HPLC STUDIES OF NATURAL PRODUCTS

A thesis

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by

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DECLARATION

This is to certify that the work incorporated in the thesis entitled "HPLC studies of Natural Products." submitted by **Mr. Kiran B. Sonawane** was carried out by him at National Chemical Laboratory under my supervision for the M. Sc. (PPPR) Degree in Chemistry of the university of Pune. Such material as has been obtained from other sources has been obtained from other source has been duly acknowledged in the thesis

B. A. Nagasampagi

Research Guide

Dedicated to my parents

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Abbreviations

HPLC ----- High Performance liquid chromatography

GC ------ Gas Chromatography

TLC ------ Thin Layer Chromatography

RP ------- Reverse phase

ABSTRACT

The thesis entitled **"HPLC STUDIES OF NATURAL PRODUCTS"** is divided into the following two chapters

Chapter I : Estimation of Camptothecin and its derivatives occuring in the plant *Nothapodytes foetida* by HPLC method

The first chapter deals with the introduction on general methods of HPLC analysis and the estimation of varius naturally occuring anticancer compounds.The new method developed by us for the estimation of the anticancer drug camptothecin, 9-methoxy camptothecin and 20-acetyl camptothecin occuring in the plant *Nothapodytes foetida* by HPLC method involves isocratic binary mixture of solvents using single wavelength UV detector and diode array detector. Analysis by UV detector has been found to give the accurate estimation of the above drugs quantitatively, whereas the diode array detector has provided the right choice of selection of the wavelength as well as peak homogenity. The developed methodology will be discussed in the detail in this chapter.

Chapter II : Standardisation of semisynthetic Camptothecin derivatives by HPLC method

This chapter deals with the introductory remarks regarding the difficulties normally encountered for the water insoluble anticancer compounds and the reported attempts for

derivatising the anticancer drugs to make them water soluble. HPLC method has proved to be very useful for checking the purity of intermediates during derivatisation stages / semisynthesis.

Standardisation of intermediate derivatives of camptothecin leading to the final commercial anticancer drug irrinotecan has been attempted . Since camptothecin is not water soluble, suitable derivatives have been reported in literature which are water soluble and hence more effective as anticancer drugs. The process development of camptothecin derivative involves ethylation, N-oxidation and rearrangement of camptothecin. The monitoring of these reactions is dificult due to poor solubility of these compounds in normal solvents. HPLC analytical method has been developed by us for monitoring the reaction process and purity of intermediates. Details of the development studies will be discussed in this chapter.

Chapter I

Estimation of Camptothecin and its derivatives occuring in the plant *Nothapodytes foetida* by HPLC method

Introduction I: High Performance Liquid Chromatoraphy

The word Chromatography in "High Performance Liquid Chromatography" indicates the greek meaning 'Chroma' means colour and 'Graphein' means to write, was introduced by Russian botanist Tswett[1] in 1903. He separated plant pigments by adsorption chromatography. In 1941 Martin and Synge were subsequently awarded the Nobel Prize for the discovery of liquid-liquid partition chromatography[2]. Noble prize awarded paper laid down the foundation of gas chromatography and HPLC. After that in 1952 James and Martin described first use of GLC[3]. Between 1967-1969 Kirkland [4] Hubber [5] and Preiss and Lipsky [6] described the first HPLC chromatographs, by operating at pressure up to 5000 psi and gave comparable analytical time with GLC. HPLC in general (fig. 1.1) consists of following parts

a) Solvent delivery system or pumps.

b) Injector valve to introduce the sample.

c) A column packed with micro particles.

d) Detector to detect eluting components.

e) Integrator to record chromatogram.

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Fig 1.1

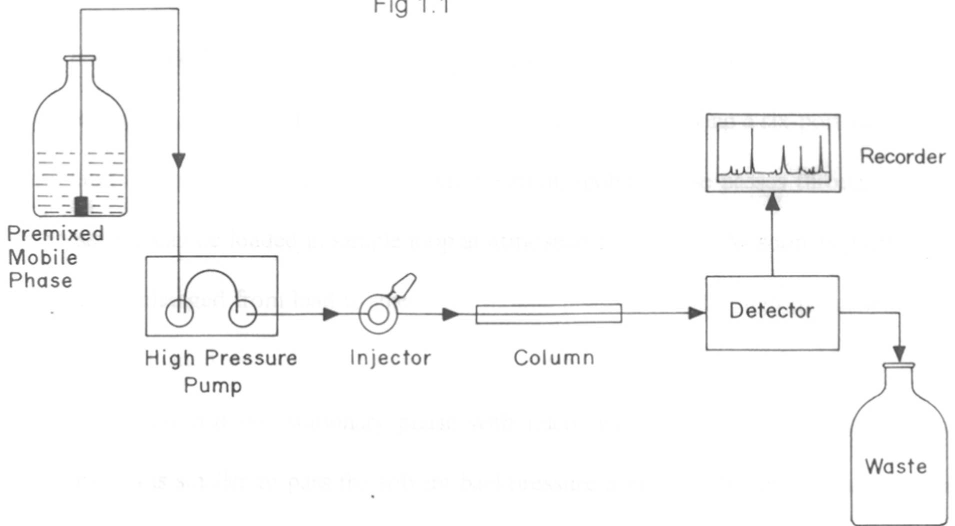


Diagram of isocratic HPLC system

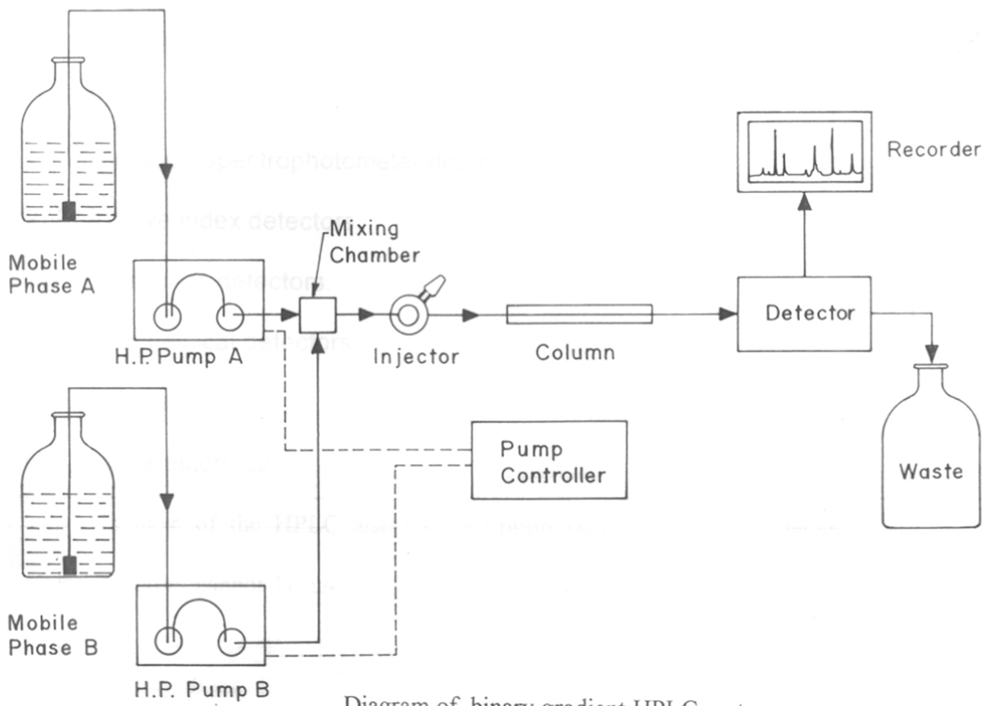


Diagram of binary gradient HPLC system

For analytical purpose generally solvent delivery system consists of pump with the flow capacity of 0. 1 ml per minute to 10 ml per minute. Injector having a six-port valve with bypass loop is used. When injector is in load position, mobile phase passes through bypass loop so sample can be loaded in sample loop at atmospheric pressure. As soon as position of the injector is changed from load to inject, the mobile phase passes through the sample loop and sample is loaded on column. Columns used are as per the nature of substrate to be analysed. Column contains stationary phase with micro particles, as space between these micro particles is smaller to pass the solvent backpressure is created. In general particle size is 5 to 10 μm. Detector output is given to integrator, which gives the information about sample components. Nowadays computer based recorders are suitable by which we can store and process data received from detector and record the chromatogram at any time as per our requirements. Various types of detectors are used for detection of eluting components.

1) Absorbance Spectrophotometer detectors

2) Refractive index detectors.

3) Fluorescence detectors.

4) Electrochemical detectors.

5) Mass detectors.

6) Chiral detectors etc.

In most of the HPLC analysis absorption spectrophotometric detectors are used. These detectors cannot be used only if sample component has very little or no absorbance

and when analyte concentration is too low for detection. UV detectors have generally deuterium light source and can detect absorption from 190nm to 400nm. If visible range (400 nm to 700 nm) is required then tungsten lamp is used. Good analytical results will be obtained only if selected wavelength is proper for detection. It requires knowledge of UV spectra of individual sample component. For this purpose pure sample is dissolved in the solvent and spectra is obtained individually for different compounds. Four types of absorption detectors can be distinguished as,

a. Fixed wavelength detectors.

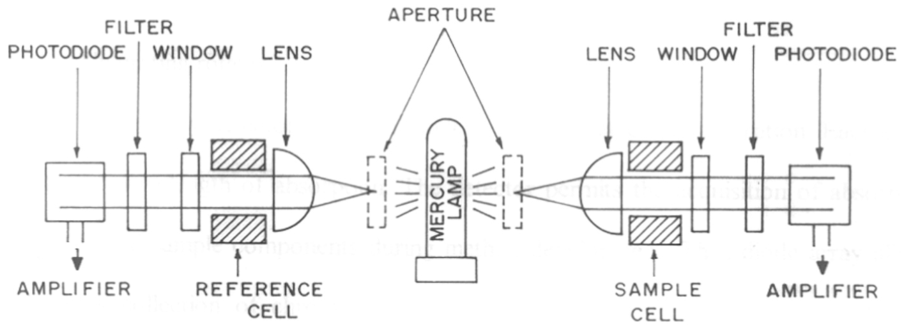
b. Variable wavelength detectors.

c. Rapid scan detectors.

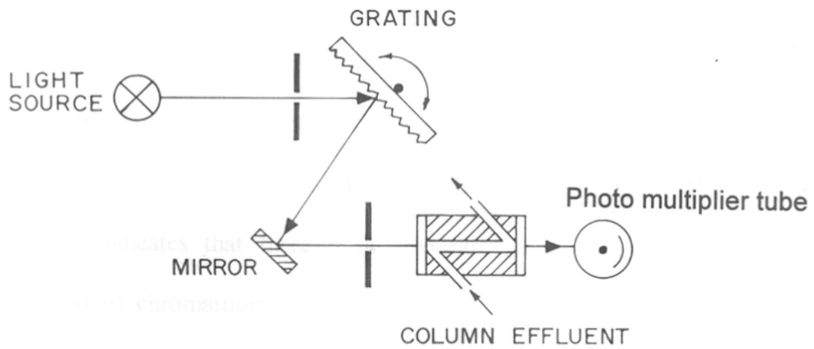
d. Diode array detectors.

Fixed wavelength detectors can operate at single wavelength only. Generally at 254nm or 280nm these detectors are less expensive detectors and possess low noise and high sensitivity compared to other detectors. In this type of detector mercury lamp is used as a light source (fig 1.2)

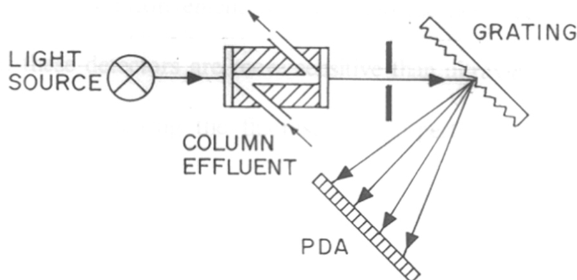
The variable wavelength detectors provide 200nm to 800nm. In this type of detector deuterium lamp or xenon, or tungston lamp is used as light source and grating is used to select the wavelength. In rapid scan detectors while compound is eluting (peak) the flow is diverted immediately before detector to waste as the peak remains in the detector flow cell. Rapid scanning of sample is started so that we can get the spectrum of that peak. In this



Simplified diagram of fixed wavelength detector



Simplified diagram of fixed wavelength detector



Simplified diagram of Photodiode array Detector

detector only grating rotates.

In diode array detectors array of diode is used to detect the absorption. Each diode has different wavelength of absorption. The detector permits the acquisition of absorption spectrum for all sample components during method development. Thus diode array allows simultaneous collection of chromatogram at different wavelengths during a single run. Chromatogram at any described wavelength can be displayed provided data is collected for that particular wavelength. This way detector provides more information on sample composition than the provided by single wavelength detector of each sample component. It provides as an important tool for selecting an optimum wavelength for the final HPLC method. If a single component is present in the peak the UV spectrum obtained across the peak should be superimposable. Peak purity is best evaluated by comparing the UV spectrum at the beginning middle and end of the band. If the spectrum at three places is superimposable, it indicates that there is no impurity overlap with peak. This can be confirmed by ratio of chromatograms of two different wavelengths. If eluting peak has impurity it shows destortion. Furthermore collecting peak manually and collecting more information by means of mass, NMR, IR, etc. can confirm purity. Because different compounds may have same UV spectrum. The refractive index detector is very sensitive for temperature so it is inconvenient to use. Fluorescence detectors are based on analyte fluorescence, these detectors are more sensitive than ultraviolet detectors. But limitation is that only the sample having the fluorescence property can be detected. Electrochemical

detectors are used mainly for ion chromatography. Mass detector cannot be used for non-volatile buffers. Chiral detectors can be used only for samples with optically active compounds.

There are five chromatographic modes in HPLC

1. Partition chromatography.
2. Liquid- solid adsorption chromatography.
3. Ion pair chromatography.
4. Ion exchange chromatography.
5. Size exclusion chromatography.

1. Partition chromatography:

In this mode stationary phase is chemically bonded silanol groups with organosilane or alkoxysilane (e.g. C_6,C_{18}). These stationary phases have Si-O-Si-R linkage which confer hydroxy stability. This partition chromatography can be carried out in normal phase or reverse phase mode.

2. Liquid- solid adsorption chromatography:

In this mode sample dissolved in solvent molecules are reversibly bonded to surface of solid by dipole–dipole interaction. Since strength of interaction is different for different compounds, residence time at the stationary phase varies, so that separation is achieved and used often for non-ionic organic compounds.

3. Ion pair chromatography:

This is alternative to ion exchange chromatography. An ionic organic compound, which forms an ion pair with sample component of opposite charge, is added in mobile phase. This is nothing but a salt, which behaves like a non ionic molecule that can be separated by reverse phase chromatography.

4. Ion exchange chromatography:

In this case stationary phase is either with acidic or basic functional groups bonded to polymer matrix. Charged species in mobile phase are attracted towards appropriate functional groups on the ion exchanger and so get separated.

5. Size exclusion chromatography:

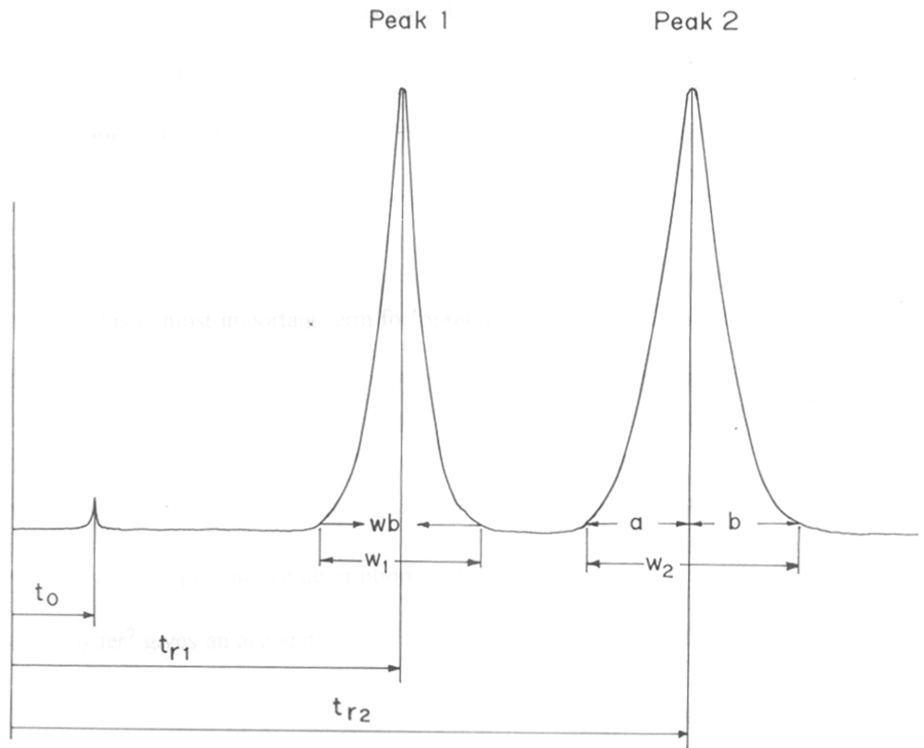
In this case stationary phase is a porous solid. Sample molecules are small enough to enter pore structure are retarded, while larger molecules are excluded and so get separated.

Chromatography theory and terms in HPLC

Peak asymmetry (As): As = b/a

Peak asymmetry can be used as criteria of column performance. With a well-packed column an asymmetry factor is between 0.9 and 1.1 with standard solutes. A maximum of 1.3 is reasonable for large-scale commercial column .Please see fig 1.3.

Fig 1.3



Capacity factor (K):

K is a measure of time spent in the stationary phase relative to the time spent in mobile phase by sample component and is given by

$$K = (tr_1 - t_o)/t_o$$

Where tr_1 is elution time of component one. t_o is elution time for unretained component. Value of K less than unity is unfavorable due to interference from non-retained peak.

Selectivity factor (α):

This is most important term for optimization of resolution useful ranges from 1.05 to 2.00. Higher values indicates waste of analytical time and is given by

$$\alpha = K_2 / K_1$$

Where K_1 is the capacity factor for peak 1 and K_2 is the capacity factor for peak 2.

Resolution (Rs): It is quantitative description of the degree of separation obtained between two peaks. Snyder[7] gives an account of how much resolution is needed for quantitative and qualitative analysis

$$Rs = \frac{t_2 - t_1}{0.5(W_1 + W_2)}$$

Where t_1 and t_2 are retention time of peak 1 and 2 respectively in minutes W_1 and W_2 are peak width of peak 1 and 2 respectively in minutes.

Efficiency (N) :It is number of theoretical plates

$$N = 16 \frac{Tr^2}{W_b}$$

Where Tr is uncorrected retention time and W_b is the peak width at the base line measured in units of time.

HETP (Height equivalent to theoretical plates):

$$H = L / N$$

Where L is length of column and N is the number of plates or efficiency. It measures the efficiency per unit length of the column.

Separation: Isocratic and gradient[8].

Isocratic separation uses the same mobile phase composition throughout the analysis, whereas in gradient elution the composition of mobile phase changes during the run. Generally the concentration of strong eluent is linearly increased. Some of the important reasons for preferring isocratic elution are

1) Gradient equipment is not available in some laboratories.
2) Gradient elution is more complicated. During the run time gradient elution varies the composition of mobile phase in different steps. Sometimes gradient may be curved, linear or segmented.

3) Gradient elution cannot be used with some detectors eg. refractive index detector.

4) Gradient elution takes more time because of column equilibration after each run for next injection.

5) Method dose not always transfer well because difference in equipment can cause change in separation.

6) Baseline problem is observed and hence highest purity solvent must be available.

Therefore gradient elution is preferred only when there is too high difference in retention times. During the new development of a method of analysis gradient elution is the best starting point, even if the final isocratic method may be possible.

Method development:

During the development of method it is necessary to see the nature of the sample and sample related information like their structure, possible impurities, number of compounds present in sample mixture, molecular weight of compounds, UV spectral characteristics, concentration range of compounds of interest in sample and its solubility. Based on these detectors and mobile phase can be selected in such a way that it is suitable to sense the all the sample components of inertest. Variable wavelength UV detector normally is the first choice. We have to see also that the sample is regular or exceptional. Exceptional sample requires different types of column and customized conditions. For inorganic compounds ion chromatograph is required. Organic isomers can be separated by normal phase HPLC or cyclodextrin column. For enantiomeric separation chiral columns are used. For

macromolecules it is necessary to have size exclusion column. These all samples come under exceptional category.

Reverse phase chromatography: In this type, stationary phase is nonpolar and mobile phase is polar. Polar compounds will elute faster than non polar compounds. Stationary phase is chemically modified silica, and mobile phase is water along with water missible organic solvents like methanol, acetonitrile etc.

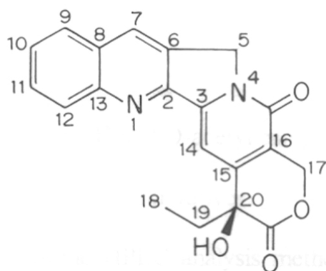
Normal phase chromatography: In this stationary phase is polar and mobile phase is non polar. Polar compounds are retained for more time. Organic solvents are used for eluting compounds

Many of organic compounds are not sufficiently volatile or are thermally unstable and hence cannot be analysed by GC without prior modification, these can be analysed directly by HPLC at room temperature. These molecules include drugs, drug intermediates, pharmaceuticals, polymers, explosives, food products etc.

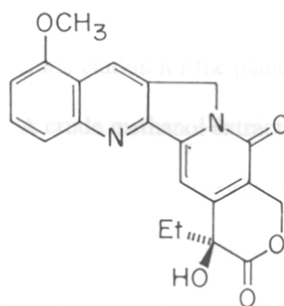
Introduction II: Present work on Estimation of Camptothecin and its derivatives.

Camptothecin **I** (s)- 4-ethyl-4-hydroxy-1-H-pyrano [3,4:6,7] indolizino [1,2-b] quinoline-3,14 (4H,12H)-dione is a pentacyclic indole antitumor alkaloid. It was first isolated from the Chinese medicinal plant *Camptotheca acccuminata* (family Nyssaceae) in 1966 by M. C. Wani and coworkers [9]. Later it was isolated from the plant *Nothopodytes foetida* previously known as *Mappia foetida* (family Icacinaceae) by Govindachari *et. al.* in 1972 in the yield ranging from 0.01% (leaves) to 0.1% (stem bark) from different parts of the plant along with 9-methoxy camptothecin **II**[10,11]. *Nothopodytes foetida* is both cultivated and found wild in Western ghats of Maharashtra. Recently O-acetylcamptothecin **III** was isolated by Tian-Shung,Wu[12] .In 1972 at National institute of health **I** was included in human trials as anticancer drug. Due to its poor water solubility it was evaluated as the water soluble sodium carboxylate of camptothecin in. initial trials [11]. Unfavorably it was found to be ineffective in patients with advanced melanoma or gastrointestinal cancer. Moreover severe toxicities were observed [13]. This terminated early human trials of camptothecin carboxylate **IV**. Later on it was found that **I** inhibits action of topoisomerase I on DNA unwinding [14]. And was found that biological activity of **I** both in vivo and in vitro is significantly greater for the lactone than for the carboxylate form.

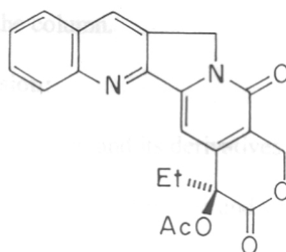
As India is one of the producer and exporter of camptothecin our aim was to develop a simple HPLC method to estimate the constituents such as, camptothecin **I**,



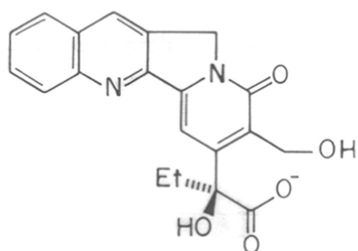
I



II



III



IV

9- methoxy camptothecin **II** and O-acetyl camptothecin **III**, so as to commercially produce I, for its conversion to soluble derivatives.

Some HPLC analysis methods have been reported, Majority of them involve analysis with fluorophotometric detectors for biological fluids[15-17] or gradient elution for natural source[18]. We wish to report a here a new quantitative method for the estimation of **I**, **II** and **III** by isocratic elution for the plant *Nothopodytes foetida*.

A crude methanol extract was prepared from *Nothopodytes foetida* hart wood. This was diluted within the linearity range and analysed for quantitaion .The best analytical result was found using C18 column at 1ml flow rate. The mobile phase used was 40:1:2:57 acetonitrile : methanol : dichloromethane : buffer pH=3. It was also found that the wavelength 355 gives the good analytical results. For sample loading on the column the results were within linear range 10ng to 300ng for **I** and **II** and 1ng to 100ng for **III** for sample loading on the column.

Result and discussion:

Camptothecin and its derivatives have been reported to give very broad peaks with isocratic elution using acetonitrile and water. Further it has been reported that analysis using trifluoroacetic acid could not be considered due to excessive retention times[16]. Experiments carried out by us using reverse phase adsorbent C18 and C8 showed that, 40% acetonitrile in buffer pH= 3 provided optimum peak shape and separation of camptothecin derivative standards. However problem of increased backpressure occurred after multiple

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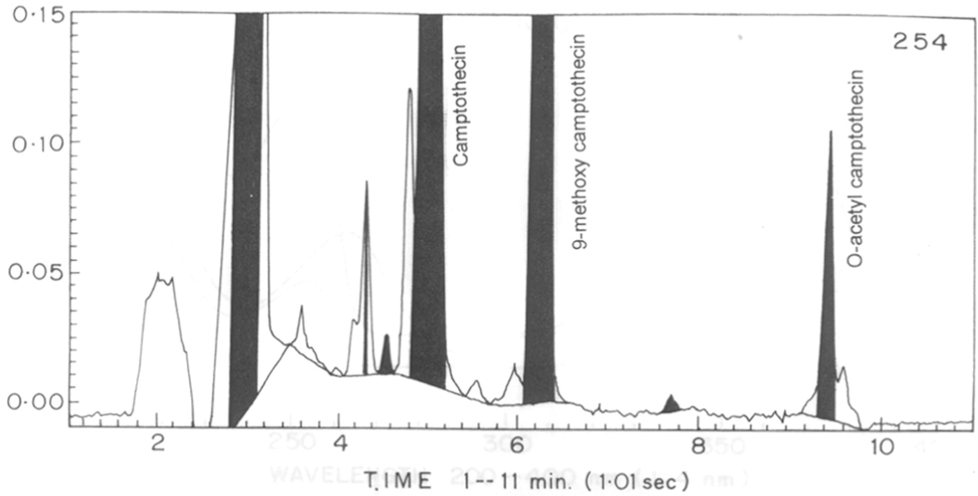
injections, probably due to the precipitation of camptothecin on the column. It is already known that the standards are insoluble in water but slightly soluble in acetonitrile and completely soluble in dichloromethane. Therefore addition of dichloromethane to the mobile phase eliminated the backpressure problem. Throughout the analysis studies the proportion of dichloromethane and methanol were kept constant at 1% and 2% respectively. Both the columns C18 and C8 provided near baseline separation of all the three camptothecin standards. It was decided to analyse the extract for quantitative estimation with isocratic conditions with both columns. Quantitative estimation for **II** and **I** on C18 column as well as for C8 was well matching for the crude extract. The O-acetyl camptothecin peak in C18 column (fig 1.4A) analysis chromatogram was also found to be relatively sharp compared to that observed in analysis chromatogram of C8 column (fig 1.4B). That means the area under the peaks corresponding to **I** and **II** were equal when analysed on both the columns. However the area under the peak corresponding to O-acetylcamptothecin **III** measured more when the crude extract was analysed on C8 column, while the same peak measured less area on the C18 column due to resolution of the impurity peaks on one side of the peak due to **III** as can be seen in Fig1.4A Thus it was clear that the peak corresponding to **III** resolved well on C18 column indicating that minor compound co-eluted on column C8. Further conformation was sought by coinjecting the standards along with the extract. Further during analysis on C18 column PDA generated UV spectrum for peak assigned to O-acetylcamptothecin in crude extract is superimposable with that of pure compound, but during the

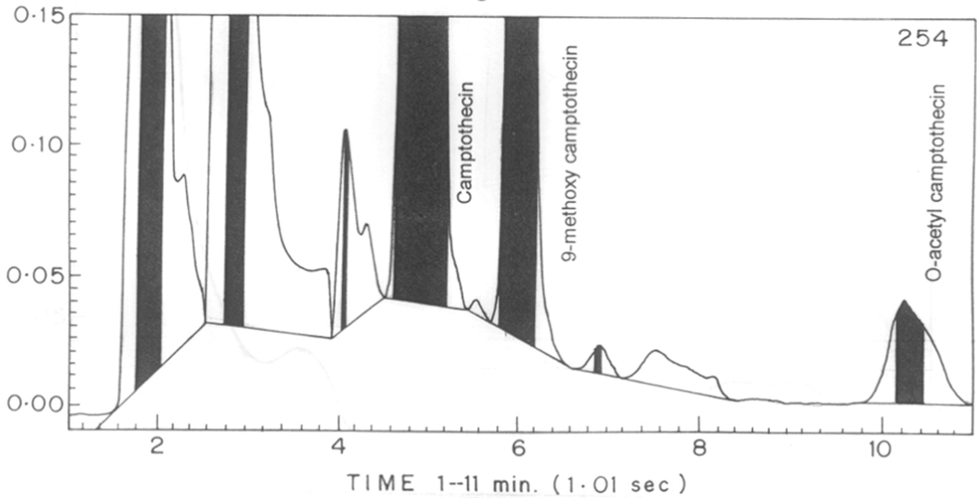
Fig 1.4 A



Peak purity chromatogram for crude extract

sample A at 254 nm using Photo diode array detector on bonded **C18** column

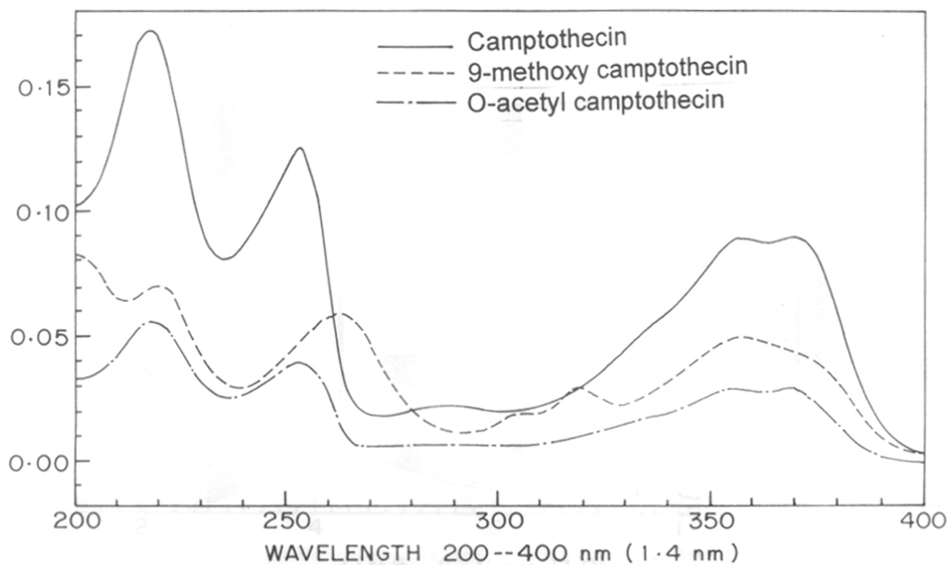
Fig 1.4 B



Peak purity chromatogram for crude

extract sample A at 254 nm using Photo diode array detector on bonded **C8** column

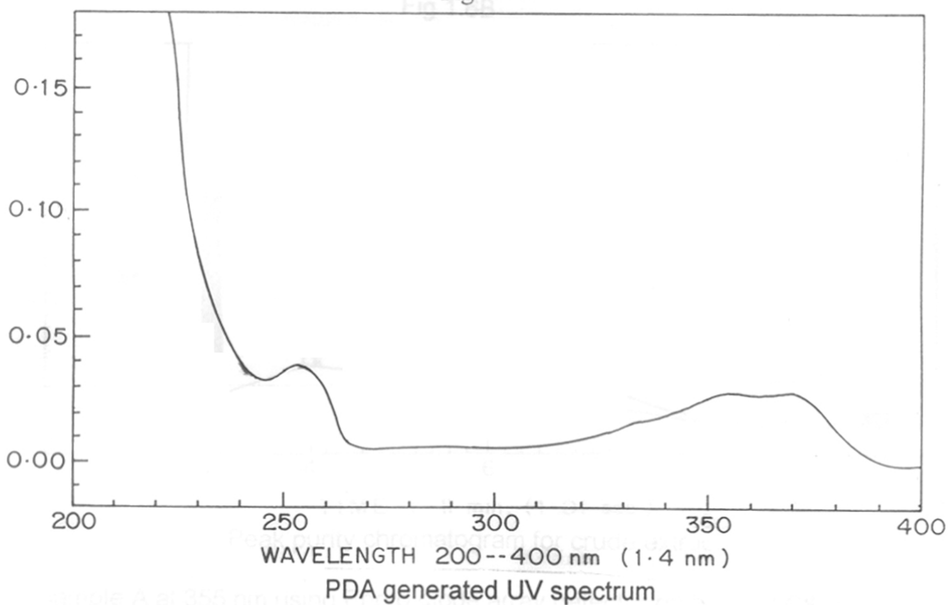
Fig 1.5 A



PDA generated UV spectrum for

peak assigned to respective standards of camptothecin on C8 column

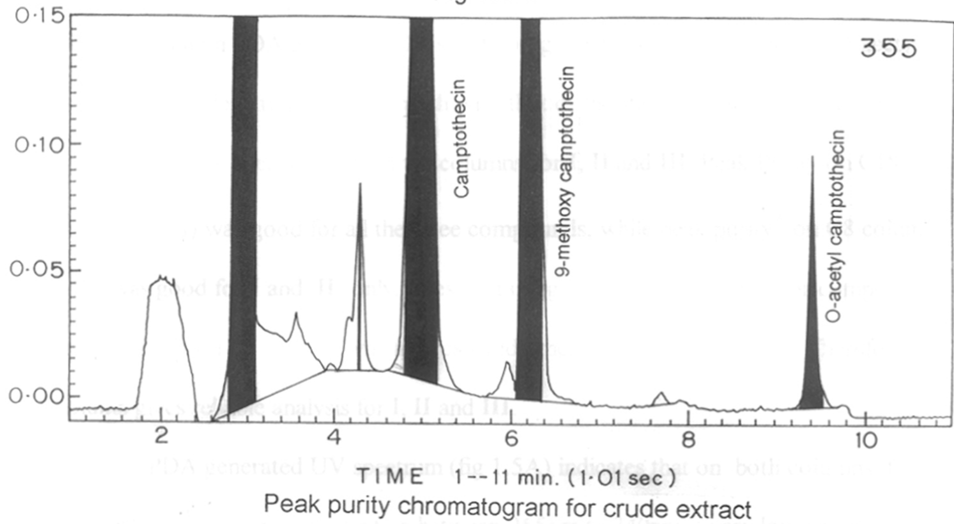
Fig 1.5 B



PDA generated UV spectrum

for peak assigned to O-acetyl camptothecin in crude extract on C8 column

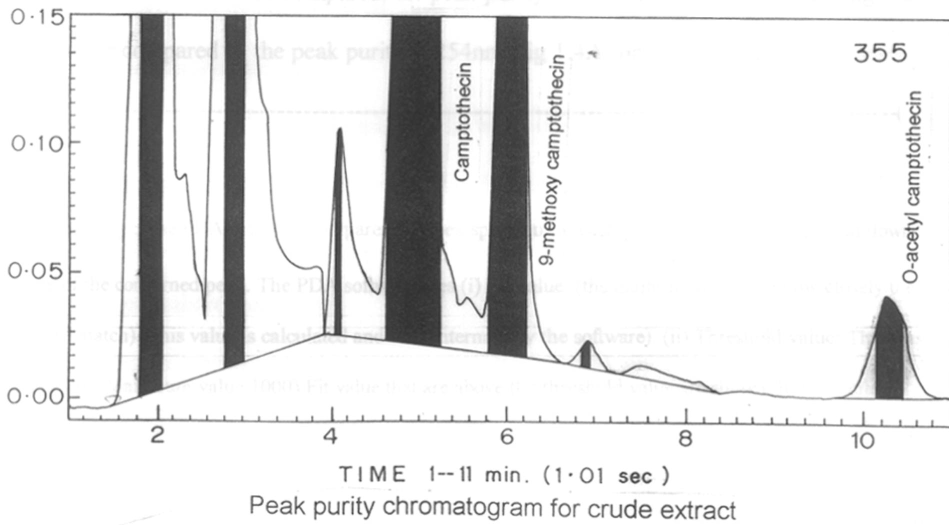
Fig 1.6 A



Peak purity chromatogram for crude extract

sample A at 355 nm using Photo diode array detector on bonded **C18** column

Fig 1.6B



Peak purity chromatogram for crude extract

sample A at 355 nm using Photo diode array detector on bonded **C8** column

analysis of C8 column PDA generated UV spectra (fig 1.5B) for peak assigned to O-acetyl camptothecin of crude extract did not match with that of the standard. In addition to this when peak purity was checked on both the columns for **I, II** and **III**. Peak Purity on C18 column (Fig 1. 4A) was good for all the three compounds, while peak purity* on C8 column (fig 1.4B) was good for **I** and **II** only. These data suggest that unknown minor compound is co eluting with **III** on C8 column which is resolved when C18 column is used. Therefore C18 column gives reliable analysis for **I, II** and **III**.

PDA generated UV spectrum (fig 1.5A) indicates that on both columns for peak assignment of standards absorption between 265nm to 340nm is very low and hence this UV range cannot be used for the analytical purpose. Spectrum also indicates that either 254nm or 355nm can be used for the detection. Maxima observed at 220 nm could not be considered due to the possible interference of other impuriries. Moreover when the peak purity of **I, II** and **III** was compared, the peak purity on chromatogram at 355nm (fig 1.6 A) was better compared to the peak purity at 254nm (fig 1.4A) on C18 column.

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* Peak Purity : The PDA software compares the apex spectrum to each point along the upslope and down slope of the concerned peak. The PDA software uses (i) Fit value (the major to determine how closely the spectra match). This value is calculated and used internaly by the software). (ii) Threshold value: This was set at 980 (Maximum value 1000).Fit value that are above the threshold value are shown in black cilour.

Peak purity at 355 is shown for C8 column (fig 1.6B) which is better than 254 (fig 1.6B). These data indicate clearly that the analysis at 355nm is ideal for analysis for all the three components in crude extracts.

Experimental:

The chromatography work was carried out on a Shimadzu, Kyoto Japan model of HPLC which consists of two solvent delivery pumps model LC-8A (duel head reciprocating) controlled by a model SCL-8A system controller, a 7725i Rheodyne injector with fixed loop volume of 20 μl and SPD-10AV model UV visible absorbance detector. Analogue output was given to CR-7A CHROMATOPAC integrator. 991 Photodiode array detector was from Millipore Watters Chromatography Division, Milford MA. U.S.A. equipped with computer and NEC 5200 printer. Column used was Merck Lichrosphere 60 RP- select B {Bonded C8} (5um) 250mm X 4mm and Lichrosphere 100 (5um) RP- C18e 250mm X 4mm with manucart. At the flow rate 1 ml per minute of mobile phase.

Chemicals and solvents:

HPLC grade acetonitrile was from Qualigence, a division of Glaxo India Ltd. A milli Q system (Millipore Corporation, Bedford, MA. U.S.A.) was used to purify water. Ortho-phosphoric acid and triethylamine analytical grade from s.d. fine chemicals and Aldrich Chemicals respectively.

Camptothecin **I**, 9-methoxy camptothecin **II** and O-acetyl camptothecin **III** were prepared in house and each compound was considered as standard. The structures were

confirmed by NMR, mass, and IR data and showed a single peak in HPLC. These standards were dried at 100^0C prior to use.

Mobile phase was prepared by mixing (40:1: 2: 57) acetonitrile : dichloromethane: methanol : buffer pH = 3. Stock buffer was prepared by addition of 140ml of triethylamine in 100ml of water and pH was adjusted to 3.0 by addition of ortho-phosphoric acid and then its volume was made to 500 ml. 1ml of this stock solution was used to make 100 ml buffer solution.

Standard Preparation:

1 mg of each standard was accurately weighed and dissolved in about 10 ml of dichloromethane and then made up to mark to 100ml with mobile phase.

Sample preparation: -

50 grams of branches and twigs (shade dried) of *Nothapodytes foetida* collected from Satara district was and powered and powder was soaked with 150 ml of 5% dichloromethane in methanol overnight (about 12 hrs.) in 500ml conical flask with a standard joint. Next day this was filtered through a filter paper in 500 ml volumetric flask and the remaining powder was refluxed with 150 ml X 2 (5% dichloromethane in methanol) for 20 minutes keeping the condenser to conical flask. It was cooled and filtered through the filter paper. All the filtrates were combined in the 500 ml flask. The volume of flask was made to mark using methanol and made homogeneous by shaking and was labeled as sample A. 5 ml of A was diluted to 100 ml with mobile phase and labeled as B.

10 μl of each sample was injected into the chromatograph. All the times, all samples were filtered through 0.45 μm pore filter before injections and quantitative results were reported as average of two injections per sample.

Columns were equilibrated with 40:1:2:57 acetonitrile : dichloromethane : methanol : buffer pH=3 at a flow rate of 1ml per min.

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CHAPTER: II

Standardisation of semi-synthetic Camptothecin derivatives

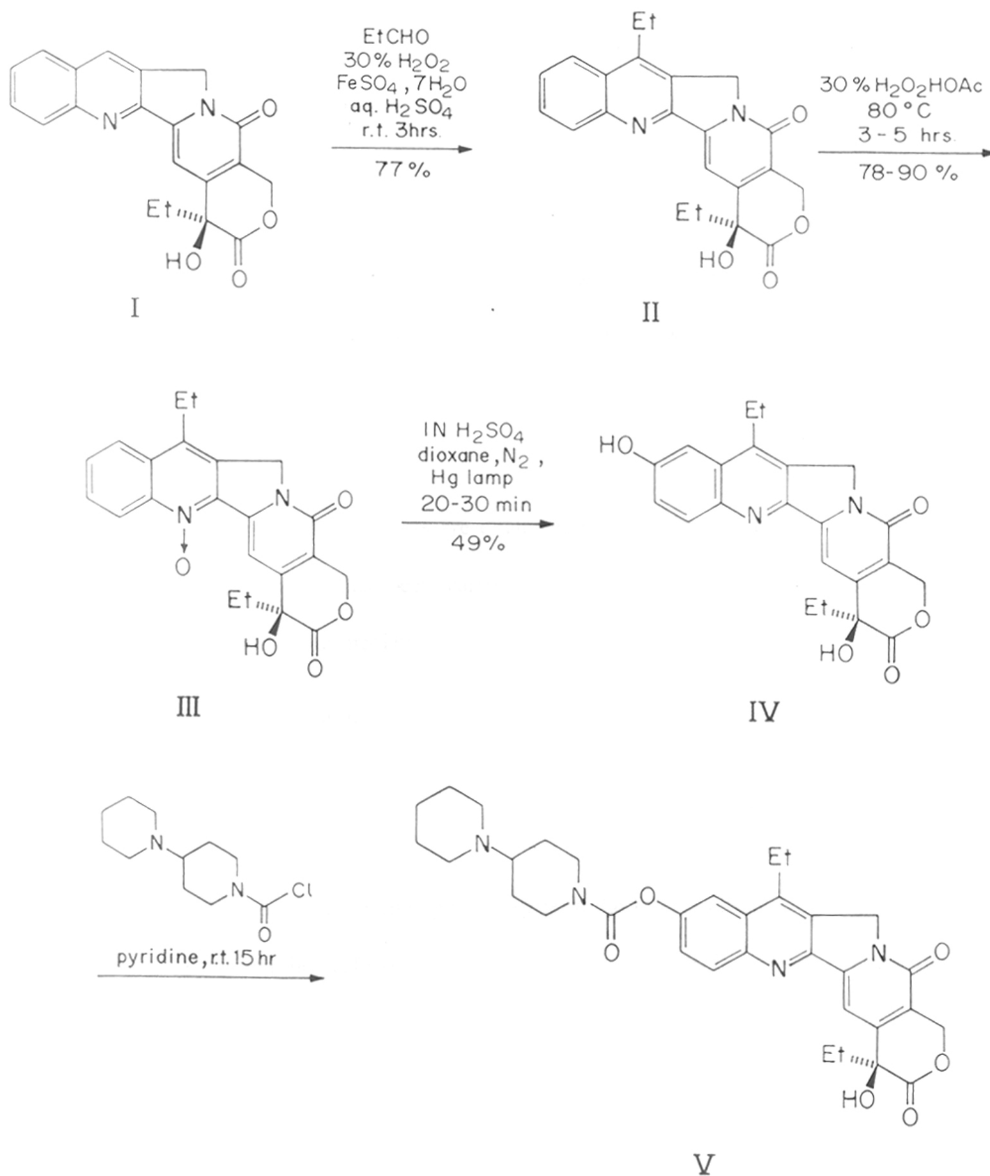
By HPLC method

Introduction I: Irinotecan

As mentioned in first chapter camptothecin **I** as an anticancer agent could not prove its efficacy due to its poor solubility in water. Structure activity relationship studies directed towards increasing activity and water solubility of camptothecin resulted in many derivatives of camptothecin. One of these derivative named as irinotecan **V** has been in the international market for the last six years. In order to make it available at cheaper rate as starting material, camptothecin is available easily from the plants cultivated in our country. A programme was initiated in our laboratory to synthesise it from camptothecin. The methodology is shown in scheme 1.

Camptothecin on treatment with propionaldehyde and 30% H_2O_2 in presence of aqueous sulfuric acid at room temperature for three hours resulted in 7-ethylcamptothecin **II** in 77%. 7-ethyl N-oxidecamptothecin **III** was prepared by oxidation of **II** using 30% H_2O_2 in acetic acid at 40 ^{0}C for 3.5 hour in the yield of 78-90 %. Introduction of hydroxyl group at C-10 position in N oxide **III** was achieved by treating **III** in 1N H2SO4 in dioxane under nitrogen atmosphere by using mercury lamp for 20-30 minutes. However the yield was only 49%. When

Scheme 1



7-ethyl-10-hydroxycamptothecin was treated with 4 -piperidino-1-piperidinecarbonyl chloride in pyridine irrinotecan was obtained.

Due to the complex structure of camptothecin and nature of medium used for carrying out reactions such as aques sulfuric acid the monitoring progress of reaction was difficult by conventional chromatographic method as TLC. We took this opportunity to develop the simultaneous HPLC method for monitoring the progress of reaction and purity of intermediates 7-ethylcamptothecin, 7-ethyl N-oxidecamptothecin, 10-hydroxy 7-ethylcamptothecin with final product irinotecan. We wish to report here the development of such a method for the quantitative estimation of the intermediates.

Although some reports are documented regarding the HPLC analysis[1-10] of irinotecan no work has been carried out towards the simultaneous quantitative estimation of the intermediates.

Results and discussion:

Most commonly used acetonitrile : water, as a mobile phase did not give proper peak shape for Irrinotecan. Similar behavior was observed when methanol : water was used as the mobile phase. For both the mobile phases the irinotecan peak was tailing and the peaks of 7- ethyl N –oxide and 10- hydroxy 7-ethylcamptothecin were merging in the irinotecan peak.

As it was reported that the irinotecan is unstable in alkaline and neutral conditions[3], trials were carried out with varying the composition of methanol acetonitrile and buffer at pH =3 using two different columns with stationary phase bonded C18 and bonded C8. When the percentage of organic phase was increased, it was observed that the compound, 7-ethyl N-oxidecamtothecin and 10-hydroxy-N-oxide were merging with each other. No resolution was observed. By increasing the percentage of methanol, the resolution value increased between these two but resulted in increased retention of 7-ethylcamptothecin (about 45 minutes). Same trials were carried out on both the columns C18 and C8. Finally it was found that the C18 column gave reliable analysis time with good resolution.

During our studies it was found that no degradation was observed up to 72 hrs, when sample was kept in freeze as a solution in mobile phase and protected from light. HPLC properties of semisynthetic irinotecan with intermediates are shown in table on page no 31 on bonded C18 column and chromatogram on page 32.

HPLC PROPERTIES OF SEMISYNTHETIC IRINOTECAN WITH INTERMEDIATES

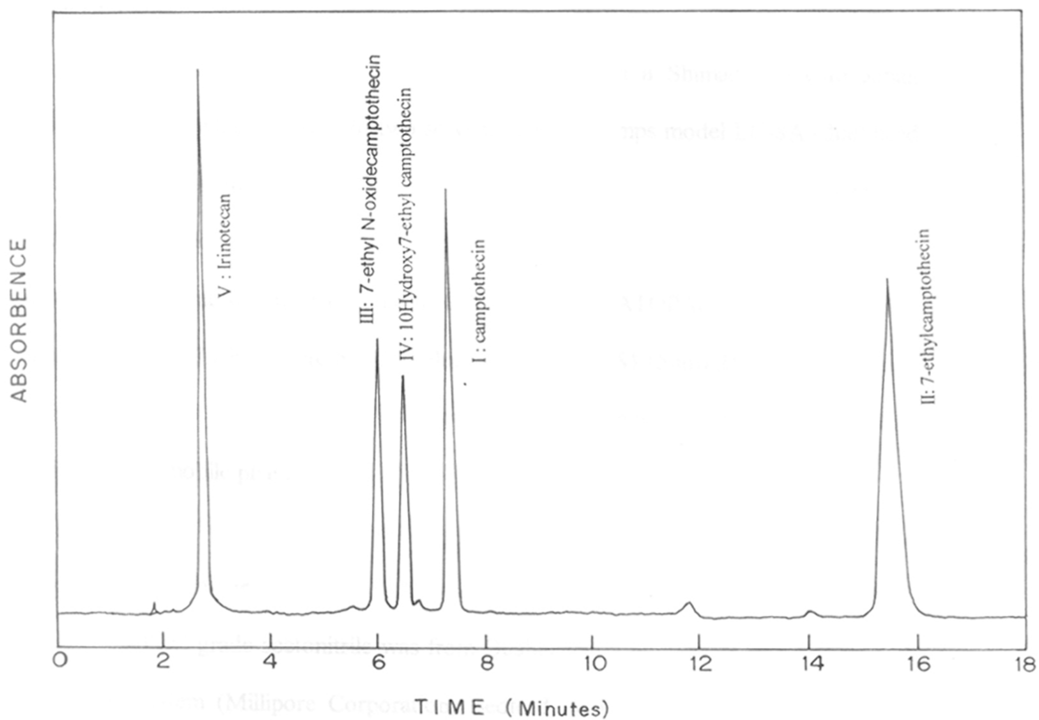
Mobile phase: (30:10: 2: 58) acetonitrile: dichloromethane: methanol: buffer pH = 3.

Flow: 1ml per minutes Wavelength: 254 nm ; Void volume (t_0) = 1.8ml

Column: Lichrosphere 100 (5um) RP- C_{18e} 250mm X 4mm with manucart

Compound	**Retention time**	**Capacity factor**	**Selectivity factor**
Camptothecin	7.32	3.06	2.49
7-ethyl camptothecin	15.52	7.62	3.31
7-ethyl N-oxide camptothecin	5.99	2.30	1.1
10- hydroxy 7-ethyl camptothecin	6.48	2.60	4.9
Irinotecan	2.75	0.53	

For all the standards the quantitative analysis of the derivatives was found between linear ranges for loading on column was 200- 400picogram. This was not optimized further.



Artificial Mixture of camptothecin standerds on C18 Column

I : camptothecin II: 7-ethylcamptothecin III: 7-ethyl N0oxidecamptothecin

IV: 10Hydroxy7-ethyl camptothecin V: Irrinotecan

Analytical conditions:

Column: Lichrosphere 100 (5um) RP- $C_{18}e$ 250mm X 4mm with manucart

Mobile Phase: 30:10:2:58 acetonitrile:methanol:dichcloromethane:buffer pH=3

Flow: 1ml per minute Pressure: 110 Kg per cm^2 Wavelength: 254nm

Experimental:

The chromatography work was carried out on a Shimadzu, Kyoto Japan model of HPLC which consists of two solvent delivery pumps model LC-8A (duel head reciprocating) controlled by a model SCL-8A system controller, a 7725i Rheodyne injector with fixed loop volume of 20 μl and SPD-10AV model UV visible absorbance detector. Analogue output was given to CR-7A CHROMATOPAC integrator. Column used was Merck Lichrosphere 60 RP- select B {Bonded C8} (5um) 250mm X 4mm and Lichrosphere 100 (5um) RP- $C_{18}e$ 250mm X 4mm with manucart. At the flow rate 1 ml per minute of mobile phase.

Chemicals and solvents:

HPLC grade acetonitrile was from Qualigence, a division of Glaxo India Ltd. A milli Q system (Millipore Corporation, Bedford, MA. U.S.A.) was used to purify water. Ortho-phosphoric acid and triethylamine analytical grade from s.d. fine chemicals and Aldrich Chemicals respectively. Camptothecin **I**, 7-ethylcamptothecin **II**, 7-ethyl N-oxide camptothecin **III**, 10-hydroxy 7-ethylcamptothecin **IV** and irinotecan **V** were prepared in house and each compound was considered as standard. The structures were confirmed by NMR, mass, and IR data and showed a single peak in HPLC. These standards were dried at under vacuum at room temperature prior to use.

Mobile phase was prepared by mixing (30:10:2:58) acetonitrile: dichloromethane: methanol: buffer pH = 3. Stock buffer was prepared by addition of 140ml of triethylamine in 100ml of water and pH was adjusted to 3.0 by addition of ortho-phosphoric acid and then its volume was made to 500 ml. 1ml of this stock solution was used to make 100 ml buffer solution.

Standard Preparation: Standards were weighed about 10 mg For Camptothecin and 7-ethylcamptothecin dissolved in 10 ml of dichloromethane. 1ml of this solution was diluted to 50 ml with mobile phase For standards III, IV and V about 10 mg weighed and then volume was made to 10 ml with mobile phase 1ml from this solution was diluted to 50 ml with mobile phase.

Sample preparation: Reaction mixtures equivalent to 1 mg of respective standards were diluted to 50ml with mobile phase and directly injected on chromatograph.

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