4.0 RESULT AND DISCUSSION

Development of Method

In this project, High Performance Liquid Chromatography (HPLC) and Gas Chromatography – Mass Spectrometry (GC – MS) were applied in identification and quantitation of Enalapril Maleate and Enalaprilat.

It was found that there are advantages inherent in GC – MS which are not available in HPLC. These advantages include the high resolution, sensitivity, accurate quantitative results, well known which can be found in GC – MS.

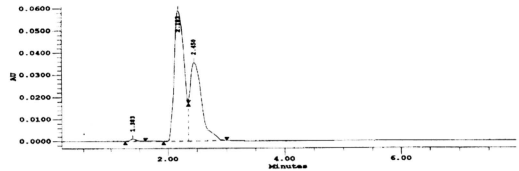
4.1 HPLC

Selection of UV detector absorption wavelength

Three different wavelengths were studied in the early stage, i.e. 214 nm, 254nm and 295 nm. From the chromatograms, enalapril maleate shows maximum absorbance at wavelength 214nm, whereas methylated enalapril with diazomethane show maximum absorbance at wavelength 295nm.

Figure 10, 11 show the UV spectra of the enalapril in methanol with wavelength 214nm & 254nm. This shows that a suitable wavelength would provide an acceptable sensitivity.

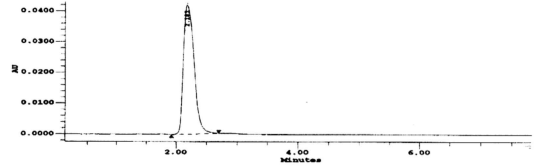
Figure 22, 23 show that the UV spectra of the methylated enalapril with diazomethane with wavelength 214nm & 295nm. This show that suitable wavelength would give much higher sensitivity and would also discriminate against the interfering peaks.



Peak Results

	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		1.383	7100	1028		BB
2		2.183	690725	59098		BV
3		2.450	492656	35476		VB

Fig 22. HPLC-PDA chromatogram of the methylated enalapril with wavelength 214nm



Peak Results

	Name	Ret Time (min)	Area (uV*sec)	Height (uV)	Amount	Int Type
1		2.183	510939	42146		BB

Fig 23. HPLC-PDA chromatogram of the methylated enalapril with wavelength 295nm

Selection of the detector

In the Photodiode Array Detector, polychromatic radiation, after passing through the sample, is dispersed by a fixed grating and then falls on to an array of photodiodes. Each diode measures a narrow band of wavelengths in the spectrum, thus the PDA has parallel data acquisition. When used as a detector for HPLC, the PDA, although more expensive than conventional UV detectors, has a number of significant advantages. The spectrum of each peak in the chromatogram can be stored and subsequently compared with standard spectra, which facilitates the identification of peaks. The optimum wavelength for single wavelength detection can easily be found, or wavelength changes can be programmed to occur at different points in the chromatogram, either to provide maximum sensitivity for peaks, or to edit out unwanted peaks, or both. The instrument can provide a contour plot, showing the relationship between absorbance, wavelength and time. This can often be used for the detection and identification of otherwise unsuspected impurities in the sample.

Figure 24, 25 show the spectra of enalapril in *trans* and *cis* form together with a chromatogram and contour plot. The lines on the contour plot join points of equal absorbance.

Since UV spectra are generally broad and featureless, it is sometimes difficult to decide if the spectra overlay properly or not, especially if the components concerned have similar spectra or if the concentration of one of them is small.

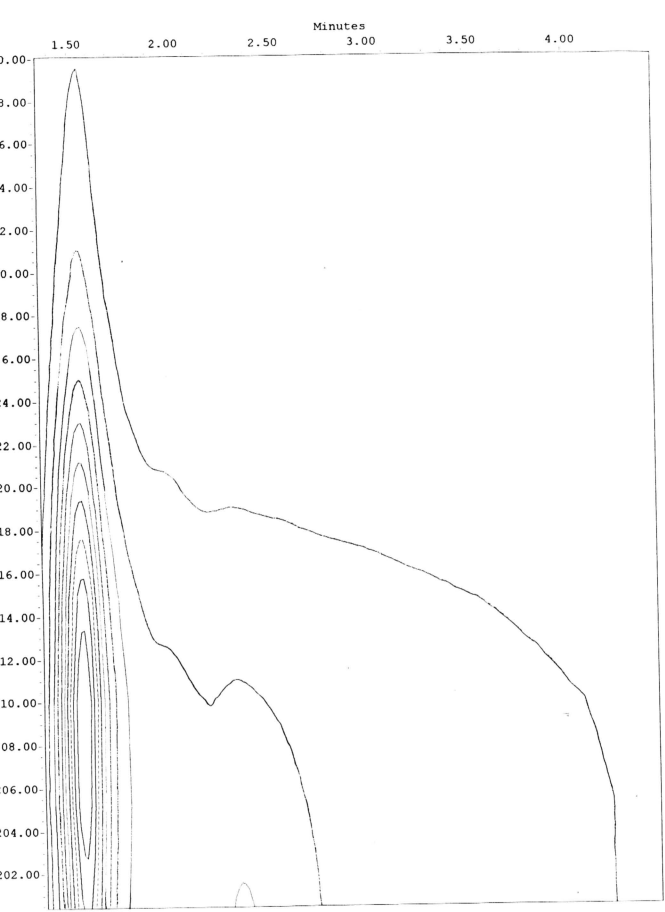


Fig. 24 HPLC – PDA contour plot of *cis – trans* enalapril isomer

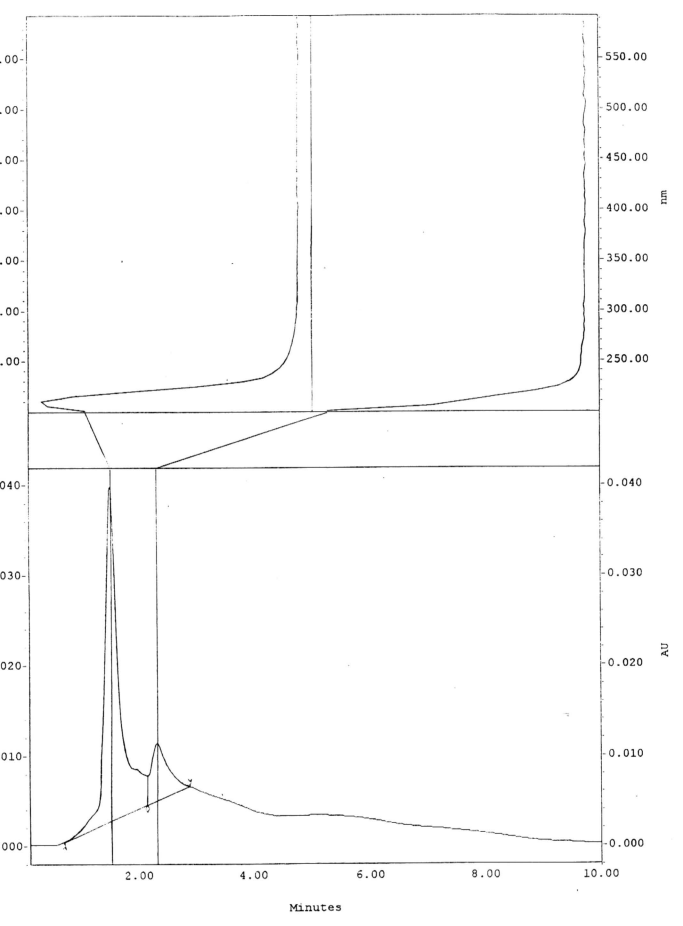


Fig. 25 HPLC – PDA spectrum index plot of *cis – trans* enalapril isomer

Choice of mobile phase and flow rate

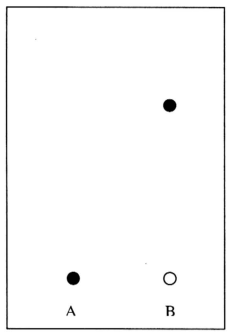
A mixture of the HPLC grade acetonitrile and glass distilled water (deionized water) was used as the mobile phase. To achieve a proper balance between analyses time and the separation of mixtures, it is important to match the polarity of the samples, with that of the stationary phase and to select an appropriate mobile phase of suitable polarity.

The resolution obtained with acetonitrile/water 70 : 30 is quite adequate, a symmetry and well defined peak shape can be obtained. (Figure 23 in p.55)

The flow rate of the mobile phase also plays a part in obtaining a good analysis.

4.2 TLC

From the figure below, pure enalapril retained at the original spot while methylated enalapril was removed from the original spot. This showed that enalapril has been derivatized by diazomethane in ether. This procedure is important as the preliminary thin – layer studies provide valuable clues for successful gas chromatography.



A : enalapril maleate

B : enalapril maleate derivatize with diazomethane

4.3 Gas Chromatography

4.3.1 General GC Performance

The retention time of the compounds is polarity dependent. The peaks eluted out according to the polarity respectively. The non–polar compound will be eluted first followed by the moderately polar compound as DB–1 and DB–5 is a non–polar packing materials.

The retention time of diazepam, enalapril maleate, enalaprilat are summarized in Table 6 and 7.

Table 6: The capillary GC retention time of diazepam, enalapril maleate, enalaprilat by using DB-1 column.

Drugs	Retention Time(min.)
Diazepam (Internal Standard)	4.94
Enalaprilat	5.56
Enalapril Maleate	5.75

Table 7: The capillary GC retention time of diazepam, enalapril maleate, enalaprilat by using DB-5 column.

Drugs	Retention Time
Diazepam (Internal Standard)	7.39
Enalaprilat	8.11
Enalapril Maleate	8.33

NOTE : Refer Figure 26

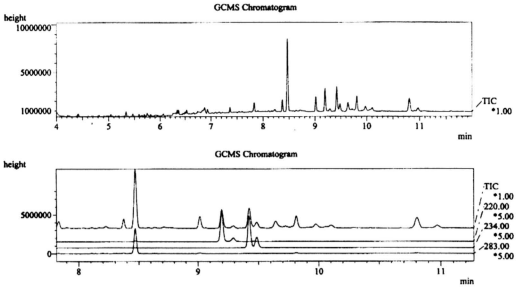


Fig 26. TIC GC-MS chromatogram of derivatised enalapril maleate, enalaprilat, diazepam.Separation done on DB – 5 column

4.3.2 Evaluation of the method

Recovery

Recovery work is essential in any analytical method. It is not only provides the degree of sensitivity and precision of a particular analytical method. The characteristic data obtained from the standardized analytical method, i.e. the recovery percentage can be used as a basis for the applicability of the analytical method on the compounds of interest. The percentage of recovery shows the method efficiency and this value must be taken into consideration during the calculation of quantitative concentration of the compounds. Besides, a recovery procedure further gives a quantitative estimate of the presence or absence of the interfering substance in a particular determination.

In this study, two sets of sample preparation were tried out: one with liquid – liquid extraction, another is solid phase extraction.

As seen in Table 8, 9, 10 from the percentage recovery, the extraction of enalapril using solid phase extraction gives the higher recovery.

Table 8: The capillary GC recovery of enalapril maleate by using liquid-liquid extraction

1ppm of Enalapril Maleate	1.12%

Note : Refer Figure 27 & Figure 28

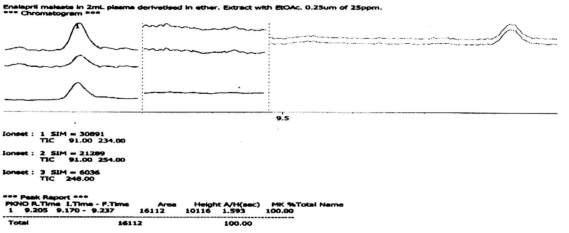


Fig. 27 GC-MS SIM chromatogram of extracted enalapril maleate from 1ml plasma by using liquid – liquid extraction after derivatisation.

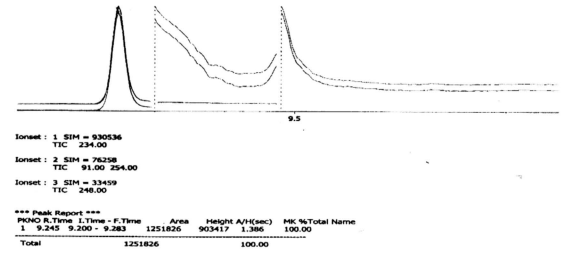


Fig 28. GC-MS SIM chromatogram of extracted enalapril maleate from 1ml plasma by using solid-phase extraction after derivatisation

Table 9: The capillary GC recovery of enalapril maleate by using solid - phase extraction analysed by using DB – 1 column

Concentration (ppm)	% of recovery		
	Diazepam	Enalaprilat	Enalapril Maleate
1.00000	74.25	70.98	70.70
0.50000	77.00	156.00	110.50
0.25000	71.88	50.76	57.33
0.12500	51.30	113.50	99.50
0.06250	43.35	87.46	60.99
0.03125	41.95	153.70	113.00

Table 10: The capillary GC recovery of enalapril maleate by using solid - phase extraction analysed by using DB – 5 column

Concentration (ppm)	% of recovery		
	Diazepam	Enalaprilat	Enalapril Maleate
1.00000	66.80	77.18	108.90
0.50000	54.97	147.00	118.00
0.25000	64.90	67.78	86.99
0.12500	28.00	91.38	72.18
0.06250	46.07	79.60	69.12
0.03125	134.50	279.00	261.00

Recovery of the sample preparation

Biological samples may contain proteins, salts and a host of organic compounds which may react with the diazomethane. Thus, traditional methods of sample preparation such as liquid – liquid extraction where enalapril was methylated with diazomethane before extraction will reduce the chances of the enalapril in the biological samples react with diazomethane. Thus, giving the result in incomplete sample recovery and also time consuming. When using the solid phase column extraction e.g. Sep –Pak C 18 cartridges, the extraction or purification of enalapril from the biological samples is done before the derivatisation by using diazomethane.

Derivatisation

The analyses of enalapril maleate is preliminary performed by HPLC because such substances are not suitable for analyses by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) without prior derivatisation. The carboxylic acids of enalapril can be converted into methylated derivatives which are more stable and volatile and therefore far more amenable to GC–MS analysis. Analytical results obtained by HPLC are frequently required to be confirmed by the GC –MS analysis of a methylated derivative[24]. By careful choice of derivatisation procedure, enalapril maleate can be converted to a more GC stable and volatile compound. Decreased polarity and increased volatility reduce adsorption on the stationary phase and result in more symmetric peaks and lower limits of detectability. Formation of derivative that is less polar than the drug itself allows the column to be operated at a lower temperature, thus prolonging its life, and reducing the possibility of thermal decomposition of the drug[25]. Derivatisation of enalapril maleate can be done by react with diazomethane. Although this procedure is simple and gives high product yields, however diazomethane is toxic and explosive and therefore should be handled with care. Enalapril derivatised with diazomethane in ether gives two symmetry cis and trans isomers. This gives difficulties in quantitative work. This can be shown in Figure 29.

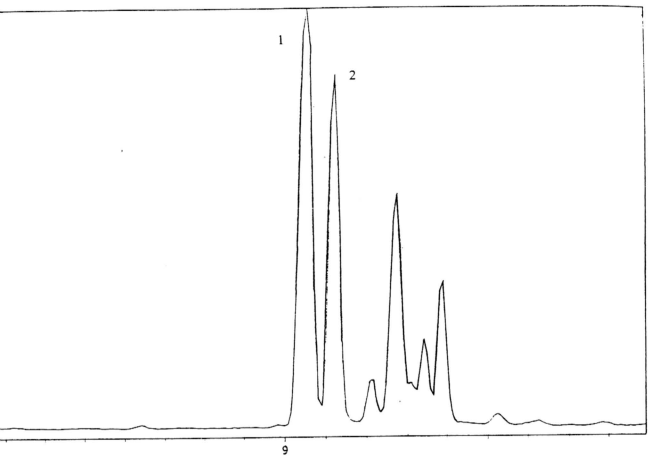


Fig. 29 Peak 1 and 2 are two symmetry *cis* and *trans* enalaprilate isomers

Calibration Curves

Calibration curves were constructed by adding known amounts of enalapril maleate, enalaprilat and internal standard (diazepam) to control plasma which was then analyzed. The peak height ratio of enalapril maleate and enalaprilat to the internal standard were plotted against the amount of enalapril maleate or the enalaprilat added. In order to calibrate the method and determine accuracy for each batch of unknown samples, standards containing 6.25, 12.5, 25, 50, 100, 200ng of enalapril maleate and the enalaprilat were added to 1mL of control samples, which were then assayed concurrently with unknown samples.

Table 11: Intensities of Enalaprilat and Enalapril Maleate in 1mL of blank plasma extracted with SPE analysed using capillary GC, DB – 1 column

Concentration (ppm)	Enalaprilat	Enalapril Maleate
1.00000	0.2580	0.3990
0.50000	0.1695	0.1960
0.25000	0.0645	0.0569
0.12500	0.0371	0.0487
0.06250	0.0169	0.0194
0.03125	0.0114	0.0128

NOTE : Refer Figure 30

Table 12: Intensities of Enalaprilat and Enalapril Maleate in 1ml of blank plasma extracted with SPE analysed using capillary GC, DB – 5 column

Concentration (ppm)	Enalaprilat	Enalapril Maleate
1.00000	0.2340	0.3303
0.50000	0.1595	0.1760
0.25000	0.0668	0.0617
0.12500	0.0400	0.0457
0.06250	0.0170	0.0188
0.03125	0.0123	0.0165

NOTE : Refer Figure 31

The calibration curves obtained are shown as in Figure 32, 33, 34, 35.

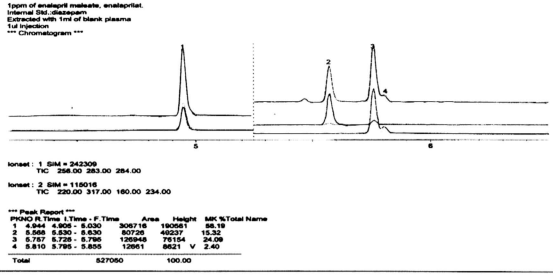
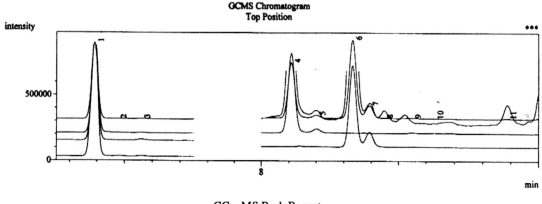


Fig 30 GC-MS SIM chromatogram of the extract (diazepam, enalapril maleate, enalaprilat)from 1ml plasma after derivatisation. Separation done on DB – 1 column

1ppm of enalapril maleate, enalaprilat
Internal standard : diazepam
Extracted with 1mL of blank plasma
1µL injection



GC – MS Peak Report

Peak	Retention Time	Area	A/H	%	Total
1	7.391	959091	618636	1.55	57.58
4	8.110	251766	144845	1.73	15.12
6	8.332	355136	104394	1.73	21.32

Fig 31 GC-MS SIM chromatogram of the extract (diazepam, enalapril maleate, enalaprilat) from 1ml plasma after derivatisation. Separation done on DB – 5 column

Figure 32 Calibration Curve of extracted Enalaprilat in 1ml plasma after derivatisation. Separation done on DB – 1 column

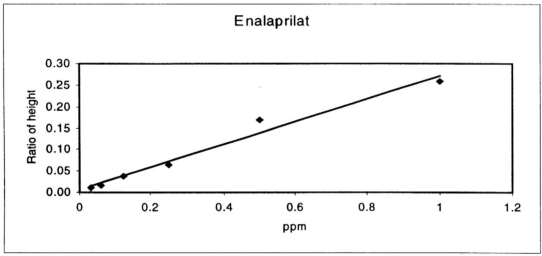


Figure 33 Calibration Curve of extracted enalapril maleate from 1ml plasma after derivatisation. Separation done on DB – 1 column

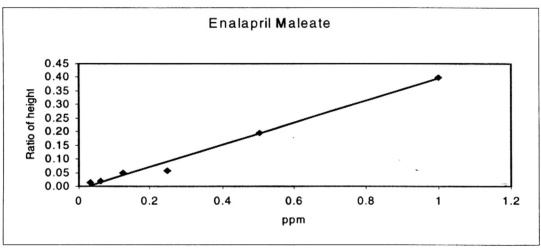


Figure 34 Calibration Curve of extracted enalaprilat from 1ml plasma after derivatisation. Separation done on DB – 5 column

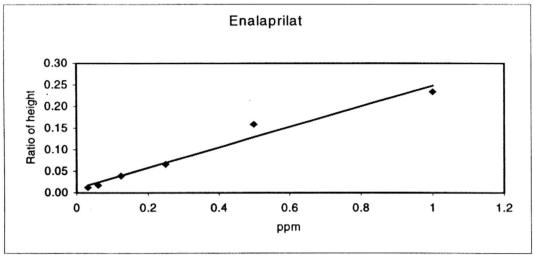
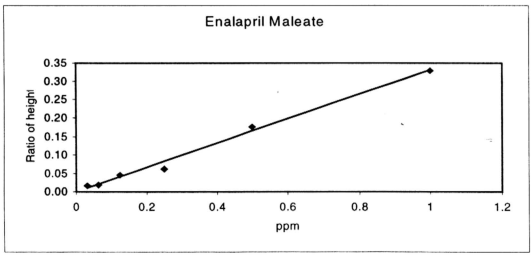


Figure 35 Calibration Curve of extracted enalapril maleate from 1ml plasma after derivatisation. Separation done on DB – 5 column.



Method Interference

Both HPLC and GC are very sensitive analytical instruments in pharmaceutical analysis. Thus, contaminants from solvent impurities, cleanliness of the centrifuge tube and through sample treatment may lead to discrete artifacts or elevated baseline problem in the chromatogram. Precautions have to be taken to ensure that the analysis is free from any interference.

The centrifuge tube were presoaked overnight in DECON and then soaked in distilled water. They were dried in an oven. The centrifuge tube were stored in a cleaned well – covered container prior to being used to prevent any accumulation of dust or other contaminants from the air.

In this analysis, HPLC grade methanol and ethyl acetate, distilled water and A.R.grade petroleum ether were used to minimize the contamination.

GC – MS determination

The mass spectra of the derivatives of enalapril maleate and enalaprilat are shown in Figure 36, Figure 37. The negative ion mass spectra have base peaks at 234 and 220, which are the fragment ion of the derivatized enalapril maleate and enalaprilat. The 234 and 220 ion were chosen for monitoring in the SIM mode.

The standard curve of enalapril maleate(EM) and enalaprilat(EN) by using DB – 5 column showed good linearity (EM : $y = 0.2375x + 0.0103$, $r^2 = 0.9694$; EN : $y = 0.3321x - 0.0008$, $r^2 = 0.992$) over the concentration of 6.25 – 200 ng/mL for 1mL plasma volume.

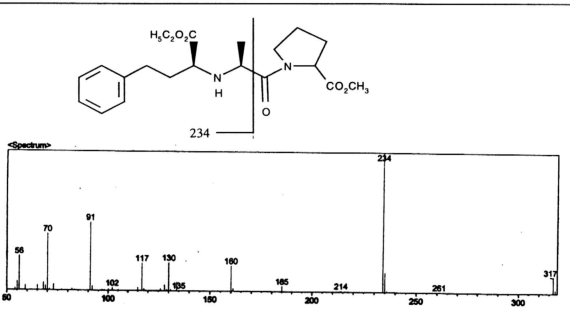


Fig 36. Mass Spectra of the enalapril methyl ester.

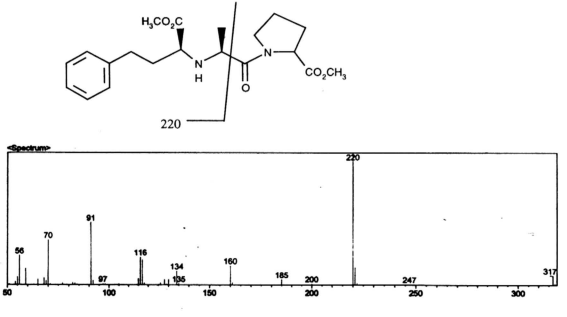


Fig 37. Mass Spectra of the enalaprilat dimethyl ester.