5. Chromatomembrane Method as Sample Preparation of Pharmaceuticals for HPLC

The coupling of the chromatomembrane method and HPLC has been first time demonstrated [104] for the determination of naphthalene, fluorene, acenaphthene, anthracene, phenanthrene, pyrene, fluoranthene, and chrysene spiked in the water. In comparison with the same procedure applied with GC, this procedure, theoretically, doesn't include the evaporation of the organic solvent by the nitrogen stream. Thus, the extract was transported directly to the separation column through the sample loop. Although the results were acceptable, some problems appeared which were caused by the desiderative miscibility of solvents for the extraction step and the eluent. The present of the non polar organic solvent in the polar eluent often causes the problem on the separation of compounds in non hydrophobic material in HPLC column.

In this research, the extraction solvent (dichloromethane) was evaporated before introducing the samples to HPLC to overcome this problem. Another aim of solvent evaporation is to achieve a significant reduction on sample volume which leads to increasing the enrichment factor.

5.1 Determination of Ethinylestradiol and Levonorgestrel

5.1.1 Introduction

At present there are three types of oral contraception available. In the sequential type, estrogen is administered alone for first week, followed by a lower dosage of the estrogen in conjunction with a progestogen for the remainder of the course. In the second, commonly used, type both an

estrogen and a progestogen are present in the tablets (as either a single dose or in three different doses). In the progestogen type, a progestogen alone is administered.

The estrogen content in such preparations, used in the management of menstrual and menopausal disorders as well as for contraception, is usually in the range 20 to 50 μg daily. As for the progestogenic content, it varies depending on the type of contraceptive. Thus, in combined oral formulations the progestogenic content is in the range 0.25 to 2 mg daily whereas in progestogen-only contraceptives it is lower (30-500 μg daily).

Other than contraception, the uses of estrogens can largely be put into three main groups: the management of the menopausal and postmenopausal syndrome (its widest use); physiological replacement therapy in deficiency states; and the treatment of prostatic cancer and of breast cancer in postmenopausal women.

Likewise, progestogens are used in the treatment of several other conditions such as infertility, endometriosis, in the management of certain breast and endometrial cancers, and either alone or in combination with estrogens in the treatment of menstrual disorders, among others.

Ethinylestradiol (ETE) is a semisynthetic estrogen female sex hormone and levonorgestrel (LEV) is a synthetic steroid with an extremely potent progestational action. Oral contraceptives have had an enormous positive impact on public health for the past three decades and there has been remarkably low incidence of troublesome side effects. Although estrogens are implicated in an increased incidence of breast and endometrial cancer, epidemiological studies have not provided convincing evidence to support

a direct correlation between the use of oral contraceptives and an increase in breast cancer. The structure of ethinylestradiol (ETE) and levonorgestrel (LEV) are presented in Figure 5.1.

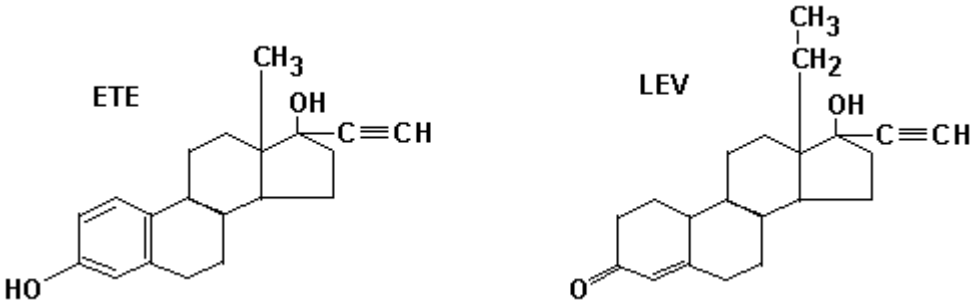


Figure 5.1 Structures of ETE and LEV

The formulation of these steroids in tablets of low dosage, i.e. 30-250 μg per tablet, presented a challenging analytical problem. A sensitive, accurate and rapid procedure is desirable for content uniformity testing of the dosage form. The most commonly encountered estrogen is ETE, which is present at very low dosage level (30-100 μg per tablet) in combination with an orally active synthetic progestin (one of the most commonly used is LEV), which is present at a level of from 5 to 30 times that of the estrogen. Progestogens are commonly added to estrogens to protect against endometrial hyperplasia and cancer because unopposed estrogen therapy may cause endometrial proliferation. Therefore, the modern low-dose oral contraceptives require a sensitive analysis method which is unaffected by the small amount of the estrogen and the large excess of progestogen.

There have been several reports [117-129] on the determination of ethinylestradiol or levonorgestrel, including the use of derivative spectrometry [117], high performance liquid chromatography with fluorescence detection [118], solid phase extraction followed by gas

chromatography-negative chemical ionization mass spectrometry of the pentafluorobenzoyl derivatives [119] and pentafluorobenzyl-trimethylsilyl derivatives [120], solid phase extraction followed by gas chromatography/MS/MS after derivatization with mixture of N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), trimethylsilylimidazole (TMSI), and dithioerytrol (DTE) [121], solid phase extraction followed by liquid chromatography-diode array detection-mass spectrometry [122], affinity chromatography with tripeptide column [123], gas chromatography-mass spectrometry [124,125], solid phase extraction followed by liquid chromatography-tandem mass spectrometry [126], and a diphasic dialysis method followed by gas chromatography-tandem mass spectrometry [127]. De Alda [128] reported a method including SPE of 500 mL of sample using C_{18} cartridges followed by HPLC-diode array detection (DAD) and HPLC-MS in series. Electrospray ionisation (ESI) in the negative ion mode was used for the MS detection of estrogens. Detection limits for DAD of estrogens were reported as 50 ng L^{-1} and for ESI-MS 500 ng L^{-1} for ethinylestradiol based on the use of 500 mL samples. Müller [129] recently proposed a method based on the semi-automated extraction using hollow fiber membrane for the GC-MS determination of ethinylestradiol. Enrichment was carried out inside a porous polypropylene hollow fiber membrane, which separated the aqueous and organic phases and regulated the transfer of analytes. n-Octanol placed inside the hollow fiber is used as the acceptor solution. However, this method is rather time consuming (1 hour extraction time), a leakage of the acceptor solution can take place, and the percent recovery is relatively low (33.8%).

Although those described methods produce high sensitivity, they still have the drawbacks. Those drawbacks are time consuming in extraction process when solid phase extraction is performed. Although SPE needs

lower amounts of organic solvents, the manual version, needed for concentrations of large sample volumes, still takes 8 to 10 hours. Furthermore, the target analytes have to be derivatized if GC-MS is used for analysis. Although some applications of SPME in connection with on-line silylation and GC-MS have been published [130], they appear unsuitable for routine analysis.

On the contrary, liquid chromatography (LC) has only been employed in a few occasions regardless of its advantages with respect to the already mentioned techniques. Thus, unlike GC-MS, LC enables the determination of steroid without derivatization and is not limited by such factors as properties of the substances (non volatile) and high molecular weight.

5.1.2. Experimental

5.1.2.1 Preparation of Standard Solutions, Reagents, and Samples

Stock standard solution of 103 μg mL^{-1} LEV was prepared by dissolving suitable amount of LEV (Schering, Berlin Germany) in 100 mL of methanol in a calibrated flask. Aliquots (5 mL and 0.5 mL) of stock solution were diluted with methanol in 50 mL calibrated flask to give the working stock solution of 10.3 and 1.03 μg mL^{-1} of LEV respectively. Two procedures of calibration standards for LEV and ETE were performed in order to investigate the feasibility of chromatomembrane method in comparison with standard method (US Pharmacopeia). Calibration standards using standard method were prepared by serial dilution of stock standards solution 103 μg mL^{-1} to produce concentrations of 2.06, 3.09, 4.12, 7.21 and 10.30 μg mL^{-1}. Meanwhile, calibration standards using

chromatomembrane method were prepared by serial dilution of stock standard solution 1.03 μg mL^{-1} to produce concentrations of 10.3, 20.6, 30.9, 7.21 and 103.0 ng mL^{-1}.

Stock standard solution of 100 μg mL^{-1} ETE was prepared by dissolving suitable amount of ETE (Schering, Berlin Germany) in 100 mL of methanol in a calibrated flask. Calibration standards using standard method were prepared by serial dilution of stock standard solution 100 μg mL^{-1} to produce concentrations of 1, 2, 4, 6, 8, 10, 15, and 20 μg mL^{-1}. Meanwhile, calibration standards using chromatomembrane method were prepared by serial dilution of stock standard solution to produce concentrations of 20, 40, 60, 80, 100, and 120 ng mL^{-1}.

HPLC-grade acetonitrile and dichloromethane (DCM) were purchased from Acros Organics (Schwerte, Germany). Methanol of analytical-grade reagent was distilled to get the HPLC-grade methanol. Tridistilled water was used throughout the experiment. Formic acid was purchased from Merck (Darmstadt, Germany).

LEV and ETE standards were supplied by Schering Deutschland GmbH (Berlin, Germany). MonoStep® (125 μg LEV + 30 μg ETE), Schering), Tetragynon® (250 μg LEV + 50 μg ETE, Schering), Minisiston® (125 μg LEV + 30 μg ETE, Jenapharm), Ethinylestradiol 25 μg® (Jenapharm), and Yasmin® (30 μg ETE, Schering) tablets were purchased from local drugstore.

Samples were prepared based on the US Pharmacopeia method [131] with little modification. In order to investigate the effect of filtration step on the preparation of the samples, two methods of samples preparation were

performed. Method 1, one tablet of Minisiston® (125 μg LEV per tablet) was finely powdered and dissolved in 5 mL of methanol by sonication for 15 min. The mixture was filtered using Care Roth disc filter holder of 25 mm diameter with 0.45 μm nylon membrane into a 10 mL calibrated flask and diluted by methanol to give LEV concentration of 12.5 μg mL^{-1}. The same procedure was applied to Tetragynon® (250 μg LEV) and MonoStep® (125 μg LEV) to give LEV concentration of 25.0 and 12.5 μg mL^{-1}, respectively. The working sample solutions were prepared by diluting those solutions with methanol to produce concentrations of 6.25 μg mL^{-1} (Minisiston®), 5.00 μg mL^{-1} (Tetragynon®), and 3.125 μg mL^{-1} (MonoStep®) of LEV.

Method 2, one tablet of Minisiston® (125 μg LEV per tablet) was finely powdered, transferred into a 10 mL calibrated flask, diluted by methanol to give LEV concentration of 12.5 μg mL^{-1} and sonicated for 15 min. The working sample solutions were prepared by diluting the solutions with methanol to produce LEV concentrations of 6.25 μg mL^{-1}. Sample preparation procedures (method 1 and method 2) were also applied for the preparation of ETE from pharmaceutical preparations so that the concentration of 3 μg mL^{-1} from Minisiston®, MonoStep®, and Yasmin®, 2.5 μg mL^{-1} from Ethinylestradiol 25 μg®, and 5 μg mL^{-1} from Tetragynon® were obtained.

5.1.2.2 Apparatus

A Knauer high-performance liquid chromatograph equipped with a Nucleosil C_{18} analytical column (250 x 4.6 mm i.d., 5 μm particle), a Knauer Variable Wavelength UV-Vis detector (Germany) and a Philip PM

8252A printer using a mobile phase of acetonitrile-methanol-water (3.5:1.5:4.5). Manual injections were carried out using a Rheodyne model 7125 injector with 20 µL sample loop. The chromatographic system was operated with a flow rate 1.85 mL min^{-1}, attenuation 0.16. The UV absorbance of effluent was monitored at 242 nm for LEV and 215 nm for ETE.

5.1.2.3 Analytical Procedure

Determination of LEV and ETE in pharmaceutical preparations consists of three steps, sample preparation, analysis, and data interpretation. Meanwhile, the sample preparation consists of extraction and separation with a chromatomembrane cell, evaporation of organic solvent, and dilution with methanol. Schematic diagram of the LEV and ETE analysis step is presented in Figure 5.2.

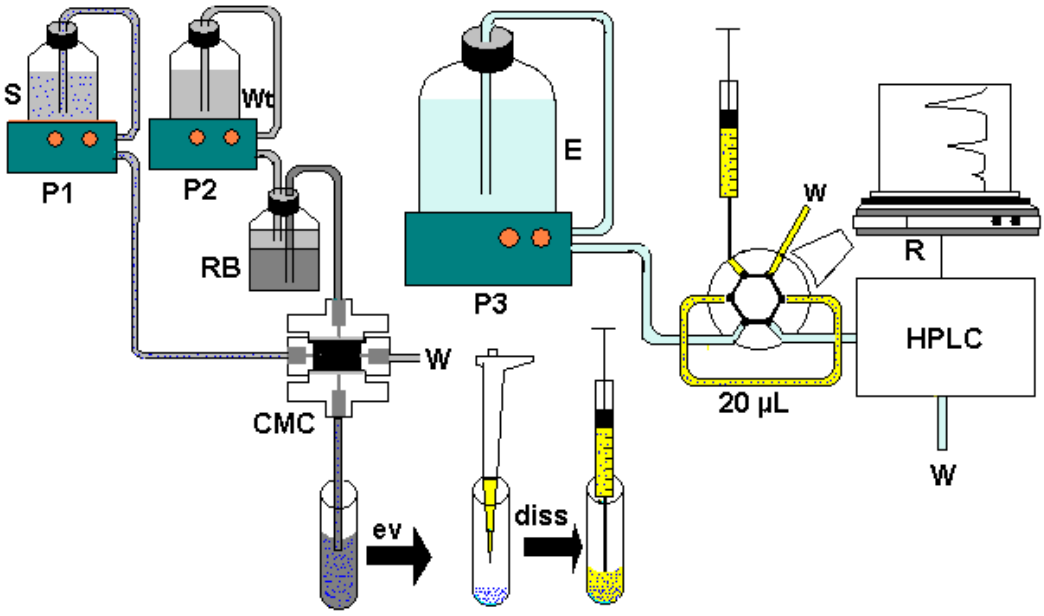


Figure 5.2 Schematic diagram of the LEV and ETE analysis

S=sample; P1/P2/P3=pumps; Wt=water; RB=replacement bottle; CMC=chromatomembrane cell; W=waste; ev=evaporation; E=eluent; diss=dissolution; R=recorder; HPLC=high performance liquid chromatograph

Extraction of LEV and ETE with a chromatomembrane cell was carried out in stop flow mode in order to achieve a significant preconcentration factor. PTFE tubing (0.8 mm i.d) was used for transferring sample and reagent. A 4-holes chromatomembrane cell was used for continuous separation, extraction and preconcentration of LEV and ETE. 0.5 mL of organic solvent (DCM) is transferred indirectly to a chromatomembrane cell by pumping water with a constant flow rate 1 mL min^{-1} through a replacement bottle using a peristaltic pump Reglo Analog MS 2/8 (ISMATEC Germany)(pump 1). After stopping the pump 1, 10 mL of sample containing LEV and ETE was pumped through a chromatomebrane cell using the same type of pump (pump 2) with constant flow rate 2 mL min^{-1} so that LEV and ETE was extracted into DCM. The pump 1 was stopped, and then LEV and ETE was eluted with 1.0 mL of DCM and collected in glass tube. LEV and ETE in DCM was then evaporated to dryness. Once dry, the residues were reconstituted in 100 µL of methanol and shaked to ensure the dissolution of LEV and ETE in methanol. The determination of those substances was performed by introducing the sample to HPLC. Manual injections were carried out using a Rheodyne model 7125 injector with 20 µL sample loop. HPLC determination was performed on a Nucleosil C_{18} analytical column (250 x 4.6 mm i.d.) containing 5 µm packing. The mobile phase was deaerated acenonitrile-methanol-water (3.5:1.5:4.5). The UV absorbance of effluent was monitored at 242 nm for LEV and 215 nm for ETE.

5.1.3. Results and Discussion

5.1.3.1. Optimization of Extraction Conditions

As mentioned in Section 3, the aim of the extraction is the isolation and enrichment of components of interest from a sample matrix. Extraction can vary in degree of selectivity, speed, and convenience and depends on the approach and conditions used. These extraction approaches are frequently easier to operate but provide optimization challenges. Optimization of this extraction process enhances overall analysis.

LEV and ETE belong to the compounds that are practically insoluble in water. With log K_{ow} (octanol-water partition coefficient) 3.67, ETE is more soluble in organic solvents and LEV as well. Ethanol, methanol, ether, acetone, dioxane, chloroform, vegetable oils, solution of fixed alkali hydroxide, methanol-acetonitrile (1:1), *n*-octanol, and DCM are known as suitable solvent for LEV and ETE. Since methanol was used as solvent to dissolve LEV and ETE in pharmaceutical preparation investigated in this research and solvent for standard solutions, the presence of methanol in samples would be an important factor on extraction efficiency.

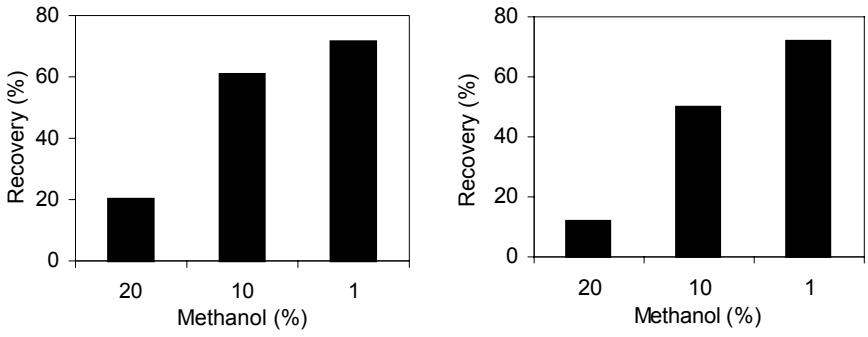
The effect of methanol in samples on the extraction efficiency was investigated by preparing a 4.12 µg mL^{-1} standard solution of LEV and a 10 µg mL^{-1} of ETE in 1, 10, 20, 50 and 100% methanol in water. Each of these solutions was pumped through the CMC, that have contained DCM as extraction solvent, with a constant flow rate (2 mL min^{-1}) while the flow of the non polar phase (DCM) was stopped. The extracted LEV was eluted from the CMC by flowing 1 mL of DCM. After DCM evaporation at room temperature, the residues were reconstituted in 100 µL of methanol to

achieve a theoretical preconcentration factor of 100. After shaking, solution was then injected into the HPLC system. The absorbance of LEV and ETE was measured by a UV detector at 242 nm and 215 nm. The percent recovery of extraction for each concentration was calculated from the mean peak height ratio of preconcentration sample relative to directly injected LEV standard solution of 4.12 μg mL^{-1} and ETE standard solution of 10 μg mL^{-1}. Effect of methanol in sample on the extraction efficiency is presented in Table 5.1 and Figure 5.3.

Table 5.1 Effect of methanol in sample on the extraction efficiency

Methanol concentration (%)	Recovery (%)*	
	Levonorgestrel	Ethinylestradiol
1	71.56	72.00
10	60.91	49.80
20	20.20	11.98

* Average of three measurements



(a) (b)

Figure 5.3 Effect of methanol in sample on the extraction efficiency
(a) LEV (b) ETE

As it can be seen, the maximum extraction efficiency could be achieved in 1% methanol solution. The extraction efficiency increased with decreasing methanol concentration in the samples. This is because of the physicochemical properties of LEV, ETE, methanol, and DCM. LEV and

ETE are soluble in methanol and DCM. Methanol is soluble in DCM. Increasing of methanol concentration in the samples increases the solubility of the LEV and ETE in the chromatomembrane cell. Thus, the extraction is more efficient in low methanol concentration. At methanol concentration 50 and 100%, methanol penetrated into micropores and flowed in the direction of non-polar liquid. This behaviour might be attributed from the ability of methanol to dissolve in DCM in the micropores. For this reason, solution of 1% methanol in water was used as solvent in further experiments.

Kirchhof [104] reported the coupling of the chromatomembrane cell with HPLC for determination of some poly aromatic hydrocarbons (PAH). In this procedure, the evaporation of the organic solvent by the nitrogen stream was not performed. Thus, the extract was transported directly to the nucleosil separation column through the sample loop. Although the results were acceptable, some problems appeared which were caused by the desiderative miscibility of solvents for the extraction step and the eluent. The presence of the non polar organic solvent in the polar eluent often causes the problem on the separation of compounds in non hydrophobic material in HPLC column. Therefore, the choice of the best organic solvent used for the extraction of LEV or ETE was also investigated.

When selecting a suitable organic solvent (extraction solvent) in the chromatomembrane method, three factors need to be taken into account. First of all, for enrichment to be effective, the analytes should be well soluble in the solvent used. Another factor is the water immiscibility. The organic solvent used must be water immiscible in order to fulfil the technical requirement of the method. Especially for the application in reverse phase HPLC, they have to have low boiling point to make them

easy to be evaporated to dryness. Among the organic solvents described above, DCM has fulfilled all the requirements because of its ability to dissolve LEV and ETE, low boiling point (40^{o}C) and water immiscibility. Thus, DCM was selected as organic solvent for extracting LEV and ETE from pharmaceutical preparations in whole experiments.

Another important factor is volume of the DCM used for elution of LEV and ETE from the chromatomembrane cell. As already mentioned in Section 4.1, 30%, on average, of the PTFE block used in a chromatomembrane cell is available for non polar phase. That means, with 1.2 cm^3 of the PTFE block used, the minimum volume of DCM used for elution of the analytes is 0.36 mL. The use of 1 mL DCM ensured the elution of analytes from the chromatomembrane cell completely. Thus, carry-over effects are negligible.

The selection of the sample volume, finally determined to be 10 mL, responded to time-saving considerations, and not to breakthrough values. In other word, the choice of the sample volume was in order to diminish the time invested during the extraction step and overall because the extraction step is often very time-consuming. Other factors involved in the extraction procedure, such as the flow rate of the sample, which could lead to lower recoveries of the compounds, or pH of the samples, were not further checked, because of the already satisfactory recoveries obtained for LEV and ETE under the optimum condition.

5.1.3.2 Optimization of Parameters for the HPLC System

The progestogens show a characteristic absorption maximum at 242 nm which arises from π→π* transitions in this case in the conjugated α,β-

unsaturated ketone (C=C-C(O)-C) at position 3[128]. In the commercial samples used in this experiment LEV presents in combination with a semisynthetic estrogen, ethinylestradiol (ETE). The effect of ETE absorption on the measurement of LEV was avoided by performing spectrophotometric detection at 242 nm, as described by Berzas et. al [117].

The HPLC method was optimised by changing three parameters: the mobile phase composition, the flow rate of eluent, and attenuation. Four different mixtures of mobile phase were investigated: acetonitrile-methanol-water (3.5:1.5:4.5), methanol-water (80:20) containing 0.5% (v/v) formic acid in water, methanol-water (81:19) containing 0.5% (v/v) formic acid in water, and methanol 100 %. The initial mobile phase investigated was acetonitrile-methanol-water (3.5:1.5:4.5), which is the mobile phase employed in the USP procedure [131]. However, the resolution between methanol and LEV peak was not so good (0.39). Then methanol-water (80:20) containing 0.5% (v/v) formic acid in water, as proposed by Wu et.al was evaluated [132]. A stable baseline could not be achieved within 9 hours; thus a slight modification using methanol-water (81:19) containing 0.5% (v/v) formic acid in water was performed, which in turn has showed no effect on decreasing the stabilization time. By using 100% methanol stabilization time was achieved in 8 hours, but the resolution was lower (0.28) and the peak shape was not smooth. Therefore, acetonitrile-methanol-water (3.5:1.5:4.5) was chosen as the optimum mobile phase.

Table 5.2 Data for mobile phase optimization

	Mobile phase	Stabilization time (hours)	Resolution
1	acetonitrile-methanol-water (3.5:1.5:4.5)	2	0.39
2	methanol-water (80:20) containing 0.5% (v/v) formic acid in water	> 9	not investigated
3	methanol-water (81:19) containing 0.5% (v/v) formic acid in water	> 10	not investigated
4	methanol 100%	8	0.28

By decreasing the flow rate from 1.85 mL min^{-1} to 1.20 mL min^{-1}, only a slight better resolution was observed. Considering the lower retention time, separation was then carried out using flow rate of 1.85 mL min^{-1}. This flow rate was also applied in determination of ETE. The retention time of ETE was 7 minutes.

Table 5.3 Data for flow rate optimization

	Flow rate (mL min^{-1})	Retention time (minute)	Resolution
1	1.20	15.3	0.39
2	1.85	10.1	0.35

A review of the UV responses to different detector attenuation indicated a linear relationship. The same behaviour was also observed for ETE.

Table 5.4 Data for attenuation optimization

Standard solution	Attenuation	Peak Height* (cm)
LEV 10.3 µg mL^{-1}	0.16	18.2
	0.32	9.3
	0.64	4.6
	1.28	2.3

* Average of two determinations

Thus, the attenuation of 0.16 was chosen for the further experiments in order to get a high response of UV detector.

5.1.3.3. Determination of Percent Recovery and Enrichment Factor

Introducing a percent recovery (R) is useful in order to investigate the amount of the analytes that can be extracted quantitatively in a chromatomembrane cell. It can be defined as a quotient between the peak of the analyte after extraction with the chromatomembrane cell (C_e) and the peak of standard solution with the same concentration (C_s).

$$R = (C_e / C_s) \times 100\ \% \quad \text{..... 5.01}$$

The term of enrichment factor consists of theoretical enrichment factor and true enrichment factor. Theoretical enrichment factor (EF_{th}) is defined as a quotient between the volume of the sample before extraction (V_s) and the volume of the sample after extraction/end volume (V_e).

$$EF_{th} = V_s/V_e \quad \text{..... 5.02}$$

True enrichment factor (EF_{tr}) is multiplying of theoretical enrichment factor with percent recovery.

$$EF_{tr} = EF_{th}.\ R \quad \text{..... 5.03}$$

Percent recovery was investigated by preparing a 4.12 µg mL^{-1} standard solution of LEV or a 10 µg mL^{-1} ETE in 1 % methanol. 10 mL of this solution was pumped through the CMC with a constant flow rate of 2 mL min^{-1} while the flow of DCM was stopped. The extracted LEV and ETE was eluted from the CMC by flowing 1 mL of DCM. The DCM containing LEV and ETE was evaporated and reconstituted with 10 mL of methanol in a calibrated flask. Solution was then injected into the HPLC system. The

absorbance of LEV and ETE was measured by a UV detector. The peak was then compared with the peak of 4.12 μg mL^{-1} standard solution of LEV or a 10 μg mL^{-1} ETE in methanol. In optimum condition as much as 71.56% of LEV and 72% of ETE could be recovered. Thus, by applying 10 mL of sample, the true enrichment factor of 71.56 for LEV and 72 for ETE can be achieved if the end volume of the sample after extraction is 100 μL. The higher enrichment factor could be expected if larger sample volume is used.

5.1.3.4 Calibration, Reproducibility, and Detection Limit

As already mentioned in Section 1, one of these research objectives is to compare the results obtained if a chromatomembrane cell is coupled with the HPLC in determination of LEV and ETE in pharmaceutical preparations with those results obtained by applying standard method (US Pharmacopeia). To achieve this research objective, the calibration curve was prepared by two different methods, without the chromatomembrane cell (standard method) and coupled with chromatomembrane cell. The calibration curves were made based on the linear relationships between the peak height and the concentration of the standard solutions of LEV and ETE.

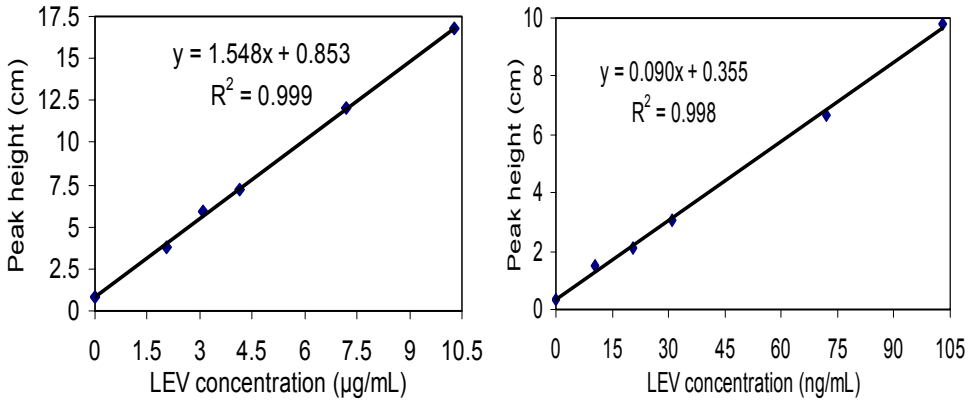
Under the optimum conditions, five series of LEV standard solutions ranging from 2.06 μg mL^{-1} to 10.3 μg mL^{-1} were examined their linearity using the standard method (without the chromatomembrane cell). All analytes showed good linearity with correlation coefficients of r^2 = 0.999 (y=1.548x + 0.853; n=3). Precision of each concentration of the standard solution was calculated based on 3 times measurements. The RSD value of 4.12 μg mL^{-1} LEV standard solution was found to be 0.81% with

detection limit of 20 ng mL^{-1}. Meanwhile, the construction of the calibration curve of LEV with the chromatomembrane cell was then performed by using concentration of the standard solutions, ranging from 10.3 to 103.0 ng mL^{-1}. Good linearity (y = 0.090x + 0.355; r^2 = 0.998; n=3) was observed. The RSD value of 10.3 ng mL^{-1} LEV standard solution was found to be 3.77% with detection limit of 0.26 ng mL^{-1} (see Table 5.5 and Figure 5.4).

Table 5.5 Calibration data for the determination of LEV

Method of determination	Concentration range	Equation	correlation coefficient (r^2)	RSD (%)	Detection limit (ng mL^{-1})
Standard method	2.06-10.30 µg mL^{-1}	Y=1.548x + 0.853	0.999	3.11[a]	20
Coupled with CMC	10.3-103.0 ng mL^{-1}	Y=0.090x + 0.355	0.998	1.07[b]	0.26

[a] standard solution of 4.12 µg mL^{-1} (n=20) [b] standard solution of 72.1 ng mL^{-1} (n=10)



(a) (b)

Figure 5.4 Calibration curve of LEV
(a) with standard method; (b) coupled with CMC

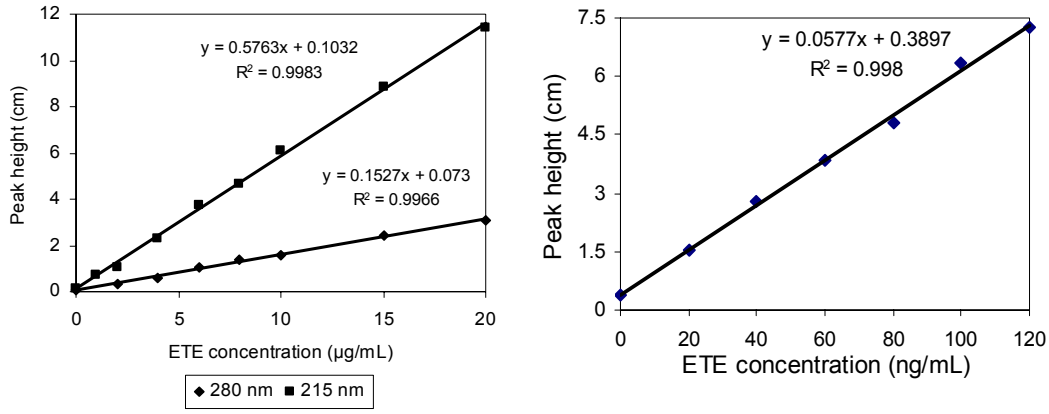
Precision or reproducibility of the method was determined by measuring a 20-fold LEV with HPLC standard method at a concentration level of 4.12 μg mL^{-1} and a 10-fold with HPLC coupled with the chromatomembrane cell at a concentration level of 72.1 ng mL^{-1}. The RSD values of 3.11% and 1.07% were observed for both methods.

In preliminary research, the absorption spectra of ETE in methanol were studied. The UV spectrum characteristic of the ETE presents two maxima, one at 215 nm and another at 280 nm, due to π→π* transitions in the aromatic ring [128]. Eight series of ETE standard solutions ranging from 1 μg mL^{-1} to 20 μg mL^{-1} were examined their linearity using the standard method (without the chromatomembrane cell) at 215 nm and 280 nm. Analysis of analytes at 215 nm showed good linearity with correlation coefficients of r^2 = 0.9983 (y=0.5763 x + 0.1032). Precision of each concentration of the standard solution was calculated based on 3 times measurements. The RSD value of 6 μg mL^{-1} ETE standard solution was found to be 2.71% with detection limit of 49.4 ng mL^{-1}. Measurement of analytes at 280 nm showed less sensitive, although a good linearity of standard curve was obtained. A linear regression was y=0.1527x + 0.073 with correlation coefficient of r^2 = 0.9966. Precision of each concentration of the standard solution was calculated based on 3 times measurements. The RSD value of 8 μg mL^{-1} ETE standard's solution was found to be 2.84% with detection limit of 188.6 ng mL^{-1}. Meanwhile, the construction of the calibration curve of ETE with the chromatomembrane cell was performed by using concentration of the standard solutions, ranging from 20 to 120 ng mL^{-1}. Good linearity (y = 0.0577x + 0.3897; r^2 = 0.998; n=3) was observed. The RSD value of 60 ng mL^{-1} ETE standard solution was found to be 1.51% with detection limit of 0.47 ng mL^{-1} (see Table 5.6 and Figure 5.5).

Table 5.6 Calibration data for the determination of ETE

Method of determination		Concentration range	Equation	correlation coefficient (r^2)	RSD (%)	Detection limit (ng mL^{-1})
Standard method	280 nm	1-20 μg mL^{-1}	Y=0.1527x + 0.073	0.9966	2.65[a]	188.6
	215 nm	1-20 μg mL^{-1}	Y=0.5763x + 0.1032	0.9983	2.78[a]	49.4
Coupled with CMC		20-120 ng mL^{-1}	Y=0.0577x + 0.3897	0.998	2.51[b]	0.47

[a] standard solution of 10 μg mL^{-1} (n=10) [b] standard solution of 60 ng mL^{-1} (n=5)



(a) (b)

Figure 5.5 Calibration curve of ETE
(a) standard method (b) coupled with CMC

Precision or reproducibility of the method was determined by a 10-fold HPLC standard method at a concentration level of 10 μg mL^{-1} and a 5-fold HPLC coupled with the chromatomembrane cell at a concentration level of 60 ng mL^{-1}. The RSD values of 2.65% (280 nm), 2.78% (215 nm) and 2.51% were observed for both methods.

Table 5.5 and Table 5.6 summarize several parameters indicative of the analytical performance of the two methodologies described, relative to the use of the CMC on the determination of LEV and ETE in pharmaceutical preparations. The sensitivity is perhaps the most important parameter for comparing the standard method and the method coupled with CMC for determination of LEV and ETE. As it can be seen, the couple of the CMC

with HPLC decreases the detection limit. The detection limit of LEV was decreased by factor of 76.92 (from 20 ng mL^{-1} to 0.26 ng mL^{-1}) and of ETE by factor 105.1 (from 49.4 ng mL^{-1} to 0.47 ng mL^{-1}). Very good relative standard deviations (lower than 3%) and recovery percentages (for ETE between 97 and 103% and for LEV between 96 and 97%) indicate better accuracy and reproducibility of both methods.

5.1.3.5 Determination of Ethinylestradiol and Levonorgestrel in Pharmaceutical Preparations

Samples were prepared based on the US Pharmacopeia method [131] with little modification. In the method described in the USP, sample solution is prepared from LEV or ETE containing tablet without further filtration. In this way, there is a risk that some solid particles in sample solution are carried over into the HPLC column and clogged inside. In order to investigate the effect of filtration step on the preparation of the samples, two methods of samples preparation were performed (see Section 5.1.2.1). The effect of filtration on recovery was examined by comparing the peak height of freshly prepared standard solution with and without filtration. The similar heights were obtained from both analyses, which implies no effect of filtration to LEV and ETE recoveries. In practice, method 1 with filtration step using nylon membrane (0.45 µm pore size) was applied throughout the experiment.

MonoStep® (125 µg LEV + 30 µg ETE), Schering), Tetragynon® (250 µg LEV + 50 µg ETE, Schering), Minisiston® (125 µg LEV + 30 µg ETE, Jenapharm), Ethinylestradiol 25 µg® (Jenapharm), and Yasmin® (30 µg ETE, Schering) tablets from local drugstore were used as pharmaceutical samples. As shown in Table 5.7, Fig. 5.6, and Fig. 5.7, the recoveries of

LEV and ETE from commercial pharmaceuticals vary between 90.27 and 107.67%, which meet the requirement for LEV and ETE tablets specified in the USP.

Table 5.7 Determination of LEV and ETE in pharmaceutical preparations

Samples	Levonorgestrel					
	Standard method			Coupled with CMC		
	Certified (µg/tablet)	Found [a] (µg/tablet)	Recovery (%)	Certified (µg/tablet)	Found [b] (µg/tablet)	Recovery (%)
Minisiston	125	115.31	92.27	125	134.16	107.11
Tetragynon	250	225.63	90.27	250	258.35	103.22
MonoStep	125	113.31	90.69	125	134.71	107.67
Samples	Ethinylestradiol*					
	Standard method			Coupled with CMC		
	Certified (µg/tablet)	Found [c] (µg/tablet)	Recovery (%)	Certified (µg/tablet)	Found [c] (µg/tablet)	Recovery (%)
Minisiston	30	27.79	92.67	30	30.56	101.62
Tetragynon	50	47.74	95.47	50	50.93	101.85
MonoStep	30	28.65	95.44	30	30.47	101.68
Yasmin	30	27.79	92.67	30	30.04	100.20
Ethinyles-tradiol 25 µg	25	24.26	96.93	25	24.89	99.54

* measured at 215 nm
[a] average of five measurements
[b] average of four measurements
[c] average of three measurements

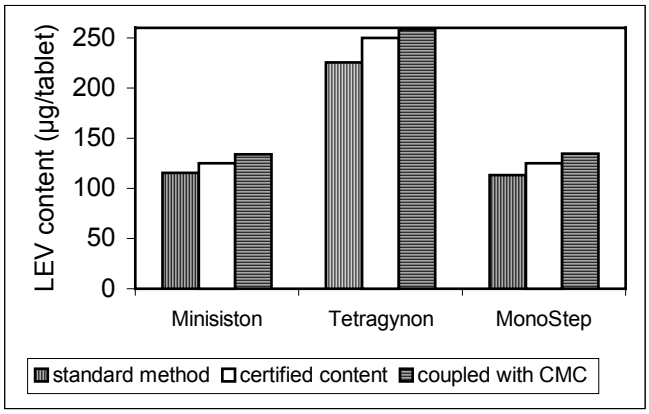


Figure 5.6 Analysis of LEV in pharmaceutical preparation

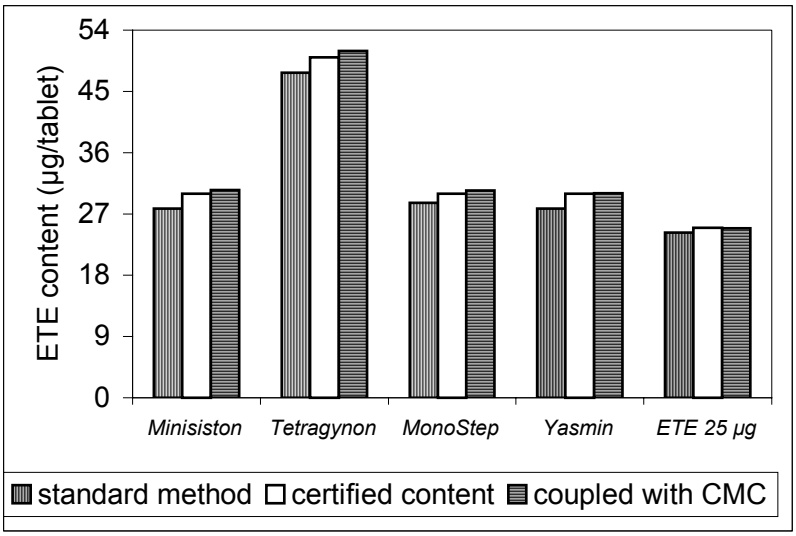


Figure 5.7 Analysis of ETE in pharmaceutical preparations

As it can be seen, the results obtained using HPLC coupled with the CMC were comparable with those obtained with HPLC standard method. The recoveries of LEV and ETE in Table 5.7 show slightly higher for the HPLC coupled with the CMC than those of the HPLC standard method. This might be because of the matrix effect. However, the effect of the matrix in pharmaceutical preparations was not investigated since the results for each pharmaceutical preparation were found to be in good agreement with the certified content.

5.1.4 Summary

In this research work, a procedure for the determination of LEV (semisynthetic estrogen) and ETE (progestogen) in pharmaceutical preparations by HPLC with uv detection coupled with the chromatomembrane cell for extraction and pre concentration manifold in flow system is described. This procedure includes liquid-liquid extraction of the compounds using dichloromethane, evaporation of extract,

reconstitution of extract in methanol, and subsequent analysis of the extract by HPLC with uv detection at 242 nm for LEV and at 215 nm for ETE. The feasibility of the chromatomembrane method has been proved by comparing the results with the HPLC method employed in United States Pharmacopeia.

The flow system was developed to provide the combination of subsequent extraction and HPLC analysis of the samples. The effect of methanol concentration in the samples, the choice and the volume of the organic solvent used for extraction, and volume of the sample used were investigated. The extraction efficiency was affected by the methanol concentration in the samples. The extraction efficiency was decreased by increasing of the methanol concentration. Extraction would be effective for the samples in 1% methanol in water. Extract was evaporated to dryness and reconstituted in methanol before manually introducing to HPLC through sample loop in order to avoid the emerging problems due to the present of non polar phase (organic solvent) in reverse phase chromatography. DCM is proved to be the best organic solvent because of its ability to dissolve LEV and ETE, lower boiling point, and water immiscible. The sample volume of 10 mL was selected in order to diminish the time invested during the extraction step. However, other factors involved in the extraction procedure, such as the flow rate of the sample, which could lead to lower recoveries of the compounds, or pH of the samples, were not further checked, because of the already satisfactory recoveries obtained for LEV and ETE under the optimum condition.

The parameters for the HPLC system were also optimized. These include the mobile phase composition, the flow rate of eluent, and attenuation. Deaerated acetonitrile: methanol: water (3.5 : 1.5 : 4.5) produced the

optimal results. The optimal resolution and chromatogram were achieved by applying 1.85 mL min^{-1} of eluent and attenuation of 0.16.

The method performance was evaluated by the determination of the enrichment factor, linearity, reproducibility, accuracy, and sensitivity of the method. In optimum condition, the enrichment factor of 71.56 (LEV) and of 72 (ETE) were achieved by introducing 10 mL of sample with the end volume of 100 µL. Good linearity (r^2>0.99) was observed. The relative standard deviation (<3%) indicated that the method has very good reproducibility. The accuracy of the method was indicated by recovery percentages. Satisfactory recoveries were observed for LEV (96.24%; n=10) and for ETE (103.13%; n=20) from measurement of a single standard solution of 72.1 ng mL^{-1} and 60 ng mL^{-1}. In general, the results obtained using the HPLC coupled with the CMC on determination of LEV and ETE in pharmaceutical preparations were comparable with those obtained by the HPLC standard method suggested by USP. However, in case of sensitivity, the HPLC coupled with the CMC showed even better sensitivity which is indicated by lower detection limit. By applying the HPLC coupled with the CMC, LEV is 76.92 times more sensitive (from 20 ng mL^{-1} to 0.26 ng mL^{-1}) and ETE is 105.1 more sensitive (from 49.4 ng mL^{-1} to 0.47 ng mL^{-1}) than the standard method. Applications of the method on the analysis of those compounds in pharmaceutical preparations were found to be in good agreement with the certified content.