Chapter III

Result and Discussion

Effect of pH

Codeine phosphate solution was prepared in various pH by adjusting with Hydrochloric acid or Sodium Hydroxide. Each solution was analyzed using ^{31}P -NMR (Figure 1). The intensity of peak from each solution was measured. First, to make the base line by drawing a straight line through the most of highest part of noise, then measure the peak intensity. Drew another line through the most of lowest part of noise and measured the peak intensity. The average of the two peak intensity above was used. The intensity of peak (c.m.) of codeine phosphate solution in various pH was shown in Table 1. The pH was plotted against the intensity of peak (Figure 2).

In table 1, the relation of intensity and pH of Codeine phosphate solution show that if the pH is less than approximately 3.55, the intensity of peak is decreased. When the pH of solution is more than approximately 6.6, the intensity of peak is also decreased.

Table I

pH	Intensity of peak (cm)
0.90	0.60
2.00	0.70
3.55	1.30
4.60	1.28
5.80	1.32
6.60	1.30
7.45	1.00
8.60	0.90
11.75	0.90

As we know that the pKa of codeine phosphate is 8.2, so at the low pH, codeine phosphate in the solution is greater than phosphate ion. The phosphorus -31 nuclear magnetic resonance can detect the amount of Codeine phosphate only in the phosphate ion, therefore, the intensity of peak is decreased.

At the high pH the intensity of peak is decreased. At the high pH the PO_4^{3-} is greater than HPO_4^{2-} and $H_2PO_4^-$. Phosphorus atoms in HPO_4^{2-} or $H_2PO_4^-$ is more easily relaxed than phosphorus atom in PO_4^{2-} (the spin-lattice relaxation mechanism). Phosphorus atom in $H_2PO_4^-$ and HPO_4^{2-} can transfer energy to hydrogen atom and oxygen atom but phosphorus in PO_4^{3-} can only transfer energy to oxygen atom. The more relaxation, the more increase in intensity, therefore at high pH the intensity of peak is decreased.

Effect of viscosity

Codeine Phosphate syrup was prepared in various viscosity. Syrup B.P. was prepared first, then adjusted the viscosity of syrup by adding proper amount of distilled water. The final solution was measured viscosity by using Ostwald viscometer. Codeine phosphate was dissolved in each syrup and analyzed by using ^{31}P -NMR. Measurement of the intensity of peak by the method mentioned above was performed. The NMR spectra were shown in Figure 3. Table 2 showed the intensity of peak of codeine phosphate syrup in various viscosity. Plotting the viscosity against the intensity of peak were showed in Figure 4.

Table 2

Viscosity (centipoise)	Intensity of peak (cm)
24.66	1.11
30.46	1.10
41.26	1.09
48.37	1.08
65.22	1.10

The experiment showed that the NMR peak of codeine phosphate syrup which had viscosity about 24 to 65

centipoise had equal intensity. It was shown that the viscosity in the range 24-65 centipoise had no effect on analyzing the Codeine phosphate syrup by ^{31}P-NMR. In general, the viscosity of syrup was in the range of 25-65 centipoise. The syrup which viscosity was more than 65 centipoise could also be analyzed by ^{31}P-NMR but the spent time was more than 10 minutes.

Effect of Concentration

Codeine phosphate was prepared to make five final concentration, 5 mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml and 25 mg/ml and analyzed by using ^{31}P-NMR. From the plotted intensity of peaks against the concentration, it was shown that the suitable concentration for making a standard curve was 5 to 15 mg/ml. (Figure 6)

Assay of Codeine phosphate injection using ^{31}P NMR

Standard solution

Codeine phosphate was dissolved in distilled water to make five final concentrations, 5.0 mg/ml, 7.5 mg/ml, 10.0 mg/ml, 12.5 mg/ml, 15.0 mg/ml. Analyzed each solution using ^{31}P - NMR. The NMR spectra were shown in Figure 5. The intensity of peak and concentration were shown in the Table 3. The concentration of solution plotted against the log intensity of peak were showed in Figure 6.

Table 3

Concentration (mg/ml)	Intensity of peak (cm)	Log intensity
5.0	1.40	0.1461
7.5	1.70	0.2304
10.0	2.50	0.3979
12.5	3.20	0.5051
15.0	5.00	0.6989

In figure 6, the plotted log intensity against concentration is linear, using linear regression, the equation of standard curve was set up as

$$y = -0.15644 + 0.055212x$$

where, y = log intensity of sample 's peak

x = concentration (mg/ml)

Codeine phosphate injection

Codeine phosphate injection 2 ml was transferred into the NMR tube and analyzed by using ^{31}P - NMR. The NMR spectra were shown in Figure 7. The intensity of peaks were measured and converted into logarithm. Table 4 showed all data obtained from experiment and the concentration of Codeine phosphate were calculated from the equation which mentioned above.

Table 4

Sample	A	B	C
Intensity of peak (cm)	4.16	4.00	4.48
Log Intensity	0.6191	0.6021	0.6513
Concentration (mg/ml)	14.0464	13.7379	14.6293

Concentration of Codeine phosphate injection = 14.1378 mg/ml

Assay of Codeine phosphate injection (U.S.P. method)

The Codeine alkaloid was extracted from the Codeine Phosphate injection and quantitative analyzed by Titration with 0.02 N. sulfuric acid. The data were shown in Table 5.

Table 5

Sample	A	B	C
Blank	0.05	0.05	0.05
H_2SO_4 (ml)	8.95	8.99	9.03
H_2SO_4 (ml)(without blank)	8.90	8.94	8.98

Each ml. of 0.02 N. sulfuric acid is equivalent to 8.128 mg. of $C_{18}H_{21}NO_3H_3PO_4 . {}^1/_2 H_2O$ (Codeine phosphate).

The amount of Codeine phosphate in each sample can be calculated as the following:

$$\text{sample A} = \frac{8.90 \times 8.128 \times 0.02}{0.0206} = 70.2322 \text{ mg/5ml}$$

$$\text{sample B} = \frac{8.94 \times 8.128 \times 0.02}{0.0206} = 70.5478 \text{ mg/5ml}$$

$$\text{sample C} = \frac{8.98 \times 8.128 \times 0.02}{0.0206} = 70.8635 \text{ mg/5ml}$$

Average concentration = 14.1095 mg/ml

In the analysis of Codeine phosphate injection using ^{31}P -NMR and U.S.P. method. The results were shown as follow:

^{31}P -NMR method   14.1378 mg/ml   (94.25% label amount).

U.S.P. method   14.1095 mg/ml   (94.06% label amount).

Labelled amount   15   mg/ml.

Since the Codeine phosphate injection was prepared in small scale by the Pharmacy Department of Central Chest Hospital in order to inject into the operation patients, thus the process of the preparation should be accurate and precision. That also means the labelled amount (15 mg/ml) should be correct.

Analysis by the U.S.P. method will have some error due to loss in the extraction process. But the sample used in analysis by the ^{31}P -NMR technique has no need to be extracted or diluted. So, the ^{31}P -NMR technique should be more accurate than the U.S.P. method. However, this technique still need further study in order

to prove the accuracy for using in analysis the codeine phosphate in injection.

Assay of Codeine phosphate syrup using ^{31}P NMR

Standard solution

Codeine phosphate was dissolved in syrup BP to make four final concentrations; 7.5 mg/ml, 10.0 mg/ml, 12.5 mg/ml and 15.0 mg/ml respectively. Each syrup was analyzed using ^{31}P -NMR. The NMR spectra were shown in Figure 8. The intensity of peaks were measured by the method mentioned above. Table 6 showed the intensity of peak, concentration, and logarithm of intensity. The plotted concentration against the log intensity were shown in Figure 9.

Table 6

Concentration (mg/ml)	Intensity of peak (cm)	Log Intensity
7.5	3.10	0.4914
10.0	3.35	0.5250
12.5	4.25	0.6283
15.0	4.90	0.6902

In figure 9, the plotted log intensity against concentration is linear, using linear regression, the equation of standard curve was set up as:

$$y = 0.26886 + 0.027987 x$$

where, y = log intensity of peak

              x = concentration (mg/ml)

Codeine phosphate syrup

Codeine phosphate syrup was prepared and analyzed by using ^{31}P -NMR. The NMR spectra were shown in Figure 10. The measurement of intensity of peak was prepared by the method mentioned above. Table 7 showed the concentration, intensity of peak and logarithm of intensity.

Table 7

Sample	A	B	C
Intensity of peak (cm)	3.44	3.40	3.42
log intensity	0.5365	0.5314	0.5340
Concentration (mg/ml)	9.5651	9.3836	9.4746

The concentration of Codeine phosphate were calculated from the equation which mentioned above.

Concentration of codeine phosphate syrup = 9.4744 mg/ml

Assay of codeine phosphate syrup using High Performance Liquid Chromatography

The sample solution was prepared by diluting the Codeine phosphate syrup with chromatographic solvent (0.05 M. KH_2PO_4 in water containing 13% (v/v) methanol) and chlorpheniramine maleate was added in the final solution to act as an internal standard. The standard solution was prepared by dissolving the codeine phosphate and chlorpheniramine maleate in chromatographic solvent. Both solutions were analyzed by HPLC. For purposes of comparison, standard solution was injected after the assay was eluted. The chromatogram of standard solution and unknown sample were shown in Figure 11 and 12. The area ratio can be calculated by using formula as the following:

$$\text{Area ratio} = \frac{\text{Area under the peak of Codeine phosphate}}{\text{Area under the peak of Chlorpheniramine maleate}}$$

The results of area ratio of standard solution were shown below; and the average area ratio of Standard solution is equal to 2.7557

Area ratio ;   Standard 1 = 2.7808

Standard 2 = 2.8473

Standard 3 = 2.6392

Table 8 showed the area ratio of unknown sample solution.

Table 8

Sample	1	2	Average
A	2.7137	2.6939	2.7038
B	3.0039	2.9344	2.9716
C	2.8161	2.9691	2.8926

The average area ratio of sample is equal to 2.856. The amount of Codeine phosphate can be calculated using the following formula;

Concentration of codeine phosphate in sample = $\dfrac{A_1 B}{A_2}$

where, A_1 = Ratio of area under the peak (sample)

A_2 = Ratio of area under the peak (standard)

B = Concentration of codeine phosphate in standard solution

Concentration of Codeine phosphate = $\dfrac{2.8560 \times 0.2}{2.7557}$

(the solution of 2 ml. sample
diluted to 100 ml.)   = 0.2072 mg/ml

Then, concentration of codeine phosphate

in the syrup   = 10.355 mg/ml

while, labelled amount   = 10 mg/ml

In the analysis of Codeine phosphate syrup using ^{31}P -NMR and HPLC method. The results were shown as the following:

^{31}P NMR method   9.4744 mg/ml   (94.74% label amount).

HPLC method   10.3550 mg/ml   (103.55% label amount).

Label amount   10   mg/ml.

The official monographs of U.S.P.XXI indicated that both Codeine Phosphate injection and Codeine Phosphate tablet contain not less than 93.0 percent and not more than 107.0 percent of the labeled amount of $C_{18}H_{21}NO_3.H_3PO_4.^1/_2H_2O$. The results of analysis of the codeine phosphate syrup by both methods are in the range of 93 to 107.0 percent label amount. Although the Codeine phosphate syrup is not official in U.S.P.XXI, these two results should be assumed to be reliable.

Analysis by using HPLC method might have some error in the process of dilution but the sample used for analysis by ^{31}P -NMR has no need for further process. Thus, the result of ^{31}P -NMR method should be more accurate than HPLC method.

Assay of codeine phosphate in Actifed Compound Linctus using ${}^{31}P$ NMR

Actifed Compound Linctus

The Actifed Compound Linctus was pipeted and then dissolved 80 mg of codeine phosphate powder into the linctus. Analyzed by using ${}^{31}P$ -NMR. The NMR spectra was shown in Figure 13. The measurement of intensity of peak was prepared by the method mentioned above. Table 9 showed the Intensity of peak, log intensity and concentration.

Table 9

Sample	A	B	C
Intensity of peak	4.06	4.00	4.10
log intensity	0.6085	0.6020	0.6127
Concentration (mg/ml)	12.1356	11.9034	12.2857

The concentration of Codeine phosphate were calculated from the equation which mentioned in assay of Codeine phosphate syrup using ${}^{31}P$ -NMR. The average of concentration (mg/10 ml) is equal to 121.0823 mg/10 ml less by 80 mg (which add into the sample).

So, concentration of codeine phosphate
in Actifed compound linctus = 4.1082 mg/ml
Labelled amount   = 2 mg/ml

Assay of codeine phosphate in Actifed compound linctus using High Performance Liquid Chromatography

Prepared sample solution by diluting Actifed compound linctus with the chromatographic solvent and adding chlorpheniramine maleate in the final solution as an internal standard. Prepared standard solution by dissolving the codeine phosphate and chlorpheniramine maleate in chromatographic solvent. Analyzed by H.P.L.C. by injecting unknown sample into the loop. For purposes of comparison, standard solution was injected after the assay was eluted. The chromatogram of standard solution and unknown sample were shown in Figure 13 and 14. Table 10 showed the area ratio of unknown sample's peak.

$$\text{Area ratio} = \frac{\text{Area under the peak of Codeine phosphate}}{\text{Area under the peak of Chlorpheniramine}}$$

Area ratio : Standard 1 = 2.8546

Standard 2 = 2.7412

Standard 3 = 2.6226

The average area ratio of Standard solution = 2.7394

Table 10

Sample	1	2	Average
A	2.5571	2.5621	2.5596
B	2.5412	2.6814	2.6113
C	2.6362	2.5778	2.6070

The average area ratio of sample   = 2.5926

Concentration of codeine phosphate in sample = $\dfrac{A_1 B}{A_2}$

where, A_1 = Ratio of area under the peak (sample)

A_2 = Ratio of area under the peak (standard)

B = Concentration of codeine phosphate in standard solution

Concentration of Codeine phosphate = $\dfrac{2.5926 \times 0.2}{2.7394}$

(the solution of 10 ml. sample
diluted to 100 ml.)   = 0.1893 mg/ml

So, concentration of codeine phosphate

in the syrup   = 1.893 mg/ml

Labelled amount   = 2 mg/ml

In the analysis of Actifed compound linctus using ^{31}P NMR and HPLC method. The results were shown as the following:

^{31}P NMR method   4.1082 mg/ml

HPLC method   1.893 mg/ml (94.7% label amount)

Labelled amount   2.0 mg/ml

The results of HPLC method were in the range of 93.0 to 107.0 percent label amount. Thus, it should be assumed that the result of HPLC method is reliable as the reason mentioned in assay of codeine phosphate syrup using ^{31}P NMR.

The concentration calculated from NMR spectra is more than label amount because Actifed compound linctus is composed of phosphate buffer. The concentration of phosphate ion from phosphate buffer that contaminate in linctus must be calculated. This amount is the correction for assay codeine phosphate in the Actifed compound linctus.

The concentration of phosphate ion (from phosphate buffer) in linctus may be calculated from the different of the amount of codeine phosphate that be calculated from the reliable assay method (such as High Performance Liquid Chromatography) and Phosphorus -31 nuclear magnetic resonance technique.