phosphate and promethazine hydrochloride were 40-72 mcg/ml, 48-87 mcg/ml and 20-36 mcg/ml, respectively. Concentration of the internal standard, ethylephedrine hydrochloride, in the solution was set to be 0.36 mg/ml.

Standard curves were constructed for each compound by plotting peak area ratio versus standard concentrations.

4.2 Precision

Intra-day precision was evaluated by ten replicate analysis (n = 10) of phensedyl cough linctus, a representative composite sample, using the proposed SPE and HPLC procedure.

Inter-day precision was similarly evaluated for three non-consecutive days (n = 3). Five replicates of the sample were used.

The mean concentration and relative standard deviation were determined.

4.3 Extraction recovery

The recoveries, at three concentration levels, of ephedrine hydrochloride, codeine phosphate and promethazine hydrochloride were evaluated. Sample solutions, at three concentrations of the three active ingredients were prepared by adding known concentrations of the drugs, internal standard and 0.5 ml carbonate buffer solution pH 11.0 to 1.0 ml syrup USP. Five replicate samples were prepared for each concentration. The mixtures were vortexed and processed according to the SPE procedure prior

to HPLC analysis. The residues were reconstituted in 25.0 ml of mobile phase. Triplicate injections of each solution onto the HPLC column.

Extraction recoveries were obtained by comparing the peak area ratios of the drugs after the SPE procedure against corresponding peak area ratios from aqueous stock solution.

5. Application

The proposed SPE procedure and HPLC method were used for analysis of five syrup formulations, representing five different manufacturers. All of these syrups contain the same concentration of ephedrine hydrochloride, codeine phosphate and promethazine hydrochloride as in phensedyl cough linctus.

Each syrup was aliquoted twice, and each was subjected to SPE procedure, then triplicate injected onto HPLC system.

The percent labeled amount of each components was calculated using the peak area ratio.