3.0 EXPERIMENTAL

3.1 HPLC

3.1.1 Reagent and Apparatus

- Enalapril maleate, enalaprilat, diazepam (pharmacology department of U.M.).
- HPLC grade acetonitrile, methanol, ethanol, acetone (Fisher Scientific).
- Water was double purified and distilled.
- HPLC grade acetonitrile, methanol and water were filtered through a 0.2μm Nylon membrane filter (Whatman).
- Acetonitrile and water were degassed using helium gas.

The liquid chromatographic system consisted of Waters Model 600 LC pumps. Samples were injected using an injection valve fitted with a 20μl sample loop. A guard – column was placed between the injection vale and the chromatographic column. Separations were performed at ambient temperature with a 150 x 3.9 mm I.D. column with 5μm particles Novapak C18 packing (Waters Association). A Waters 996 Photodiode Array detector operates exclusively with Waters Millennium software was used.

HPLC analysis

The HPLC operating condition :

Column : Nova – Pak C 18 (3.9 x 150mm)

Eluent : acetonitrile : water (70 : 30)

Method development analysis:

Method development 1:

1mg of enalapril maleate was dissolved in 1ml of ethyl acetate gave 1μg/μl enalapril. 1 μl was injected into the chromatograph. The compound was detected by using UV detector at 254nm, A.u.f.s is 0.02. at a flow rate of 0.5ml/min, the retention time found. t_r = 3.4 min for ethylacetate and t_r = 2.4 min for enalapril maleate.

- Ethyl acetate show the solvent peak in UV detector. Thus ethylacetate is not the ideal solvent.

NOTE : Refer Figure 9

Method development 2:

1mg of enalarpil maleate was dissolved in 1mL of methanol gave 1µg/µl enalapril. 0.5µl was injected into the chromatograph. The compound was detected by using UV detector at 254 nm, A.u.f.s. = 0.02. At a flow rate of 0.5 ml/min, the retention time found. $t_r = 2.5$ cm for enalapril maleate.

- With the wavelength 254nm, enalapril show single peak.

NOTE : Refer Figure 10

When the compound was detected at 214nm. Two peaks were found, $t_r = 2.7$,4.2 min for *cis, trans* isomer enalapril.

- With the optimum wavelength 214nm, enalapril show the *cis–trans* isomerization.

NOTE: Refer Figure 11

Method development 3:

1 mg of enalapril maleate was added with 50µl of methanol and 4µl of pyridine. It was evaporated to dryness under nitrogen gas. The residue was reconstituted with 1ml methanol gave 1µg/µl enalapril maleate. 0.5µl was injected into the chromatograph. The compound was detected by using UV detector at 214nm, A.u.f.s. = 0.02. At flow rate of 0.3ml/min. A peak with $t_r = 5.0$min for enalapril was detected.

- With the use of pyridine, enalapril show single peak.

NOTE: Refer Figure 12

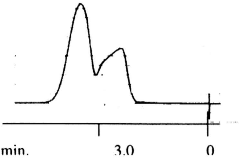


Fig 9. HPLC-UV chromatogram for the enalapril maleate dissolve in

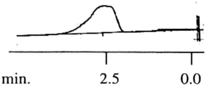


Fig 10. HPLC-UV chromatogram for the enalapril in methanol detected at wavelength 254nm.

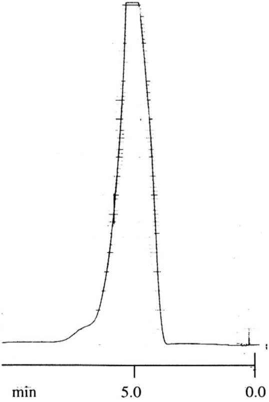


Fig 12. HPLC-UV chromatogram for the enalapril liberated by pyridine detected at 214nm

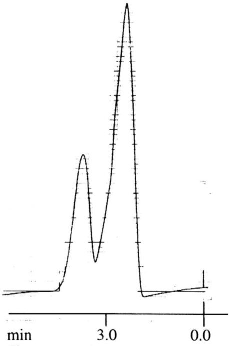


Fig 11. HPLC-UV chromatogram for the enalapril in methanol detected at wavelength 214nm

In the preliminary measurement, the retention for 4μL pyridine in 50μL of methanol was detected at $t_r = 7.1$ min.

Testing for sensitivity

0.5μL of 0.1μg/μl enalapril was injected at different A.u.f.s.

Table 1: Peak height for different A.u.f.s.

A.u.f.s.	Peak height
0.01	8.5 cm
0.005	13.2cm
0.003	> 22.5 cm

- ➢ Lower A.u.f.s. gives higher sensitivity

NOTE: Refer Figure 13,14,15

1μl of 0.01μg/μl enalapril was injected into the chromatograph. At A.u.f.s. = 0.004, two retention time were found, $t_r = 4.2, 5.2$ min for *cis* & *trans* enalarpil.

- ➢ At the lower A.u.f.s., the peak splitting caused by *cis* & *trans* isomer still can be observed.

NOTE : Refer Figure 16

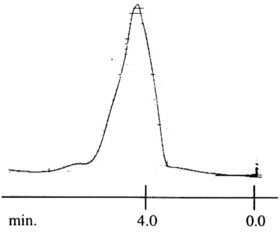


Fig 13. HPLC-UV chromatogram of 0.5μL of 0.1μg/μL enalapril at 0.01A.u.f.s.

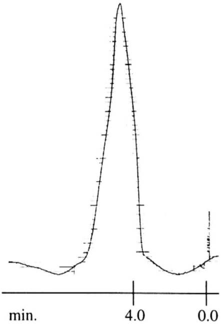


Fig. 14. HPLC-UV chromatogram of 0.5μL of 0.1μg/μL enalapril at 0.005 A.u.f.s.

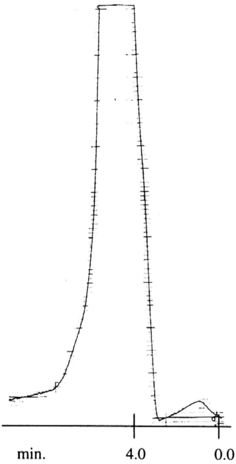


Fig 15. HPLC-UV chromatogram of 0.5µL of 0.1µg/µL enalapril at 0.003 A.u.f.s.

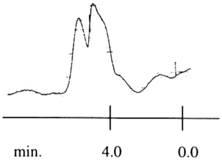


Fig 16. HPLC-UV chromatogram for 1µL of 0.001µg/µL enalapril at A.u.f.s.= 0.004

Method development 4:

1mg enalapril maleate was added with 10µl distilled pyridine and was shaken for 10 minutes at room temperature. 50µl of methanol was added and then evaporated to dryness under nitrogen gas. The residue was reconstitute with 0.5ml methanol.

Between each injection, inject 2µl methanol to clean the column for 5 minutes.

The HPLC system was equilibrated with the mobile phase acetonitrile: water 70:30 at a flow rate of 0.8 ml/min by injecting methanol. The detection was by PDA absorption at 214nm.

0.5µl of 2µg/µl enalapril was injected.

Table 2: Retention time for Trial sample at 0.7ml/min flow rate

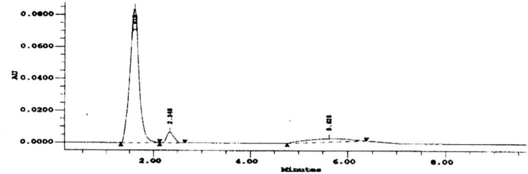
Sample	Retenion Time (min)
Trial sample 1	$t_r = 1.590$
Trial sample 2	$t_r = 1.552$
Trial sample 3	$t_r = 1.612$ $t_r = 2.345$
Trial sample 4	$t_r = 1.552$ $t_r = 2.335$

Table 3: Retention time for Trial sample at 0.8ml/min flow rate

Sample	Retention Time (min)
Trial sample 1	$t_r = 1.445$ $t_r = 2.128$
Trial sample 2	$t_r = 1.505$ $t_r = 2.120$
Trial sample 3	$t_r = 1.412$ $t_r = 2.078$
Trial sample 4	$t_r = 1.408$ $t_r = 2.108$
Trial sample 5	$t_r = 1.400$ $t_r = 2.117$

- with different flow rate, *cis* & *trans* isomer of enalapril still can be observed.

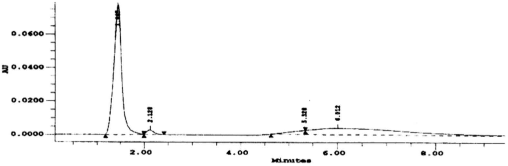
NOTE : Refer Figure17, Figure 18



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		1.612	1120782	83915		BV
2		2.345	67604	6671		VB
3		5.628	132723	2120		BB

Fig 17. HPLC-PDA chromatogram for enalapril at flow rate 0.7ml/min



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		1.445	925818	78016		BV
2		2.128	28896	2789		VB
3		5.328	53001	2364		BV
4		6.012	497094	3769		VB

Fig 18. HPLC-PDA chromatogram for enalapril at flow rate 0.8ml/min

Method development 5:

A series of concentration of enalapril were prepared to check the detectability of solute in the sample by using HPLC.

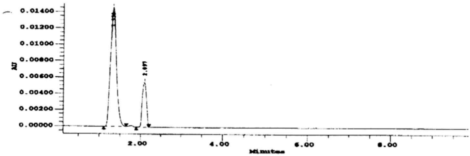
1 µl of 100ppm, 10ppm , 1ppm of enalapril were prepared as method above was injected into the chromatograph.

Table 4 : Retention time for 100ppm,10ppm,1ppm of enalapril at 0.8ml/min flow rate.

Concentration of the sample (ppm)	Retention time
100	1.330
10	1.323
1	1.237

- The retention time for 1ppm enalapril was different from other. So, we were not sure whether the retention time at 1.237 is for enalapril maleate or not.

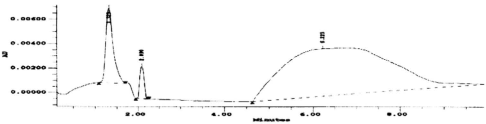
NOTE : Refer Figure 19, Figure 20, Figure 21



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		1.330	142832	14619		BB
2		2.097	38292	5460		BB

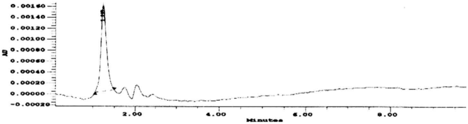
Fig 19. HPLC-PDA chromatogram of 100ppm enalapril



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		1.323	72174	6148		BB
2		2.090	18542	2652		BB
3		6.223	676426	3898		BB

Fig. 20 HPLC-PDA chromatogram of 10ppm enalapril



Peak Results

#	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		1.237	14732	1563		BB

Fig 21. HPLC-PDA chromatogram of 1ppm enalapril

3.2 Thin Layer Chromatography (TLC)

TLC of enalapril is performed on silica gel coated chromatoplate. The developing solvent system is hexane : ethyl acetate (4 : 1) by volume. Development is carried out in a lined container, and the solvent is allowed to migrate about two thirds of the length of the plate, the plate is removed from the container and dried. The plates are visualized by examing under ultraviolet light at 254nm.

Two spots of the sample, enalapril and methylated enalapril with diazomethane, were applied to the chromatoplate. The plate is developed as above.

3.3 GC – MS

3.3.1 Apparatus and Reagent

Apparatus

- Shimadzu's GC – MS QP 5000 compact.
- Shimadzu's GC – MS QP – 5050A compact.
- High performance quadrupole mass spectrometer
- Microsyringe
- Sep – Pak C18 cartridge [International Sorbent Technology (Mid Glamorgan, UK)]

Reagent

- Solvent: HPLC ethyl acetate (Fisher Scientific)
  HPLC methanol (Fisher Scientific)
  Chloroform (Fisher Scientific)
  Diethyl ether (Fisher Scientific)
  Redistilled water
  0.02M hydrochloric acid
  petroleum ether (boiling range 40 – 80 °C) (Fisher Scientific).
- Drugs: Enalapril maleate
  Enalaprilat
  Diazepam
- N–Methyl–N–nitroso–p–toluenesulfonamide (Diazald), Di(ethylene glycol) ethyl ether for diazomethane generation, were purchased from Aldrich (Milwaukee, USA)

Instrumental condition as follow:

Instrument (GCMS) settings

Shimadzu's GC – MS – QP 5000

- Carrier gas: He
- Sample size: 1μL
- Capillary column

  Type: DB – 1, 30m X 0.25mm I.D., 0.25μm film thickness
- Oven temperature: 150°C
- Oven equilibrium time: 0.1min
- Injector temperature: 280°C
- Interface temperature: 295°C
- Sampling time: 1.00min
- Control mode: splitless
- Column pressure: 52.1kPa
- Column flow: 1.7ml/min
- Linear velocity: 49.5
- Split ratio: 12
- Total flow: 23.8ml/min
- Carrier flow: 23.8ml/min
- Acquisition Mode : SIM
- Oven temperature program

Rate	Temperature (°C)	Keep Time (min)
-	150.0	0.00
35.0	290.0	10.00

Shimadzu's GC – MS – QP 5050 A

- Carrier gas: He
- Sample size: 1μL
- Capillary column

  Type: DB – 5, 30m X 0.25mm I.D., 0.25μm film thickness
- Sampling time: 2.00min
- Inj. initial temperature: 280°C
- Interface temperature: 300°C
- Control mode: splitless
- Column inlet pressure: 114.9 kPa
- Column flow :1.8mL/min
- Linear velocity: 48.9cm/sec
- Split ratio :12
- Total flow: 27.1mL/min
- Oven temperature program

Rate	Temperature (°C)	Keep Time (min)
-	70.0	0.00
45.0	300.0	15.00

- Pressure program:

Rate	Pressure (kPa)	Keep Time (min)
-	114.9	5.00

- Acquisition Mode: SIM
- Interface temperature: 300.00°C
- Solvent cut time: 6.00 min
- SIM table:

Start time (min): 7.25
End time (min): 7.75
Ch1 – m/z: 256.00
Ch1 – m/z: 283.00
Ch1 – m/z: 284.00
Start time (min): 8.00
End time (min): 9.00

Ch1 – m/z: 220.00
Ch1 – m/z: 234.00
Ch1 – m/z: 160.00

3.3.2 Procedure

- **Diazomethane preparation**

The reagent used to esterify carboxylic acids of enalapril is diazomethane. Diazomethane in ether can be prepared by the following procedure[23]:

1. Assemble the following glassware:
   a) 100ml round bottom flask in a 75°C hot water bath
   b) Dropping separatory funnel
   c) Water condenser assembly
   d) 100ml conical flask in an ice bath
2. Distilled and collected the middle fraction ether at 35°C.
3. Add to the two neck round bottom flask in the water bath;
   a) 3ml of carbitol
   b) 0.66g potassium hydroxide in 1ml of distilled water
   c) 5 ml anhydrous ether
4. Add to the dropping funnel;
   a) 1.084g diazald in 30ml anhydrous ether
5. Add to the receiving flask 10 ml of anhydrous ether
6. Make certain the condenser water is on and the receiving flask is in an ice bath.
7. Warm the hydroxide solution on a hot plate and slowly add diazald over a period of 20 min.
8. The rate of addition should equal the rate of distillation.
9. When all the diazald is used up, add an additional 10 ml ether and continue until the distillate is colorless.

- ❑ **Enalapril maleate, Enalaprilat, Diazepam Standard Stock Solution preparation**

1mg/ml Enalapril maleate, Enalaprilat, Diazepam standard solution

1mg of Enalapril maleate was dissolved and diluted with 1ml HPLC methanol

1mg of Enalaprilat was dissolved and diluted with 1ml HPLC methanol

1mg of Diazepam was dissolved and diluted with 1ml HPLC methanol

0.1mg/ml Enalapril maleate, Enalaprilat, Diazepam standard solution

0.1ml of 1mg/ml Enalapril maleate was dissolved with 0.9ml HPLC methanol

0.1ml of 1mg/ml Enalaprilat was dissolved with 0.9ml HPLC methanol

0.1ml of 1mg/ml Diazepam was dissolved with 0.9ml HPLC methanol

0.01mg/ml Diazepam standard solution

0.1ml of 0.1mg/ml Diazepam was dissolved with 0.9ml HPLC methanol

0.04mg/ml Enalapril maleate, Enalaprilat standard solution

0.4ml of 0.1mg/ml Enalapril maleate was dissolved with 0.6ml HPLC methanol

0.4ml of 0.1mg/ml Enalarprilat was dissolved with 0.6ml HPLC methanol

0.02mg/ml Enalapril maleate, Enalaprilat standard solution

0.5ml of 0.04mg/ml Enalapril maleate was dissolved with 0.5ml HPLC methanol

0.5ml of 0.04mg/ml Enalaprilat was dissolved with 0.5ml HPLC methanol

0.01mg/ml Enalapril maleate, Enalaprilat standard solution

0.5ml of 0.02mg/ml Enalapril maleate was dissolved with 0.5mlHPLC methanol

0.5m of 0.02mg/m Enalaprilat was dissolved with 0.5m HPLC methanol

0.005mg/m Enalapril maleate, Enalaprilat standard solution

0.5ml of 0.01mg/ml Enalapril maleate was dissolved with 0.5ml HPLC methanol

0.5ml of 0.01mg/ml Enalaprilat was dissolved with 0.5ml HPLC methanol

0.0025mg/ml Enalapril maleate, Enalaprilat standard solution

0.5ml of 0.005mg/ml Enalapril maleate was dissolved with 0.5ml HPLC methanol

0.5ml of 0.005mg/ml Enalaprilat was dissolved with 0.5ml HPLC methanol

0.00125mg/ml Enalapril maleate, Enalaprilat standard solution

0.5ml of 0.0025mg/ml Enalapril maleate was dissolved with 0.5ml HPLC methanol

0.5ml of 0.0025mg/ml Enalaprilat was dissolved with 0.5ml HPLC methanol

3.3.3 Calibration

- **Working Standard solution**

1. 6 working standards (0.04, 0.02, 0.01, 0.005, 0.0025, 0.00125mg/ml) and a blank were prepared.
2. 5.0μl of the low enalapril maleate, enalaprilat standard solution was added into each 6 (except blank) centrifuge tube.
3. 5.0μl of the 0.01mg/ml diazepam(internal standard) was added into all working standards.
4. 0.5ml of diazomethane in ether was added into all working standards.
5. The standard solutions were evaporated by using nitrogen gas.
6. The residue was reconstituted with 200μl ethyl acetate.
7. 1μl of each standard was injected into the column.

These can be illustrated in Table 5

Table 5: Preparation and concentration of working standard solutions

(a)[Enalapril Maleate]/ [Enalaprilat] mg/ml	Volume of Enalpril Maleate /μl	Volume of Enalaprilat /μl	Volume of 0.01mg/mL Diazepam /μl	Volume of Diazomethane in ether /μl	Concentration of (a) working standard, (ppm)
0.00000	-	-	5	500	0.00000
0.00125	5	5	5	500	0.03125
0.00250	5	5	5	500	0.06250
0.00500	5	5	5	500	0.12500
0.01000	5	5	5	500	0.25000
0.02000	5	5	5	500	0.50000
0.04000	5	5	5	500	1.00000

3.3.4 Method Development

- *Method Development 6*

➢ **Derivatization before extraction**

2mL of blank plasma was placed in a screw – cap culture tube. Standard stock solution of enalapril maleate and enalaprilat were added. The sample was methylated by adding 2mL of diazomethane solution at room temperature for 15 min. Ethyl acetate(4mL) was added. Samples were vortex for 1 minute, mixed using a gentle tilting action for 10 min. and the organic and aqueous phase were separated by centrifugation. Collected the extracted organic layer portion. Repeat the extraction procedure for 3 times. The extracted layer was evaporated to dryness at 40°C under nitrogen gas. The residue was taken up in 200µl ethyl acetate. 1 µl of which was injected into the column.

- ➢ Recovery too low.

- ☐ *Method Development 7 (successful method)*

- ➢ **Derivatization after extraction**

The internal standard (diazepam, 50ng), different concentration of standard solutions enalapril maleate and enalaprilat (0.04, 0.02, 0.01, 0.005, 0.0025, 0.00125mg/ml) were added to 1ml of plasma in a centrifuge tube. The sample was applied to a Sep – Pak C18 cartridge, which had been preconditioned with methanol (5ml) followed by redistilled water (5ml). Subsequently the column was washed with 5mL 0.02M hydrochloric acid and 6 ml petroleum ether. The sample was eluted with 6 ml methanol. The eluant was evaporated by nitrogen gas and methylated by adding diazomethane solution at room temperature for 15 min. It was evaporated at 40°C under nitrogen gas. The residue was taken up in 200µl ethyl acetate. 1µl of sample was injected into column.

3.3.5 Recovery of Enalapril Maleate and Enalaprilat

Small amounts of enalapril maleate and enalaprilat may be lost during the solvent extraction process or solid phase extraction process, and may occur even in the HPLC and GC analyses. The percentage recovery of enalapril maleate and enalaprilat was determined to evaluate the efficiency of the method employed.

The recovery procedure is as follows: the enalapril maleate and enalaprilat standards of known concentrations were spiked into 1mL of blank plasma. It was then followed by the solid phase extraction, GC analytical procedures. The percentage of recovery was calculated by the formula stated below:

$$\% \text{ recovery} = D/E \times 100\%$$

where D = peak height of recovered enalapril maleate and enalaprilat contained after the extraction procedure.

E = concentration of enalapril maleate and enalaprilat