HPLC ANALYSIS OF DIGOXIN AND DIGITOXIN: DEVELOPMENT OF METHODS FOR DOSAGE FORM ASSAY AND SEPARATION OF POTENTIAL IMPURITIES AND METABOLITES

by

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ABSTRACT

The objective of this investigation was to develop quantitative isocratic HPLC methods for the analysis of digoxin and digitoxin. An HPLC system that employs a reverse-phase column, UV detection at 220 nm and solvent systems consisting of various proportions of water, methanol, isopropanol and dichlormethane was developed for the separation of digoxin, digitoxin and their potential degradation products and metabolites. HPLC separations of the above compounds by isocratic, solvent switchover and gradient elution modes were carried out in chromatographic times of 27, 16 and 13 minutes, respectively.

For purposes of monitoring the separation of dihydro metabolites of digoxin, a 100% fluid recovery system was developed for use in the HPLC analysis of digoxin and its metabolites after fluorogenic post-column derivatization using the air-segmentation process.

As an evidence of selectivity, the isocratic HPLC systems were utilized for the separation of a mixture of ten closely related steroids and the isolation of digitoxin from Digitalis purpurea leaf.

The isocratic HPLC systems were found to be applicable for the quantitative analysis of digoxin and digitoxin in their respective dosage forms. The HPLC assay of digoxin and digitoxin dosage forms was carried out in less than forty-five minutes. These methods were found to be precise, accurate, sensitive enough for single tablet assay, and capable of simultaneously monitoring the potential degradation products or metabolites of digoxin and digitoxin.

A comparison of the assay of digoxin and digitoxin dosage forms

by HPLC and USP methods indicated that: (a) the precision and accuracy of both methods were comparable and within acceptable limits; (2) analysis by HPLC can be completed in less than forty-five minutes whereas the USP methods require over four hours; and (3) the HPLC methods have the advantages of higher sensitivity, selectivity and simplicity over the USP methods.

The HPLC methods were used for the stability study of digoxin and digitoxin in their respective dosage forms. Lanoxin^{R} and digitoxin tablets were found to be stable under all the conditions of storage used in this study. Natigoxin^{R} tablets, Lanoxin injection and elixir were found to be subject to varying degrees and patterns of degradation. On the basis of the stability results it was observed that the assortment of pathways that may be involved at different conditions and times of storage would make it difficult to estimate digoxin shelf-life from data obtained by accelerated aging.

From the results of this investigation, it was concluded that the isocratic HPLC methods were suitable for the assay of digoxin and digitoxin dosage forms as well as for purposes of stability testing and simultaneous monitoring of degradation products or metabolites.

This abstract represents the true contents of the thesis submitted.

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ABBREVIATIONS

a.u.	-	absorbance units
a.u.f.s.	-	absorbance units full scale
°C	-	degrees centigrade
cm	-	centimeter
ECD	-	electron capture detector
eV	-	electron volt
Fig.	-	figure
g	-	gram
GLC	-	gas-liquid chromatography
HFB	-	heptafluorobutyrate
HPLC	-	high-performance liquid chromatography
i.d.	-	internal diameter
IR	-	infrared
LC	-	liquid chromatography
m	-	meter
M	-	molar
mcg	-	microgram
min.	-	minute
ml.	-	milliliter
mm	-	millimeter
MS	-	mass spectrometer
ng	-	nanogram
nm	-	nanometer
ODS	-	octadecylsilane

Psi	-	pounds per square inch (= 0.07 atmosphere)
RIA	-	radioimmunoassay
$T_{\frac{1}{2}}$	-	biological half-life
TLC	-	thin-layer chromatography
μ	-	micron (= 10^{-4} cm)
μm	-	micrometer
USP	-	United States Pharmacopeia
UV	-	ultraviolet

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DEDICATED

TO

THE PHILOSOPHY OF ETHICS

IN THE AFFAIRS OF MAN

INTRODUCTION

Ever since the publication in 1785 of William Withering's treatise entitled, "An Account of the Foxglove and some of its Medical Uses" (Withering (1937)), there has been a gradual increase in the use of digitalis glycosides. Digoxin and digitoxin are cardiac glycosides obtained in purified form from the leaves of Digitalis lanata and Digitalis purpurea, respectively and are commonly used in the treatment of congestive heart failure. These drugs belong to the Cardenolide C and A series, respectively, and are members of a large class of closely related compounds collectively known as the digitalis glycosides. A national prescription survey in the United States has been quoted (Doherty and Kane (1975)) to have found that digoxin, digitoxin and digitalis leaf were, respectively, fourth, sixteenth and nineteenth among the most frequently prescribed drugs in the country in 1971. The situation might also be the same in Canada.

The extremely low unit dosages, narrow toxic to therapeutic dosage ratios along with intersubject variations of sensitivity require a high degree of content uniformity of digoxin and digitoxin dosage forms, especially in tablet formulations. The presence of pharmaceutical excipients and probable formation of degradation products introduce additional requirements of assay specificity. Hence, analysis of digoxin and digitoxin in their respective dosage forms calls for methods that are sensitive enough to monitor unit dose amounts of digoxin (0.125 mg per tablet) and digitoxin (0.1 mg per tablet) with sufficient selectivity to prevent any interference from other compounds. It would also be

advantageous if the methods could allow the simulatneous determination of possible degradation products.

Most of the earlier methods reported for the quantitative analysis of these drugs were either colorimetric of fluorometric. Many investigators have later used thin-layer, gas-liquid and high-performance liquid chromatography methods. The United States Pharmacopeial methods also employ colorimetric, fluorometric and chromatographic techniques. The colorimetric methods generally lack sensitivity. Moreover, they have no selectivity because the color forming derivatizing reagents react with the sugar moiety or the lactone ring, both of which are present in all cardenolides. Since the fluorometric methods are based on the reaction of the derivatizing agents with the steroid moiety of the glycoside molecule, they are non-specific with respect to other digitalis glycosides. Gas-chromatographic methods involve derivatization procedures that again render them non-specific.

The lack of precise and readily quantifiable parameters of therapeutic response of digoxin and digitoxin has made it necessary that individual titrations be done on each patient. This requires highly sensitive and specific methods of analysis that would be able to indicate concentration/therapeutic response correlations by monitoring serum concentrations of the drugs in the presence of their metabolites. A variety of analytical techniques, including a number of HPLC methods have been reported in the literature culminating in a method that employs a two-step procedure in which the cardiac glycoside is separated by HPLC and quantitated by radioimmunoassay (RIA). The literature indicates that this two-step procedure has been necessary because of the non-specificity of the RIA technique and the lack of a sensitive HPLC method for the

analysis of digoxin and digitoxin in biological fluids. Moreover, it would be necessary that the HPLC method should ensure non-interference from all possible metabolites.

In order to assure identity, safety, efficacy and monitor stability of digoxin and digitoxin, it is clear that there is a need for methods of analysis that are sufficiently sensitive to allow single tablet assay and selective enough to permit simultaneous analysis of degradation products. This investigation, therefore, will attempt to provide some HPLC data which may help to satisfy this need. Attempts will also be made to establish the relative merits of the HPLC methods in comparison with those of USP XX.

I. LITERATURE SURVEY

In a study of the evolution of the analytical methods of a drug, one would, of necessity, have to examine the nature and unique characteristics of both the technique and the drug so as to appropriately locate them in the overall picture of related facts. Analysis is a step-wise process that may be divided into: the preparatory process, the separatory process and the determination process. By far the highest proportion of the time of analysis is taken by the separatory step e.g. extraction, precipitation, filtration, centrifugation etc. The major techniques in the separation procedures have been extraction and chromatography.

Since its discovery by Tswett (1903), the development of chromatography has progressively facilitated the separation process. Many have contributed to this development. These include: L.S. Palmer (1922) who represents the link between Tswett and the next generation; Edgar Lederer (1972) who fifty years ago brought back the technique from oblivion (Kuhn et al., 1931); Laszlo Zechmeister (1937) who probably did the most to make classical column chromatography a simple tool, easily accessible to everybody; Erika Cremer (1950) who built the first gas chromatograph similar to our present day systems; and Martin and Synge (1941) who laid the theoretical foundations on which liquid-liquid partition chromatography is based. High-performance liquid chromatography (HPLC) is just one more recent refinement of the chromatographic technique.

1. High Performance Liquid Chromatography

Chromatography is a physical process of separation of the components of a mixture due to the differences in their equilibrium distribution between a mobile phase and a stationary phase. The various branches of chromatography are shown in the schematic diagram in Fig. 1. HPLC encompasses the techniques that are shown in the solid boxes. Hence in essence it is a refinement of what was formerly known as liquid or column chromatography. HPLC may now be defined as a liquid chromatographic technique in which the liquid mobile phase is pumped through a column of microparticulate stationary material, under high pressure, and the separated components are detected and recorded in the form of individual peaks.

(A) Theory

(a) Mechanism of Retention

The basis of HPLC separations can be due to any of the following mechanisms of retention:

(i) Partitioning. This is the distribution of the sample between a liquid mobile phase and a liquid stationary phase in which the later liquid is coated on solid support material. The retention of the sample by the liquid stationary phase is dependent on the partition coefficient of the sample. The distribution equilibrium is:

$$\text{mobile phase} \rightleftharpoons \text{stationary phase} \qquad \text{(Eq. 1)}$$

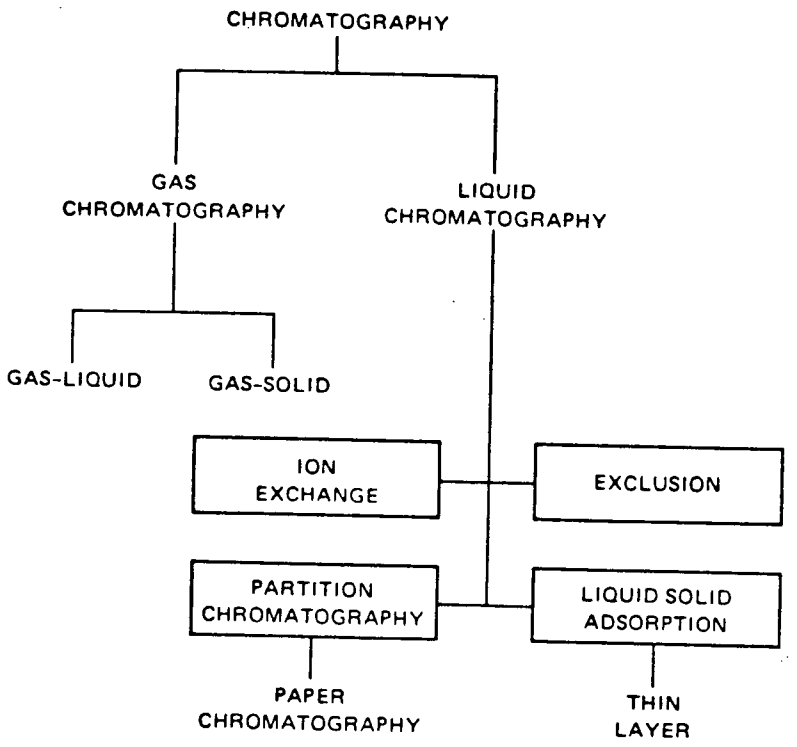


Fig. 1. Diagrammatic Representation of Branches of Chromatography (Farris (1976))

(ii) Adsorption. This is the retention of a sample by a solid stationary phase (un-coated) due to adsorption of the sample onto the solid support material. The distribution equilibrium of the sample is due to adsorption-desorption:

$$\underset{\text{(stationary phase)}}{\text{Adsorption}} \rightleftharpoons \underset{\text{(mobile phase)}}{\text{Desorption}} \qquad \text{(Eq. 2)}$$

(iii) Ion Exchange. This is the retention of a sample ion by an ion-exchanger resin (stationary phase) due to sorption of the sample ion onto the resin in exchange for the mobile ion. The distribution equilibrium is:

$$\underset{\text{(onto the Exchanger resin)}}{\text{Sorption}} \rightleftharpoons \underset{\text{(into the liquid mobile phase)}}{\text{Desorption}} \qquad \text{(Eq. 3)}$$

e.g. $RH^+ + \text{sample ion}^+ \rightleftharpoons \text{R-sample ion}^+ + H^+$

(cationic exchanger resin)

(iv) Gel permeation (Exclusion). This is the retention of a sample by a porous stationary phase due to the relative molecular size of the sample. Larger molecules excluded from all or a portion of the pores, by virtue of their physical size, elute from the column before the smaller molecules. The distribution equilibrium is:

$$\underset{\text{(into the pores of the stationary phase)}}{\text{Permeation}} \rightleftharpoons \underset{\text{(into the mobile phase)}}{\text{Diffusion}} \qquad \text{(Eq. 4)}$$

The equilibrium constant in Equations 1, 2, 3 and 4 may generally be expressed (Farris (1976)) as:

$$K = \frac{C_s}{C_m} \qquad \text{(Eq. 5)}$$

where, K = equilibrium constant

C_s = concentration of sample in stationary phase

C_m = concentration of sample in mobile phase

(b) Theoretical Plate

The path of a sample molecule through a chromatographic bed may be depicted as a number of minute jumps between the stationary and mobile phases. These jumps approximate a "drunkard's walk" as a mathematical model. It follows from statistical analysis that a Gaussian distribution (normal distribution) should be observed for a chromatographic band, and this is found experimentally to be true within limits. Using this analysis, according to Martin and Synge (1941), given an experimentally determined chromatographic band as shown in Fig. 2, the number of theoretical plates N, can be calculated from the retention volume R', and the band half-width, σ, as shown in Equation 6 and 7. The significance of

$$N = \left(\frac{R'}{\sigma}\right)^2 \qquad \text{(Eq. 6)}$$

$$\text{or,} \quad N = 16\left(\frac{R'}{w}\right)^2 \qquad \text{(Eq. 7)}$$

where R' = retention volume

σ = band half-width at 0.607 the height of the band (= standard deviation)

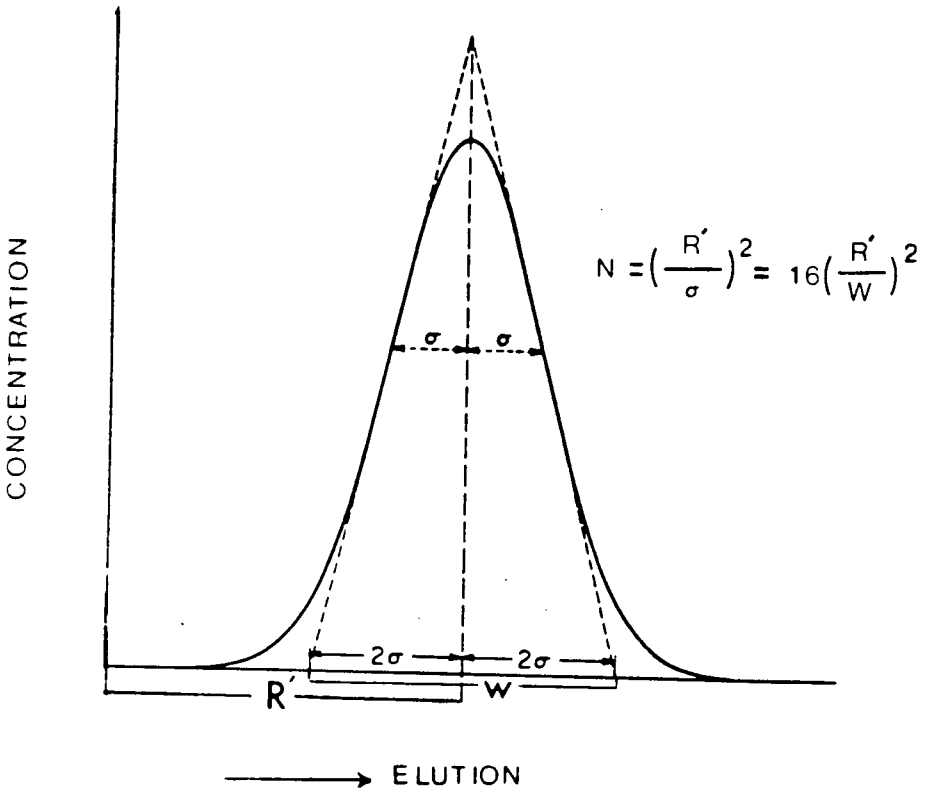


Fig. 2. An Idealized Elution Chromatogram of a Single Component. R' = retention time; w = baseline width of the band; σ = band half-width at 0.607 of peak height (Walton (1975))

N = number of theoretical plates

w = baseline width of the band ($w \simeq 4\sigma$)

the number of theoretical plates is that it is equal to the average number of equilibrations of the sample between the mobile and stationary phases. Hence the greater the number of plates (or cycles) the sharper the band. Factors that affect the number of theoretical plates include: type and size of adsorbant, nature and flow rate of solvent and dimensions of the column.

A term that is usually encountered in the literature relating column length (L) to number of theoretical plates (N) is known as height equivalent to a theoretical plate (HETP). This relationship is expressed as:

$$HETP = \frac{L}{N} \qquad \text{(Eq. 8)}$$

(c) Band widening

The Theoretical Plate model of Martin and Synge can be used for the explanation of the effects of band widening during sample migration. Statistical analysis predicts that a band, in moving along an even chromatographic bed, will double in width when its migration distance is quadrupled. The upper half of Fig. 3(a) shows the appearance of a band of substance A, after it has migrated 1, 2 and 4 units through a bed with corresponding band widths of 0.25, 0.35 (i.e. $0.25 \times \sqrt{2}$) and 0.5 (i.e. $0.25 \times \sqrt{4}$) units, respectively. In Fig. 3(b), one can see the result of chromatographing a mixture of the same substance A, with another substance B, where the latter moves 0.75 units when A has traveled the initial unit. At this point, the bands overlap badly, but on migrating

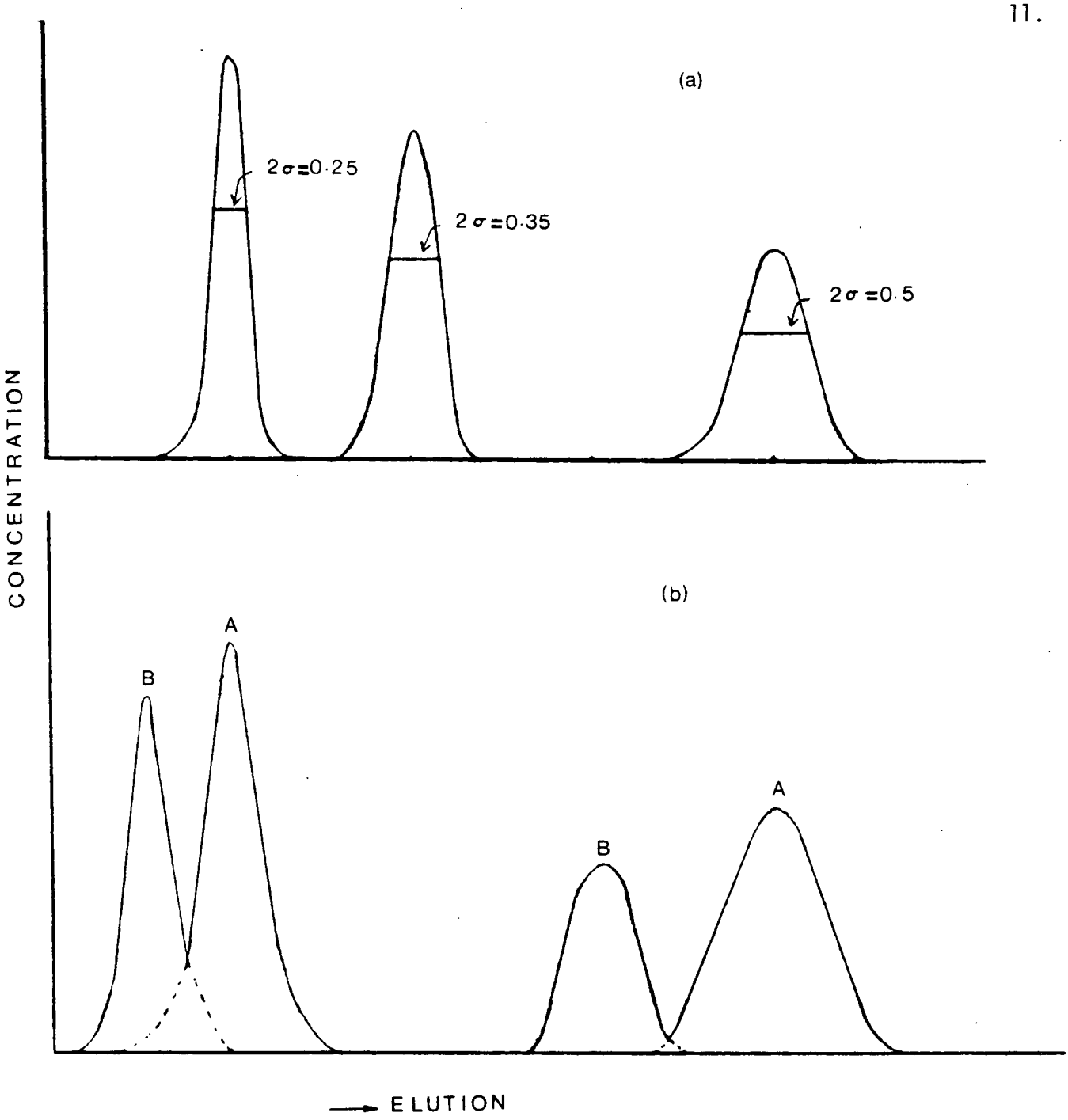


Fig. 3. Chromatogram showing: (a) Appearance of a Chromatographic band after migrating 1, 2 and 4 units from the origin in an even chromatographic bed; and (b) Separation of a mixture of substances (A) and (B). 2σ = band width at 0.607 of peak height.

four times the initial distance the bands corresponding to A and B are almost completely separated. This is because the band centers have moved apart by a factor of four, whereas they have widened by only a factor of two (Giddings, 1975). This fact is the fundamental principle behind chromatographic separations.

(d) Resolution

Resolution is the degree of separation of two adjacent peaks and is expressed by Equation 9 with reference to Fig. 4.

$$R_s = \frac{d_2 - d_1}{2(\sigma_1 + \sigma_2)} \qquad \text{(Eq. 9)}$$

where, R_s = resolution (or resolution factor)

d_1 = distance traveled by peak 1

d_2 = distance traveled by peak 2

σ_1 = band half-width of peak 1

σ_2 = band half-width of peak 2

The units for the distance between bands can be in terms either of volume or of linear dimensions provided that the widths 2σ are also given in the same units. Fig. 4 illustrates the case where R_s is equal to 1, i.e., when the band separation $(d_2 - d_1)$ is just equal to the sum of the band widths $(2\sigma_1 + 2\sigma_2)$. A fraction cut at the minima between the peaks contains each component in almost 98% purity. If the value of the resolution factor (R_s) is increased to 1.5, it would mean that the components can be isolated in 99.9% purity (Snyder and Kirkland (1979)).

The distance between bands is related to the ratio of the partition coefficients or the distribution coefficients in general of the corres-

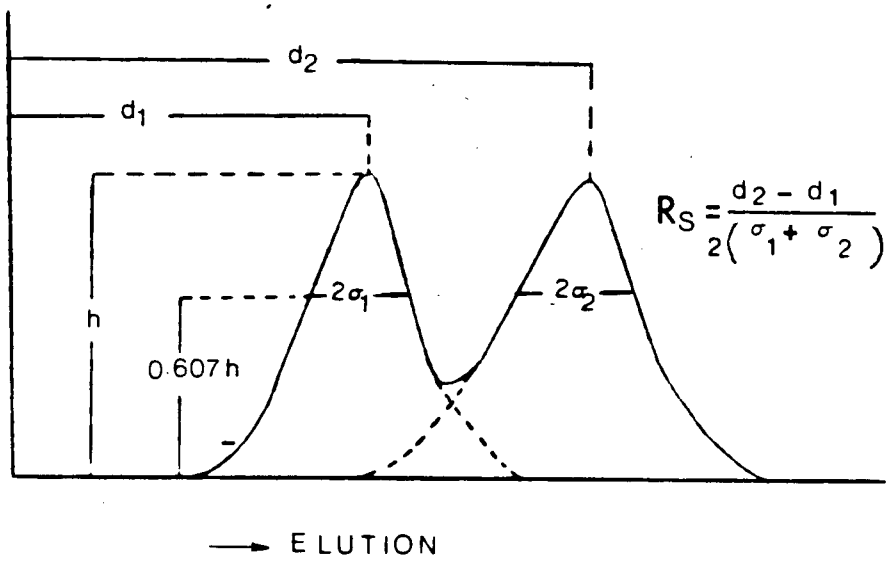


Fig. 4. Chromatogram showing the parameters used for the calculation of resolution (R_S). d_1 = retention value of component 1; d_2 = retention value of component 2; h = peak height; σ = half band-width.

ponding compounds. By substituting this and certain other relations into Equation 9, it is possible to show that the resolution factor (R_S) is a product of three different terms:

$$R_S = \tfrac{1}{4}\sqrt{N}\,\left(\frac{\alpha - 1}{\alpha}\right)\,\left(\frac{K'}{K' + 1}\right) \qquad \text{(Eq. 10)}$$

(a) (b) (c)

where, a = the column efficiency term (random dispersion)

b = the column selectivity term

c = the capacity ratio factor

N = number of theoretical plates

α = selectivity

K' = capacity factor (retention)

(i) Efficiency (number of theoretical plates, N)

This is a measure of the sharpness of a chromatographic band (see Fig. 5) and its quantitative expression is given in the equation:

$$N = \left(\frac{T_R}{\sigma}\right)^2 = 16\left(\frac{T_R}{w}\right)^2 = 5.5\left(\frac{T_R}{w_{1/2}}\right)^2 \qquad \text{(Eq. 11)}$$

where N = efficiency (number of theoretical plates)

T_R = retention time

σ = standard deviation of the band (measured at 0.607 of peak height)

w = peak width at baseline

$w_{1/2}$ = band width at half peak

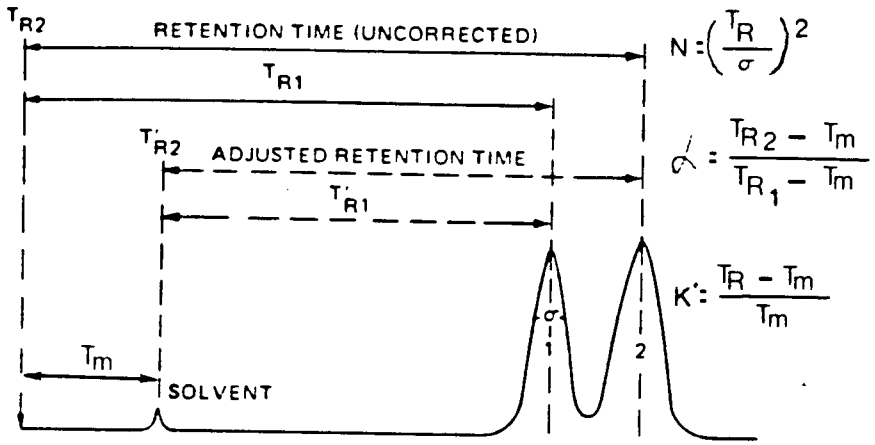


Fig. 5. Chromatogram showing the parameters used for the calculation of Efficiency, Selectivity and Capacity Factor. T_{R1} = retention time of compound 1; T_{R2} = retention time of compound 2; T'_{R1} - adjusted retention time of compound 1; T'_{R2} = adjusted retention time of compound 2; σ = band half-width at 0.607 of peak height.

Equation 11 is similar to Equations 6 and 7 except that the distance in this case is measured in terms of retention time. (Equations 5-11 are from Farris (1976)).

(ii) Selectivity (α)

Selectivity is a measure of the elution time of a compound relative to that of the other compound (adjacent band), as shown in Fig. 5. This is expressed in Equation 12 (Fallick (1975)):

$$\alpha = \frac{T_{R_2} - T_m}{T_{R_1} - T_m} = \frac{T'_{R_2}}{T'_{R_1}} = \frac{K_2}{K_1} \qquad \text{(Eq. 12)}$$

where, α = selectivity

T_{R_1} = retention time of compound 1

T_{R_2} = retention time of compound 2

T_m = solvent front in dimensions of time

T'_{R_1} = adjusted retention time of compound 1

T'_{R_2} = adjusted retention time of compound 2

K_1 = distribution coefficient of compound 1

K_2 = distribution coefficient of compound 2

Selectivity is a function of the stationary phase and the solvent system and can be improved by changing the distribution coefficient K and/or the stationary phase volume V_s, as shown in the Equation 13 (Yan et al. (1979)):

$$V_R = V_m + KV_s \qquad \text{(Eq. 13)}$$

where, V_R = retention volume of a compound

V_m = mobile phase interstitial volume (void volume) and dead volume of the instrument (t_m x flow rate)

K = distribution coefficient

V_s = - stationary phase volume (Partition) or,
- pore volume (exclusion) or,
- surface area (adsorption) or,
- ion-exchange capacity (ion exchange)

For purposes of effecting better selectivity, the distribution coefficient (K) in Equation 13 can be altered by:

- changing the mobile phase, which may increase or decrease the polarity, pH or ionic strength
- changing stationary phase which may mean changing pore size of gels, modifying surfaces of adsorbants, or changing the liquid used as stationary phase.
- changing temperature
- changing the nature of the solutes: e.g., by eliminating the charge of an amino acid by changing the pH of the mobile phase so as to reduce affinity for ion-exchange resins; or by forming an ion pair.

It is to be noted that the factor of selectivity confers on HPLC a decided advantage over gas-liquid chromatography (GLC), because the mobile phase in the latter technique is inert.

(iii) Capacity ratio factor (K')

This is a measure of the degree of retention of a compound and is expressed as (Fallick (1975)):

$$K' = \frac{T_R - T_m}{T_m} = \frac{V_R - V_m}{V_m} = \frac{KV_s}{V_m} \qquad \text{(Eq. 14)}$$

Where K' = capacity ratio factor

T_R = retention time

T_m = retention time of un-retained compound (solvent front)

V_R = retention volume

V_m = void volume (mobile phase)

K = distribution coefficient

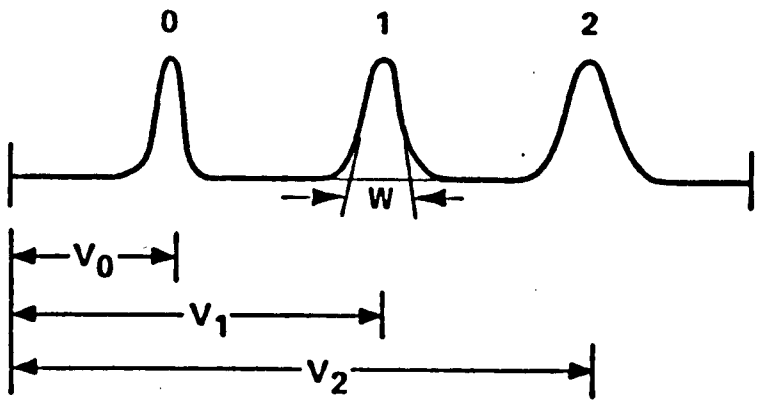
V_s = stationary phase volume

Retention and resolution can be increased by increasing the amount of stationary phase (V_s). This is done by:

- changing the loading of liquid phase in partition packings;
- changing the charge density in ion-exchange resins;
- changing the pore volume in exclusion gels;
- changing the surface area of adsorbants

According to the literature, capacity ratio values of 2 to 6 are associated with the greatest efficiency of HPLC columns.

A more concise graphical and mathematical presentation of theoretical plates, selectivity and retention is shown in Fig. 6.



$$\text{RETENTION, } k' = \frac{V_1 - V_0}{V_0}$$

$$\text{SELECTIVITY, } \alpha = \frac{k'_2}{k'_1} = \frac{V_2 - V_0}{V_1 - V_0}$$

$$\text{PLATES, } N = 16\left(\frac{V_1}{W_1}\right)^2$$

Fig. 6. Chromatogram depicting the parameters that are employed for calculating Retention, Selectivity, and Theoretical Plates. V_0 = solvent front; V_1 = retention volume of compound 1; V_2 = retention volume of compound 2; w = band width at baseline (Fallick (1975)).

(B) Instrumentation

The high-performance liquid chromatograph, as schematically shown in Fig. 7, is basically a column chromatograph with accessories that have been introduced for purposes of solvent delivery and mixing, sample injection and detection and data processing.

(a) Solvent reservoir

Containers that are used as solvent reservoir are made of type 304 or 316 stainless steel, glass or an inert polymer like polytetrafluoro-ethylene. The solvent usually leaves the reservoir via a stainless steel frit filter.

(b) Pumps

Solvent delivery in HPLC can be achieved by using a variety of pumping mechanisms that are classified as follows:

(i) Mechanical pumps

- Screw-driven syringe type. This is a mechanism in which the solvent contained in a syringe is slowly pushed by means of a plunger resulting in a pulse-free flow of solvent. However, the limited solvent capacity of the syringe necessitates frequent stoppings for puposes of refilling and resulting.
- Reciprocating piston. This set-up consists of a sapphire or ruby plunger that has slow forward and fast backward movements that result in a pulsating but continuous supply of solvent. Pulsations cause periodic "noise" that is

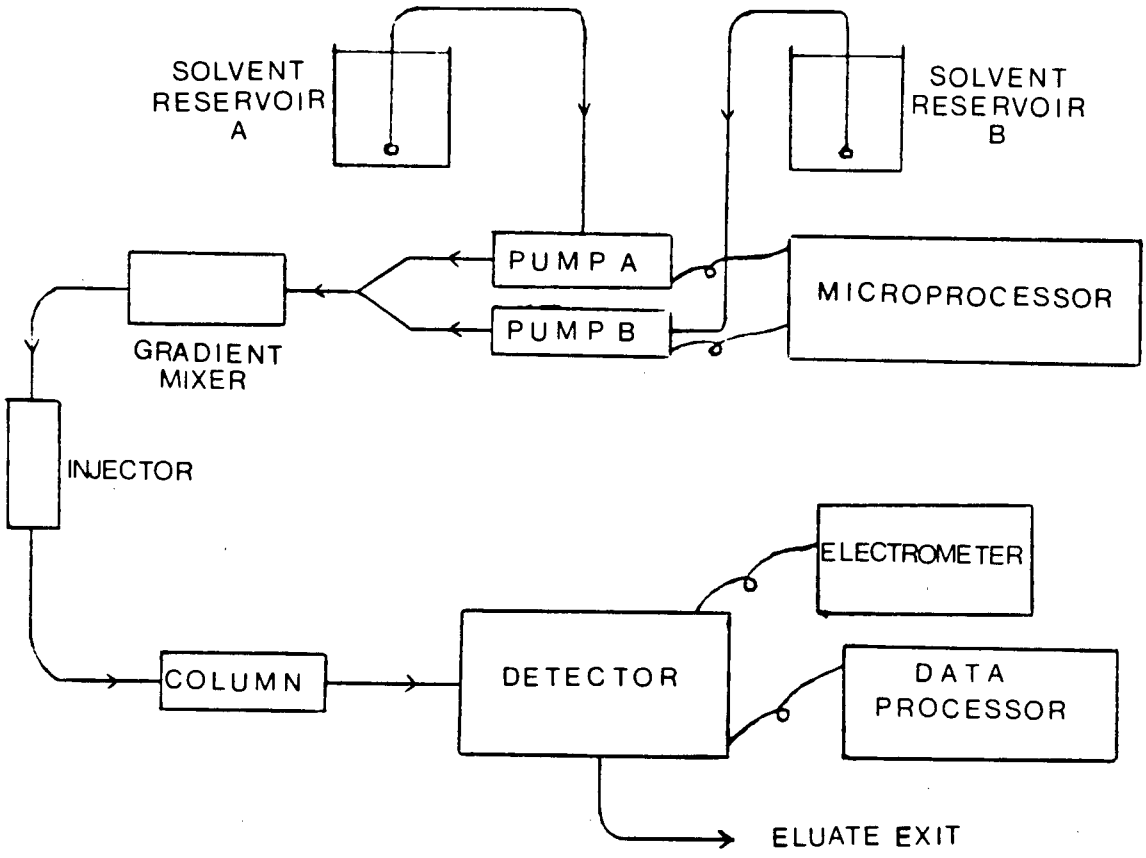


Fig. 7. Schematic Diagram of a Gradient High-Performance Liquid Chromatograph.

especially disturbing to the bulk-property detectors. A dampening device, consisting of a narrow-bore coil of stainless steel tubing, is used to reduce detector "noise" by eliminating the pulsations. Another variation of the reciprocating piston is known as the reciprocating diaphragm pump which also supplies a pulsating mobile phase.

(ii) Pneumatic Pumps

These pumps use gas-pressure which is applied on a suitable collapsible container or piston that pressurizes the mobile phase resulting in a pulse-free flow.

(c) Sample Introduction Devices

Mechanisms by which the sample is introduced into the liquid chromatograph fall into the following categories:

(i) Injection ports. These are classified into on-column injection ports and swept injection ports. The former is a set-up in which the syringe needle extends through the septum into the column packing where it deposits the sample. The latter type involves the deposition of the sample just before the column inlet after which the sample is swept into the packing by the mobile phase.

(ii) Sample valve (six-port injection valve). Sample introduction utilizes a valve arrangement in which the ports that lead into the loop and waste are connected while being cut off from the port that introduces the solvent and the port

that leads into the column. Sample injection into the flow system is done by closing the port that introduces the sample and the other port that leads into the waste, in which case the solvent will flow through the loop into the column. This type of injection valve is used in situations where the solvent is pumped at pressures of more than 2000 pounds per square inch (psi). A schematic diagram of a six-port injection valve is shown in Fig. 8.

(d) Columns

(i) Column Material. Most HPLC columns are constructed of precision-bore stainless steel or Trubore^{R} glass. Even though ordinary stainless steel tubing can be used, it has been shown by Kirkland (1969) that the smooth inside surface of the wall of the precision-bore stainless steel and Trubore^{R} glass apparently reduces the band spreading due to wall effects.

(ii) Solid Support Materials. The solid support materials with which HPLC columns are packed are alumina or silicious in nature and are commercially available in a variety of particle sizes. These packing materials may be grouped into two categories.

- Small Porous Particles. These are typically 5-10 microns in diameter and fully porous and therefore offer relatively high surface area. Provided that they are uniformly packed these materials make good columns with small inter-particle void spaces and outstanding efficiency. However, the small particles have the disadvantages of high resistance to liquid flow and difficulty in packing.

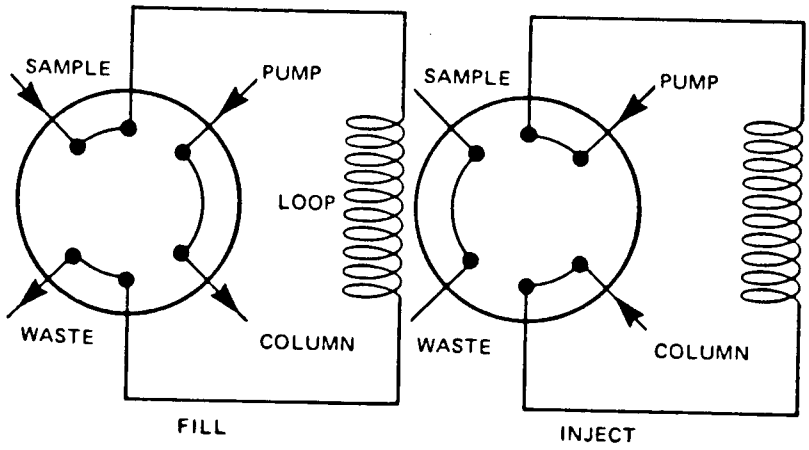


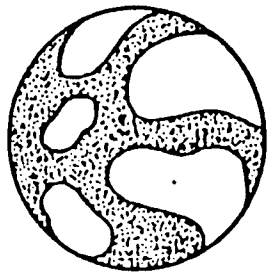
Fig. 8. Diagrammatic Representation of a Six-Port Injection Valve (Farris (1976)).

- Pellicular Supports. These are non-porous support particles uniformly coated with thin porous layers of liquid phase or ion exchange resin. The porous surface makes the stationary phase readily available for solute interaction. These materials usually have particle diameters of 37-44 microns and the coating accounts for 1/30th of the radius of the particle. Pellicular supports are also known in the literature as Porous Layer Beads (PLB) or Controlled Surface Porosity Packings (CSP).

A diagramatic presentation of the structures of the packing materials discussed is shown in Fig. 9.

HPLC columns are commonly classified into Normal Phase (Forward Phase) and Reverse Phase types depending upon the nature of the packing material.

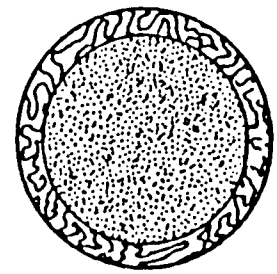
- Normal Phase Column. This type of column is packed with silicon or alumina and has polar characteristics. Examples of commercially available adsorbents along with their functionality and surface area are given in Table I.
- Reverse Phase Columns. The solid support materials in this case are classified into two:
  - Physically Bound. The packing in this column consists of silica or alumina material that has been coated (physically bound) with liquid stationary phases like triethylene glycol, ethylene glycol, hydrocarbon polymers etc.
  - Chemically Bonded. This type of stationary phases is currently available in two different forms which are known as Esterified Silicious Supports and Chemically Bonded Silicone Polymers. The former are prepared by esterification of silicious supports



PREPARATIVE SEPARATIONS

FULLY POROUS
HIGH CAPACITY

PORASIL
STYRAGEL
DURAPAK/PORASIL
BONDAPAK/PORASIL



ANALYTICAL SEPARATIONS

PELLICULAR
HIGH EFFICIENCY

CORASIL
DURAPAK/CORASIL
BONDAPAK/CORASIL

ANALYTICAL & PREPARATIVE SEPARATIONS

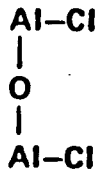
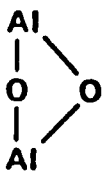
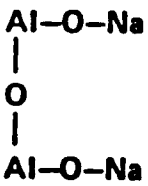
FULLY POROUS
HIGH SPEED
HIGH EFFICIENCY

μPORASIL
μSTYRAGEL
μBONDAPAK

Fig. 9. Diagrammatic Representation of the Structures of Column Packing Materials used in Analytical and Preparative Separations (Waters Associates Publications Ref. No. 2584).

TABLE I. NAME, FUNCTIONALITY AND SURFACE AREA OF COMMERCIALLY AVAILABLE ADSORBENTS USED IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY.[a]

ADSORBENTS

NAME	FUNCTIONALITY	SURFACE AREA (m^2/g)
PORASIL A, B, C, D, E, F	–SiOH	400 to 2
PORASIL T	–SiOH	300
μPORASIL	–SiOH	350
CORASIL I	–SiOH	12 - 15
CORASIL II	–SiOH	25 - 30
WOELM ALUMINA ACID		200
WOELM ALUMINA NEUTRAL		200
WOELM BASIC BASIC		200

[a]Waters Associates Publications Ref. No. 2571.

with a monomolecular organic layer of alcohols, as described by Halasz and Sebastian (1969). The latter are obtained by reacting silane reagents with the surface of the porous shell of Zipax^{R} support and then polymerizing the reagents to give the desired silicone coating, as reported by Kirkland and Destefano (1970). Silicious support materials bonded with long chain hydrocarbons (e.g., octadecylsilane) are at present amongst the most commonly used reverse phase columns. A diagram that shows the structure of bonded packing material, functionality and the separation process is presented in Fig. 10. The versatility of a reverse phase column is demonstrated in Fig. 11 in which manipulation of pH and introduction of ionic reagents like tetrabutyl ammonium hydroxide can facilitate separation as a result of ion-pair formation.

A summary of some characteristics of Normal and Reverse Phase Columns is given in Table II. Design and operational parameters that are associated with Analytical and Preparative HPLC are listed in Table III.

(e) Detectors

The detector is a device that continuously monitors the concentration of the solute as it leaves the column. The detectors commonly used in HPLC fall into two categories: Bulk property detectors and solute property detectors.

(i) Bulk Property Detectors. These devices monitor the change in the overall physical property of the mobile phase and are also known as universal detectors. Examples of this type of detectors are refractive

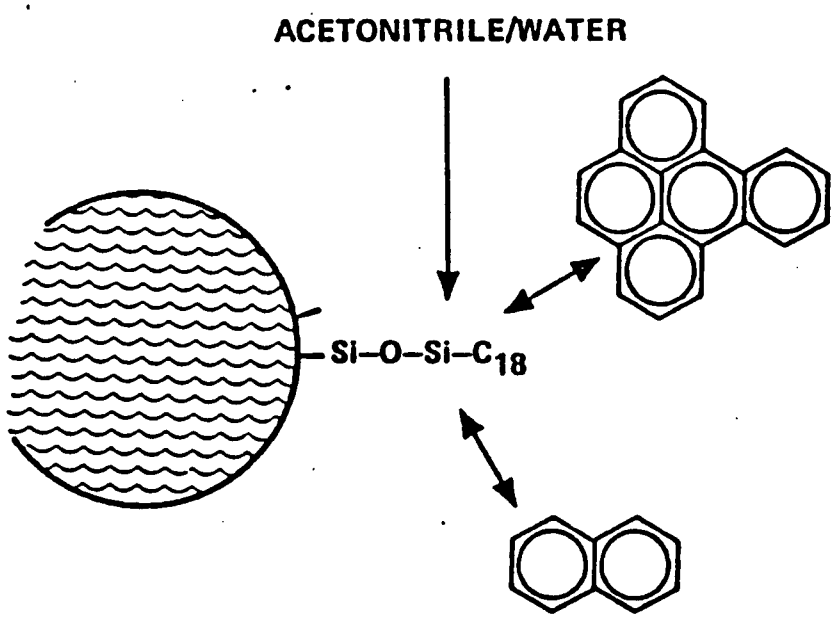


Fig. 10. Diagrammatic Representation of the Structure and Functionality of Bonded-Packing Material and the Separation Process (Waters Associates Publications Ref. No. 2248)

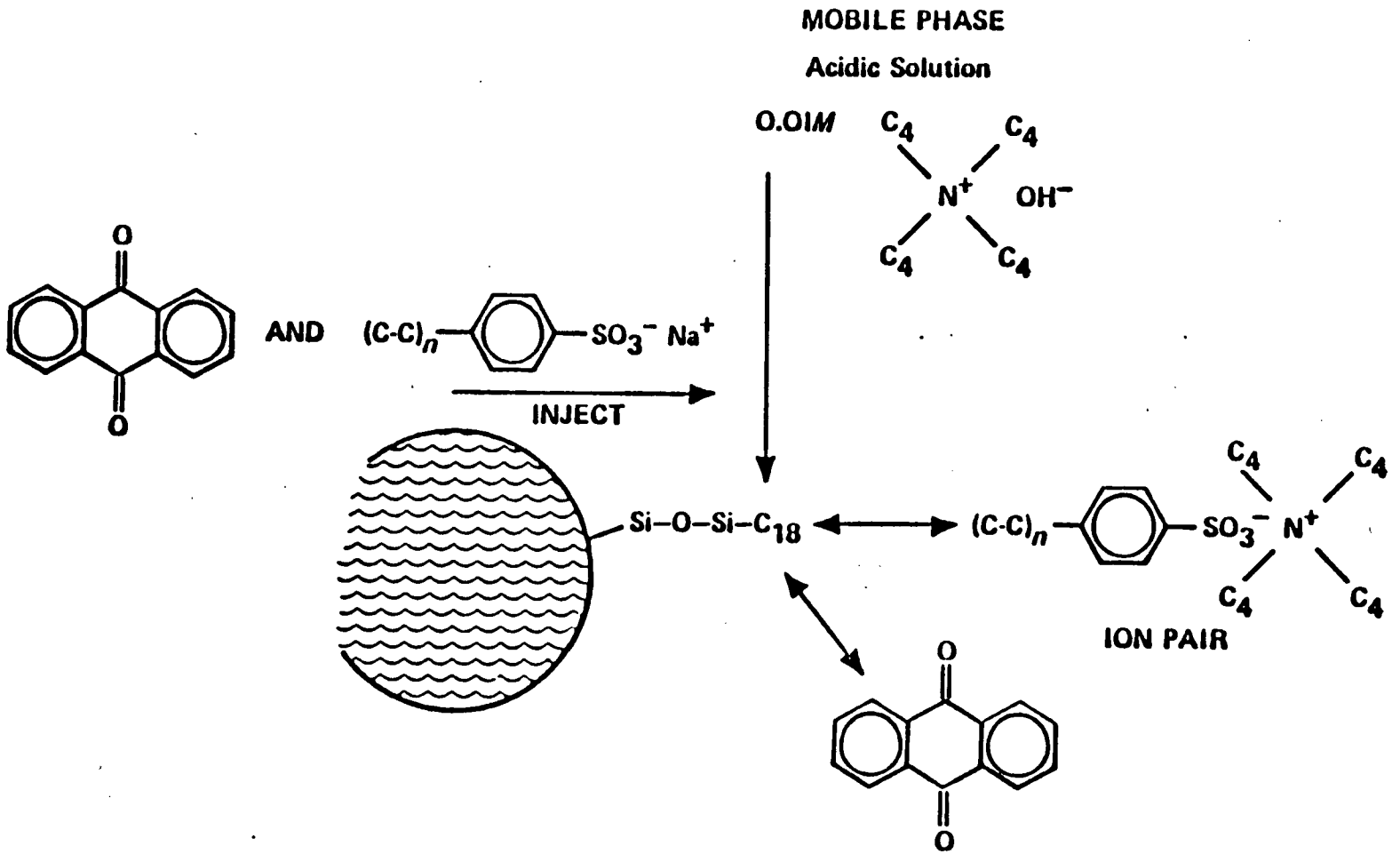


Fig. 11. Diagrammatic Representation of Ion-Pairing for Reverse Phase Separations (Waters Associates Publications Ref. No. 3100).

TABLE II. SUMMARY OF SOME CHARACTERISTICS OF NORMAL AND REVERSE PHASE COLUMNS[a]

CHARACTERISTICS	NORMAL PHASE	REVERSE PHASE
PACKING POLARITY	**High**	**Low**
SOLVENT POLARITY	**Low to Medium**	**Medium to High**
SAMPLE ELUTION ORDER	**Least Polar First**	**Most Polar First**
EFFECT OF INCREASING SOLVENT POLARITY	**Reduces Elution Time**	**Increased Elution Time**

[a]Waters Associates Publications Ref. No. 2583.

TABLE III. DESIGN AND OPERATIONAL PARAMETERS ASSOCIATED WITH ANALYTICAL AND PREPARATIVE HPLC[a]

Design Parameters	A Analytical HPLC	B Preparative (High Sample Capacity)
Length, cm	25–100	25–200
i.d., mm	2 to 4	~8 mm
Shape	Straight	Straight
Support:		
Diameter, microns	5–40	10–20 μ
Surface area	Moderate to high	High
Mobile Phase	Nonviscous Spectrograde	Volatile Spectrograde
Stationary Phase		
Partition	Thin Film	Higher loading bonded phase
Adsorption	Uniform activity	Higher surface area
Ion Exchange	Thin film	Higher capacity, less cross-linking
Exclusion	Rigid gel	Porous gel
Operational Parameters		
Gradient	Complex samples only	Complex samples only
Flow rate, ml/hr	30–120	200–400
Pressure, psig	500–5000	500–5000 psi
Temperature	Optimize for reproducibility and resolution	Increase for solubility
Sample size, μg	0.050 to 500 Often higher than optimum	Up to 500 milligrams per injection

[a]Pascott (1976)

index, conductivity and dielectric constant measuring devices. All of these detectors are temperature sensitive and most lack the sensitivity needed for HPLC.

(ii) Solute Property Detectors. Selectors of this type measure physical properties of the solute which are not exhibited to any significant extent by the mobile phase. UV absorption, fluorescence, polarographic and radioactivity detectors are examples of this type.

The two most widely used detectors are the UV absorption and the refractive index. The former is one of the most sensitive detectors in liquid chromatography and can detect samples in nanogram range. The latter type is fairly easy to use and can detect solutes in the microgram range. Unlike the UV absorption detector, however, the refractive index detector is sensitive to changes in temperature and solvent flow.

(f) Data Processors

Detector response in HPLC is normally presented in the form of a chromatogram showing a number of peaks corresponding to different compounds that have been separated. The chromatogram may be obtained by using a simple pen recorder, digital integrator and printer or electronic computer-calculator. Quantitation of peak area or peak height measurements is done by the latter types. Electronic data processors with computational features that make them capable of printing out directly in concentration terms are now available.

(C) Technique

The primary purpose of HPLC, as in all chromatographic work, is separation (resolution) of the components of a mixture. Two other factors that should also be considered are speed and capacity (sample load). It is the appropriate balance of the above three factors - the so called chromatographer's triangle, that results in an optimal chromatogram. The triangle, as shown in Fig. 12, represents the possibility that high speed and high capacity, high capacity and good resolution, and good resolution and high speed may be at the expense of resolution, speed and capacity, respectively. The unique characteristics of HPLC that enable it to optimize these factors so as to obtain satisfactory chromatograms are:

- porous microparticulate packings with dimensions of 5-37 microns.
- small bore columns (2.6 - 4.0 mm i.d.)
- introduction of high-pressure pumps
- availability of relatively high-sensitivity on-line detectors

(a) HPLC Mode Selection

A knowledge of the molecular weight range, solubility and functional groups of the compounds to be analysed by HPLC is essential for the appropriate choice of HPLC mode. A molecular weight of over 2000 would require exclusion HPLC with an aqueous or non-aqueous phase depending on whether the compounds are soluble or insoluble in water. For compounds that have molecular weights of less than 2000 and are water insoluble, the choice can be partition, adsorption or exclusion HPLC depending on whether they are homologs, isomers or of different sizes. Water soluble compounds

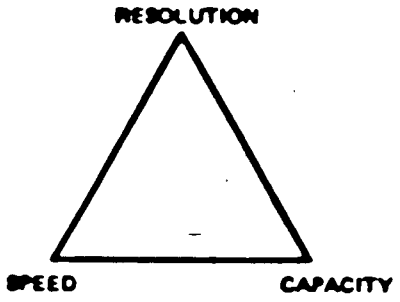


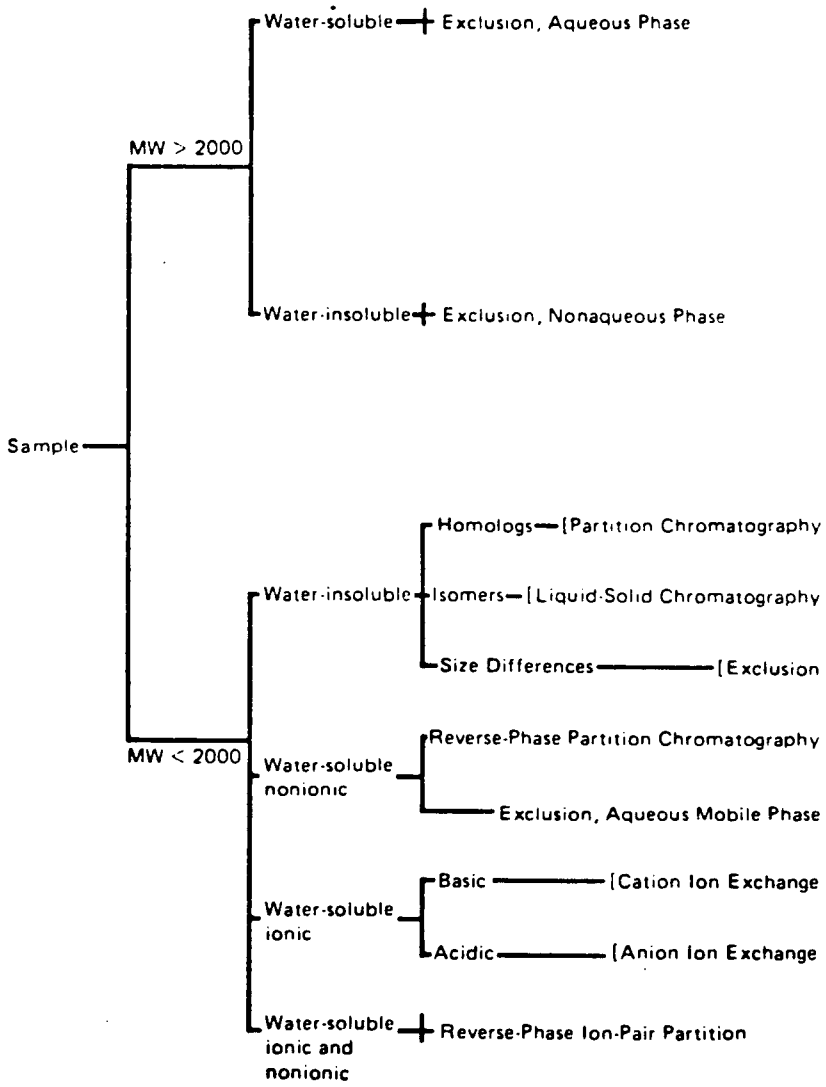
Fig. 12. Triangle of Resolution, Speed and Capacity representing the Balance necessary for Chromatographic Efficiency.

of low molecular weight may require reverse phase, exclusion, ion-exchange or reverse-phase ion-pair partition HPLC according to whether they are non-ionic, ionic or both. A schematic guide to HPLC mode selection is given in Table IV.

(b) Optimization of Resolution

(i) Isocratic Elution. This is a process in which one solvent or a solvent system of fixed composition is used as the mobile phase. In order to conveniently locate the peaks in a chromatogram having reasonably short retention times, it would first be necessary to manipulate the capacity ratio factor (K'), as shown in Fig. 13. This is normally achieved by changing the solvent strength of the mobile phase. Then efficiency (N) can be altered to see if there can be better separation by making peaks sharper. This is brought about by choosing the right type (having high sample retention capacity) and length of column. Finally the selectivity factor (α) may be changed to selectively move one of the peaks and effect separation. Manipulations of selectivity, while more or less maintaining the same capacity factor, may be accomplished by slight alterations of polarity. Selectivity and capacity ratio factor are functions of mobile phase characteristics like polarity, viscosity, solubility factors etc. Changes in the above factors are brought about by changes in the type of solvent or composition of solvent systems. The solvents that are used as the mobile phase are usually chosen from a table where they are listed on the basis of empirical scales of relative solvent polarities. An example of such a scale, that of Macek and Prochazka, is shown in Table V, as reported by Hais and Macek (1963). The role of the mobile phase in liquid chromatography has been discussed

TABLE IV. GENERAL GUIDE FOR HPLC MODE SELECTION[a]



[a] Baumann (1971)

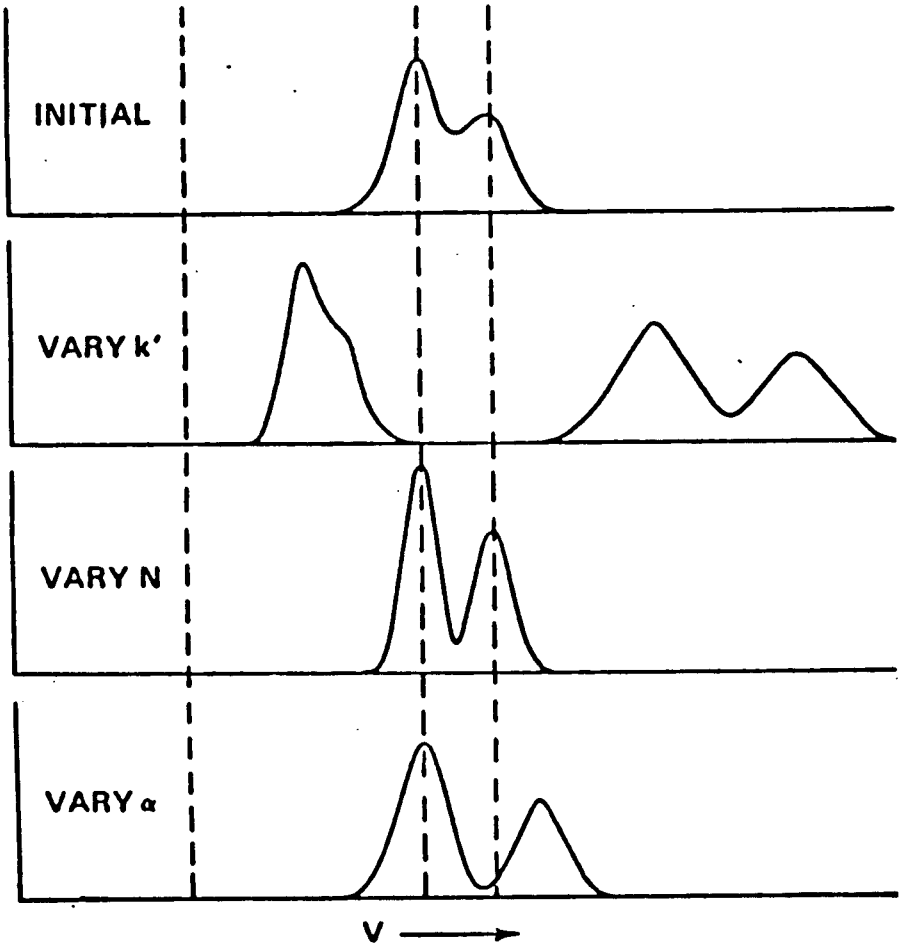


Fig. 13. Diagrammatic Representation of Effects of Varying Capacity, Efficiency and Selectivity Factors on Resolution (Snyder and Kirkland (1979)).

TABLE V. PARTIAL LISTING OF SOLVENTS USED IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN ORDER OF DECREASING POLARITY[a]

Water (most polar)	CH_2Cl_2
Formamide	$CHCl_3$
Acetonitrile	1,2-Dichloroethane
Methanol	Bromobenzene
Acetic acid	Ethyl bromide
Ethanol	Benzene
Isopropanol	Propyl chloride
Acetone	Toluene
Dioxane	Xylene
Tetrahydrofuran	CCl_4
t-Butanol	CS_2
Methyl ethyl ketone	Cyclohexane
Phenol	Hexane
n-Butanol	Heptane
n-Pentanol	Kerosine (least polar)
Ethyl acetate	
Ethyl ether	
n-Butyl acetate	
Nitromethane	
Isopropyl ether	

[a] Hais and Macek (1963)

at length by Snyder (1971).

(ii) Gradient Elution. This type of elution involves a gradual change of solvent composition to effect a corresponding change (increase or decrease) in the polarity of the mobile phase, which in turn results in a significant reduction of the retention times of slowly eluting components. Gradient elution, which may be programmed in a linear or non-linear mode, is especially useful for a mixture of compounds with a wide range of polarities. In the case of UV detectors, even though this method of elution does increase detection sensitivity for late-eluting components, it can only be used in cases where the solvents do not have any significant UV absorbance. Moreover, following separation by gradient elution, the last portion of the solvent gradient has to be washed out and the column has to be re-equilibrated with the solvent of initial composition. The details of experimental optimization of gradient elution have been reported by Snyder and Saunders (1969). Other methods that have been employed for purposes of optimizing resolution include flow programming, repeated separations and coupled columns.

(c) Advantages of HPLC

The advantages of HPLC over liquid and gas-liquid chromatography may be summarized as follows -

over liquid chromatography:

- speed and resolution
- sensitivity of on-line detectors
- re-usable columns
- easy sample recovery
- solvent programming

over gas-liquid chromatography:

- non-destructive to sample
- amenable to biological systems, since the column packing can be neutral, anionic or cationic
- the selectivity factor of the liquid mobile phase
- ideal for large molecules (non-volatile compounds)

(d) Disadvantages of HPLC

Even though HPLC offers many advantages as indicated above, it has also drawbacks that include the following:

- high equipment cost
- limited sensitivity of available detectors towards certain types of compounds
- the experience necessary to obtain good results

The development of new and more sensitive HPLC detector systems may bring further advances in resolution, speed and convenience. Computers interfaced with HPLC can play a significant role in the selection of LC systems as appropriate factors can be incorporated into mathematical models suitable for computer calculations. It appears, therefore, that HPLC will play an increasingly important role in the separatory and quantitation steps of analysis.

2. The Test Drugs: Digoxin and Digitoxin

(A) Chemistry

(a) Description

Digoxin and digitoxin are cardiotonic secondary glycosides obtained in purified form from the leaves of Digitalis lanata Ehrhart and Digitalis purpurea Linné, respectively. These difficultly purified glycosides occur very widely in a variety of plant families as well as in the venoms of certain toads, and more than one hundred and fifty individual compounds belonging to the digitalis-strophanthus group have been described (Wilson, 1960). Commonly employed cardiac glycosides are obtained from digitalis, strophanthus and squill. However, the term digitalis is often used to designate the entire group of cardiac glycosides rather than those from digitalis alone.

(b) Names

Digoxin is known by names that include the following (Merck Index, 1976): Cordioxil, Davoxin, Digacin; Dilanacin, Dixina, Lanocardin, Lanicor, Lanoxin, Rougoxin, Vanoxin. Digitoxin has been known by registered names that include the following (Jakovljevic, 1974): Cardigin, Crystodigin, Digicoryl, Digilong, Digimerck, Digipan, Digisidin, Digitaline Nativelle, Digitora, Digitoxoside, Digitrin, Lanatoxin, purodigin, purpuren, purpurid.

The chemical name of digoxin is: 3β-[(O-2,6-Dideoxy-β-D-ribo-hexopyranosyl-(1→4)-O-2,6-dideoxy-β-D-ribo-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-ribo-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide.

Similarly, the chemical designation of digitoxin is: 3β-[(O-2,6-Dideoxy-β-D-ribo-hexopyranosyl-(1→4)-O-2,6-dideoxy-β-D-ribo-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-ribo-hexopyranosyl)oxy]-14β-hydroxy-5β-card-20(22)-enolide.

(c) Appearance

Digoxin is an odorless, white crystalline powder and its crystals appear as radially arranged, four-and five-sided triclinic plates from dilute alcohol or dilute pyridine. Digitoxin is a white or pale buff crystalline powder, and the crystals from dilute alcohol are very small elongated, rectangular plates.

(d) Synthesis

Successful synthesis of digoxin and digitoxin has not yet been reported and therefore these drugs are commercially obtained by ethanolic extraction of digitalis leaves and subsequent chromatographic purification.

(e) Solubility

Digoxin is soluble in pyridine, dilute alcohol or a mixture of chloroform and alcohol; almost insoluble in ether, acetone, ethyl acetate, chloroform; and practically insoluble in water (Merck Index, 1976). Solubility of digoxin and its metabolites has been reported to be maximum in solvents such as alcohols, chloroform and methylene chloride (Gault et al., 1976). According to Merck Index (1976), one gram of digitoxin dissolves in about 40 ml chloroform, in about 60 ml alcohol and in about 400 ml ethyl acetate. Digitoxin is soluble in acetone, amyl alcohol, pyridine; and sparingly soluble in ether, petroleum ether,

water (1 g/100 liter at 20°C).

(f) Stability

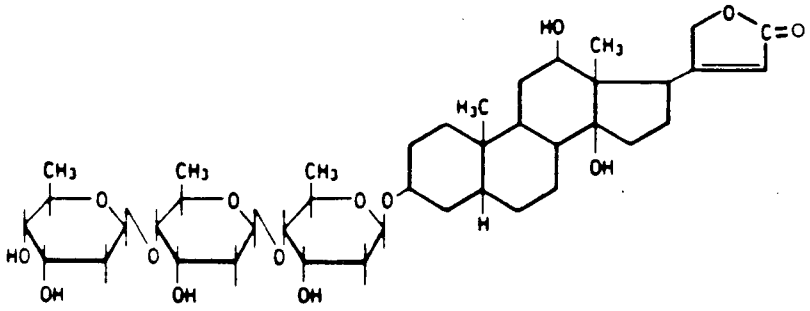
Digoxin has been reported to be stable for an indefinite period of time when kept in well closed containers and protected from light (Foss et al., 1980). It has also been observed that no degradation occurs in tablets or neutral solutions of digoxin in ethanol and propylene glycol for periods of up to five years. Storage of digoxin under intensive light for long periods of time may result in degradation due to opening of the lactone ring.

Digitoxin is also reported to be relatively stable under optimal conditions. It has been shown that no degradation of digitoxin in tablets, injections or solutions was found when stored for five years in the dark at temperatures of up to 30°C (Samuelson, 1964).

(g) Chemical Structure

(i) Formula, Molecular Weight and Classification

The empirical formulas, molecular structures, conformational arrangements and molecular weights of digoxin and digitoxin are shown in Figs. 14 and 15. Like any of the cardiac glycosides, the digoxin or digitoxin molecule is made of a sugar portion and an aglycone (genin) portion, as indicated in the molecular structure. Cardiac glycosides are generally classified according to the type of genin. Two types of genin may be distinguished according to whether there is a five- or six-membered lactone ring. These genins, as shown in Fig. 16, are known respectively as cardenolides (e.g. digoxigenin or digitoxigenin) and bufadienolides



$C_{41}H_{64}O_{14}$ DIGOXIN Mol. Wt. 780.92

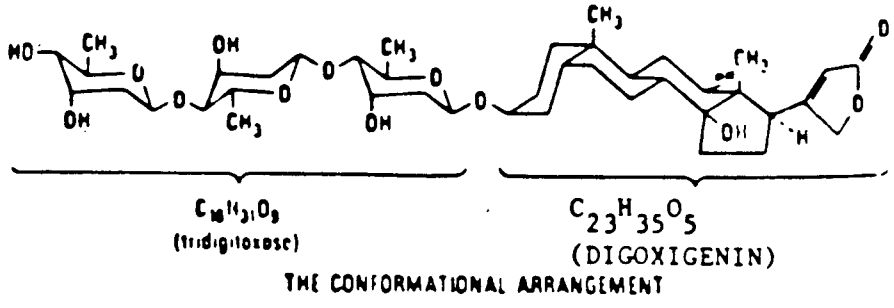
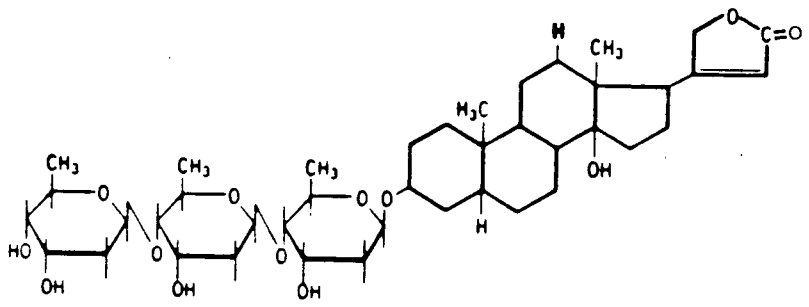
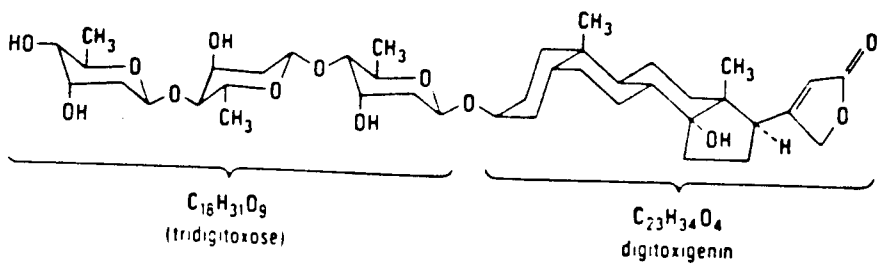


Fig. 14. Chemical Structure of Digoxin.



$C_{41}H_{64}O_{13}$ DIGITOXIN Mol.Wt. 764.92



THE CONFORMATIONAL ARRANGEMENT

Fig. 15. Chemical Structure of Digitoxin.

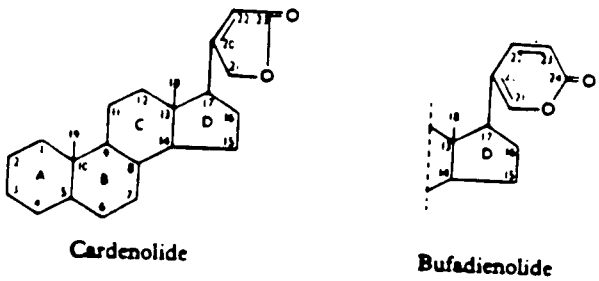


Fig. 16. Chemical Structures of the Cardenolide and Bufadienolide Groups of Genins.

(bufanolides, e.g. scillarenin). Digitalis lanata and Digitalis purpurea leaves contain a mixture of cardioactive glycosides which are classifed into five cardenolide series on the basis of the individual aglycone. The cardenolide "C" and "A" series of cardiac glycosides, among which digoxin and digitoxin are two secondary glycosides, are the most abundant and important.

As shown in Table VI, a stepwise hydrolysis of the primary glycoside lanatoside C, brought about by reagents or enzymes, yields a number of secondary glycosides (including digoxin) and varying number of digitoxose sugars. Cleavage of the last digitoxose molecule results in the aglycone, digoxigenin. Digitoxigenin can also be obtained from lanatoside A, in the same way. In general, mild alkaline hydrolysis results in the loss of the acetyl group of the lanatoside molecule; enzymatic hydrolysis cleaves glucose; and subsequent acid hydrolysis splits off digitoxose yielding the corresponding aglycones (Cobb (1976)).

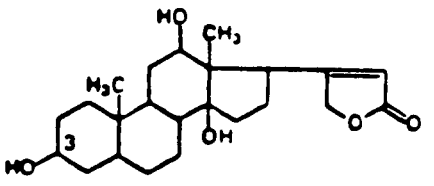
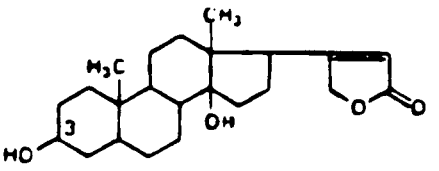
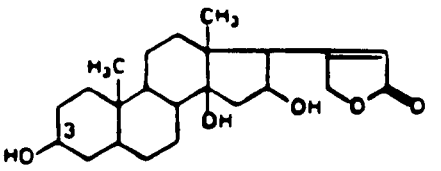
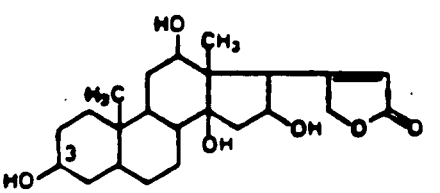
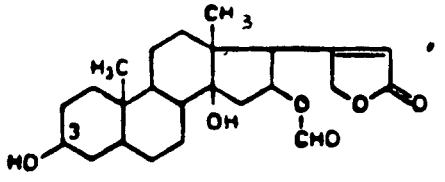
(ii) Structure-Activity Relationship

The basic structure of the aglycone portion of digoxin or digitoxin, like all of the other cardiac glycosides, is a cyclopentane-phenanthrene nucleus to which is attached a lactone ring. And the essential structures for biological activity are contained in the aglycone portion.

The unique structural characteristics of cardiac glycosides may be summarized as follows (Wilson et al. (1971)):

- all of the cardiac glycosides are complex steroids
- in all known cases, one or more carbohydrate residues are attached through the oxygen at position 3 of the steroid nucleus, and almost without exception the oxygen bond to the steroid nucleus is in the β-configuration

TABLE VI. GENINS OF THE CARDENOLIDE SERIES OF DIGITALIS GLYCOSIDES (Cobb (1976))

Series	Aglycone	Compound	Glycosidically linked residue at C_3
C		Digoxigenin	—
		Digoxigenin monodigitoxoside	-D
		Digoxigenin bisdigitoxoside	-D-D
		Digoxin	-D-D-D
		α-Acetyldigoxin β-Acetyldigoxin	-D-D-D Ac
		Lanatoside C	-D-D-D-G Ac
A		Digitoxigenin	
B		Gitoxigenin	
D		Diginatin	
E		Gitaloxin	

- position 17 of the steroid nucleus is invariably substituted with an unsaturated lactone ring, also in the β-configuration
- all active compounds carry a β-hydroxyl group on carbon 14
- unlike most other steroids, the C/D ring junction in the cardiac glycosides is always cis; with the exception of some glycosides (e.g. uzarigenin, urezigenin, etc.) ring junction A/B is always cis.
- in all cases the B/C relationship is trans.
- most of the cardiac glycosides may be related to the coprostane series, whereas those of the uzarigenin group are related to the cholestane series.
- the α,β-unsaturated, five-membered lactone ring (butenolide) in position 17 is a distinguishing feature of the digitalis-strophanthus group.
- most of the digitalis glycosides contain angular methyl groups at positions 10 and 13, but an aldehyde or primary alcohol at position 10 is characteristic of the strophanthus glycosides.

(B) Pharmacology

Digoxin and digitoxin, like any of the digitalis glycosides and certain closely allied drugs, have a specific and powerful action on the myocardium that is unrivaled in value for the treatment of congestive heart failure. The main pharmacodynamic property of these drugs is their ability to increase the force of myocardial contraction. The useful effects of the drugs in congestive heart failure - increased cardiac output; decreased heart size, venous pressure and blood volume; diuresis and relief of edema - are all explained on the basis of increased contractile force, a positive inotropic action.

As a result of the clinical observations of many workers in the field (Wenckebach, 1910; Pratt, 1918; Christian, 1919; Luteu, 1924 etc.) it has been established that the digitalis glycosides are effective in congestive heart failure regardless of cardiac rhythm, and that they bring relief not by virtue of cardiac slowing but by their direct action to increase the force of myocardial contraction.

It has also been pointed out that cardiac glycosides exert their inotropic stimulation by increasing the rate at which tension or force is developed, and not by prolonging the duration of the contractile process (Wallace et al. 1963). Digitalis glycosides reduce the ventricular rate in atrial fibrillation through vagal and extravagal influences that increase the effective refractory period of the A-V transmission system and through a vagally mediated increase in the atrial frequency. Hence, the decrease in heart rate upon digitalization, is secondary to the improvement of the circulation, and is not the primary therapeutic action of the drug.

Some evidence suggests that "therapeutic" concentrations of digitalis potentiate the activity of the Na-K-dependent ATPase enzyme system (Palmer et al., 1966). At present, circumstantial evidence seems to indicate that digitalis toxicity is related to inhibition of ATP-ase (Moe and Farah, 1967).

(a) Mechanism of Action

As suggested by Kahn (1963) and Page (1964), cardiac glycosides may exert a direct effect on the myocardium by conceivably acting at one or more of the following sites of the cardiac muscle: the cell membrane, the T system, the sarcoplasmic reticulum, directly on proteins associated with contractile structures, mitochondria, the nucleus, soluble enzymes and even, perhaps, unknown but important structures within the cell.

The unique combination of inhibition of atrioventricular impulse to the otherwise salutory inotropic effect of digoxin (and other cardiac glycosides) still remains to be an unfortunate disadvantage.

(b) Absorption, Fate and Excretion

Orally administered digoxin is adequately absorbed from the intestinal tract, even in the presence of vascular congestion of the enteric mucosa, hypoxia and diarrhea - conditions that may exist in patients with cardiac failure. By a comparison of the oral and intravenous dose, digoxin is found to be absorbed to the extent of fifty per cent or more; and gastro-intestinal absorption is usually completed within approximately two hours (Moe and Farah, 1967). Metabolic transformation of digoxin occurs chiefly, but not solely in the liver (Abel et al., 1965; Katzung and

Meyers, 1966). And digoxin is said to be excreted largely in unchanged form, chiefly through the kidneys (Ashley et al., 1958).

It is generally recognized that lipid soluble digitoxin is 100% absorbed (Takanashi et al., 1978). In contrast to digoxin, digitoxin is bound to serum albumin to the extent of over 90% (Lucas and Martino, 1969). It is also widely acknowledged that over 90% of absorbed digitoxin is metabolized in the liver (Doherty, 1973) and excreted mainly by the kidneys. Because of enterohepatic recirculation, approximately 25% of the metabolic products appear in the stool. The conversion of digitoxin into water soluble compounds in the liver occurs through two pathways (Doherty, 1973):

(1) conversion to digoxin by 12β-hydroxylation and

(2) sugar cleavage by hydrolysis followed by conjugation reactions.

(C) Pharmacokinetics and Therapy

Pharmacokinetic studies with digoxin have shown that its distribution is rapid and corresponds to an open two-compartment model (Nyberg et al., 1974). Doherty (1968) reported digoxin half-lives of approximately 33 hours. The results of many experiments agree sufficiently to indicate a mean $t_{\frac{1}{2}}$ of the terminal slope following intravenous administration of digoxin of 40-50 hours (Koup et al., 1975; Kramer et al., 1976).

Some evidence suggests that the pharmacokinetics of digoxin follow linear processes (Otten et al., 1976; Bodem et al., 1977). The $t_{\frac{1}{2}}$ of urinary excretion obtained from a multiple dose study of digoxin was reported to be 51.2 hours (Dengler et al., 1978). The latter investi-

gators have reported a clearance of 102.5 milliliters per minute; and a volume of distribution (at steady state) of 421 liters.

It has been reported that digoxin is bound only to the extent of 23 per cent; and that the protein binding of the digoxin metabolites is lower than digoxin (Smith et al., 1978). This would, then, mean that the effect of albumin concentration on digoxin plasma level is less important than in the case of digitoxin.

During maintenance therapy, the day-to-day variation of digoxin blood levels and of urinary digoxin excretion show fluctuation to an extent that can not be explained by analytic or other experimental errors. These are not convincingly explained and need further investigation (Dengler et al., 1978).

Mean serum or plasma digoxin concentrations in groups of patients without evidence of toxicity average about 1.4 ng per milliliter (from data involving well in excess of 1000 patients - Smith et al., 1978). The above authors report that mean serum concentrations tend to be two to three times higher in patients with clinical evidence of digoxin toxicity; and the difference in mean levels was statistically significant in the vast majority of cases. It has also been reported, however, that overlap of levels between groups with and without evidence of toxicity was observed in most series and that it tends to be more pronounced in prospective, blind studies than in retrospective studies (Beller et al., 1971).

Since as much as 97% of digitoxin is bound to plasma albumin (Lukas and Martino, 1969), its total plasma concentration is from 15 to 20 times higher than that of digoxin at comparable therapeutic levels (Smith and Haber, 1973). Digitoxin has a therapeutic plasma concentration of 14-26 ng/ml and is usually reported to have a plasma half-life of

5-7 days. Total digitalizing oral dose for digoxin and digitoxin is usually in the range of 2-3 mg and 1.2-1.6 mg, respectively. And the daily oral maintenance dose range for digoxin and digitoxin is 0.25-0.75 mg and 0.05-0.2 mg, respectively.

According to the experience of many investigators in the field, it is apparent that no serum concentration of digoxin and digitoxin can be selected that clearly separates toxic and non-toxic states in the usual clinical setting (Smith et al., 1978). Hence, judgement of optimal doses and serum concentrations must be based, to a large extent, on assessment of each individual clinical response.

According to Smith et al. (1978), the following factors may influence individual sensitivity to cardiac glycosides:

(i) Type and severity of underlying cardiac disease.

(ii) Serum electrolyte derangements
- hypokalemia and hyperkalemia
- hypomagnesemia
- hypercalemia
- hyponatremia

(iii) Acid-base imbalance

(iv) Concomitant drug administration
- anaesthetics
- catecholamines and sympathomimetics
- antiarrhythmic agents

(v) Thyroid status

(vi) Renal function

(vii) Autonomic nervous system tone

(viii) Respiratory disease

3. Development of Methods of Analysis of Digoxin and Digitoxin

(A) Analysis of Samples in Plant Extracts, Standard Mixtures and Dosage Forms

As far back as the nineteenth century, a colorimetric method was published by Lafon (1885) who used equal amounts of sulphuric acid and ethanol with the addition of ferric chloride.

Amongst the earliest chemical methods of analysis of digoxin that subsequently appeared were probably those of Baljet (1918), Morel (1935) and Warren et al. (1948), in which alkaline picrate, m-dinitrobenzene and 2-naphthoquinone-4-sulphonate were used as reagents, respectively. Various other colorimetric assays for digoxin have also been reported. 1,3,5-trinitrobenzene in alkaline medium was used as reagent by Kimura (1951); Xanthydrol by Pesez (1952); acetone-phosphoric acid by Dequeker and Loobuyck (1955); 3,5-dinitrobenzoic acid by Tattje (1957); 2,4-dinitro-diphenylsulfone by Tattje (1958); m-dinitrobenzene by Houk et al. (1959); and thiobarbituric acid by Mesnard and Devaux (1961).

Along with the development of colorimetric procedures, fluorometric methods of analysis were reported by Petit et al. (1950) and Jensen (1952) who used syrupy phosphoric acid and hydrochloric acid-glycerol (1:1), as reagents, respectively. Hydrogen peroxide, hydrochloric acid and methanol were used as reagents by Jensen (1953) and Wells et al. (1961). Tattje (1954) used a mixture of sulphuric and phosphoric acids with the addition of ferric chloride. The fluorophor obtained with a mixture of acetic anhydride, acetyl chloride and trifluoroacetic acid has been reported by Jakovljevic (1963), to give a low yield of a highly conjugated compound of substituted, 3,4-benzpyrene.

An automated method based on acid induced fluorescence was proposed by Khoury (1967). Another automated fluorogenic procedure using a standard Technicon automatic analyzer system for the unit dose analysis of digoxin and digitoxin in tablets was reported by Cullin et al. (1970).

Meanwhile, the development of chromatographic methods of analysis has tremendously facilitated the qualitative and quantitative determinations of cardiac glycosides. And chromatography was later to be recognized as the most valuable tool in the resolution and quantitative evaluation of cardiac glycoside mixtures.

The method of Zaffaroni et al. (1949) for the separation of steroidal mixtures on filter paper impregnated with formamide or ethylene glycol was used by Reichstein and Schindler (1951) for the chromatography of Digitalis glycosides. Many investigators (Jensen, 1956; Tantivatana and Wright, 1958; Potter, 1963; Wolf and Karacsony, 1963; and Dzyuba et al., 1971) have subsequently used paper chromatography to determine quantitatively digitalis glycosides by elution of the resolved compounds followed by various colorimetric assay procedures.

Rabitzsch et al. (1969) have reported a butenolide ring specific method of quantitative paper chromatographic analysis of cardiac glycosides using 2,4,2',4'-tetranitrodiphenyl as reagent.

Thin-layer chromatography on silica gel was used by Stahl and Kaltenbach (1961) in order to separate low loadings of cardiac glycoside mixtures. Thin-layer chromatographic separations have also been reported by Heusser (1965); Hauser et al. (1968) and Potter et al. (1972) in which the separated components were eluted and quantitated using colorimetric methods. Evans et al. (1974) resolved the components of extracts of Digitalis purpurea by hydrolysing the glycosides to the parent aglycones,

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which they then measured by densitometry of thin-layer chromatograms.

Hoeke et al. (1969) reported a rapid separation of digoxin from digitoxin and acetyldigitoxin using kieselgel G plates, a solvent system of chloroform/methanol (9:1) and hydrochloric acid as a detecting agent.

Separation of digoxin from digitoxin by the use of silica Gel G plates, followed by detection with one per cent iodine in chloroform, extraction and spectrophotometric quantitation after treatment with dixanthylurea reagent was described by Bican-Fister et al. (1969) and Johnston et al. (1966). Other TLC methods involving the formation of colored derivatives have been reported by Bell and Krantz (1948); Jensen (1973); Zurkowska and Ozarowski (1964); Myrick (1969); and Wichtl and Dexler (1966).

Carvalhas and Figueira (1973) described a comparative study of thin-layer chromatographic techniques (various solvent systems) for separation of digoxin, digitoxin and their main metabolites.

A spectrofluorometric method for the direct quantitative evaluation of digoxin, digitoxin and acetyldigitoxin on TLC using hydrochloric acid as the fluorogenic reagent was reported by Frijns (1970). The sensitivity mentioned was about 0.25 mcg..

Separation of digoxin and digitoxin from their 20,22-dihydro derivatives, by multiple TLC on cellulose films was accomplished by Rabitzsch (1968). Earlier, Jelliffe et al. (1967) had described an ultramicrofluorescent spray reagent for detection and quantitation of digitoxin and other cardiac glycosides on TLC. Their reagent consisted of ascorbic acid, methanol, hydrochloric acid and hydrogen peroxide. They claimed a limit of detection of 0.01 mcg..

Jensen (1954); Wells et al. (1961); Lugt (1973); and Britten and

Njau (1975) have reported other TLC methods involving the formation of fluorescing products of cardiac glycosides.

Sabatka et al. (1976) described the separation of digoxin and dihydrodigoxin by thin-layer and paper chromatography and their subsequent quantitation after fluorogenic derivatization. They reported limits of detection of one nanogram and ten nanograms for digoxin and dihydrodigoxin, respectively. A two-dimensional thin-layer chromatography of digitalis cardenolides using a continuous development technique has recently been reported by Clarke and Cobb (1979).

Gas-Liquid chromatographic methods of separating simple mixtures of cardiac glycosides after forming trimethylsilyl derivatives were first reported by Jellife and Blankenhorn (1963) and subsequently, by Wilson et al. (1967) and Wilson et al. (1969).

Tan (1969) developed a gas chromatographic method of identification of digitalis cardenolides as their anhydro derivatives. He presented spectral evidence showing that the tertiary 14 β-OH group is neither affected by esterification nor etherification.

Bhandari and Walker (1969) have used gas-liquid chromatography qualitatively as a means of identifying cardiac glycosides. Watson et al. (1972) have described identification of submicrogram amounts of digoxin, digitoxin and their metabolic products by GLC-ECD analysis of genin-diheptafluorobutyrate (-HFB) derivates after separation by paper chromatography. They reported a sensitivity of 25 pg. for both digoxigenin-HFB and digitoxigenin-HFB. A rapid quantitative analysis of digoxin was reported by Kibbe and Araujo (1973), by using gas-liquid chromatography with a flame ionization detector. They used no derivatization and claimed a sensitivity of about 1 mg. per milliliter.

Identification by gas-chromatography - mass spectroscopy of dihydrodigoxin - a metabolite of digoxin in man, has been reported by Watson et al. (1973).

Stroll et al. (1951) used the differences in polarity of the cardiac glycosides to separate them on silica-gel columns, and column chromatography has been used for the enrichment of cardiac glycoside extracts prior to their evaluation by paper chromatography (Kaiser, 1966) or thin-layer chromatography (Hauser et al., 1969).

The advent of high-performance liquid chromatography (HPLC) has introduced a powerful technique for the resolution of complex mixtures of compounds. Evans (1974) has used ion-exchange HPLC in order to separate the components of the "A" series cardenolides, and Lotsher et al. (1975) have described the use of reversed phases for the separation of a number of cardiac glycosides and aglycones.

Castle (1975) has reported the use of HPLC for the quantitative determination of low levels of digoxin, digitoxin and their metabolites. He described: (1)isocratic separation of (a) digoxigenin, digoxigenin mono-digitoxoside, digoxigenin bis - digitoxoside and digoxin; (b) digitoxigenin, digitoxigenin mono-digitoxoside, digitoxigenin bis-digitoxoside and digitoxin; (2) gradient elution separation of (a) digoxin, digitoxin and their metabolites and (b) gitoxin from digoxin and its metabolites. He utilized a variable wavelength detector set at 220 nanometers and a reverse phase column with various mixtures of acetonitrile and water as the mobile phase. This paper indicates a minimum detectable amount of digoxin of 39 ng (isocratic conditions) and 14 ng (gradient conditions) at a signal to noise ratio of two and attenuation of 0.01 a.u.f.s.. Total separation is claimed to be complete in less than thirty minutes.

Lindner and Frei (1976) have described partition high-performance liquid chromatographic systems for the separation of digitalis glycosides of the cardenolide group on silica gel. They used non-polar solvent systems of variable composition and an ultra-violet detector set at 220 nm. A sensititivy of about 15 ng and a separation time of four to twenty minutes have been reported therein.

A high-resolution procedure for digitalis glycoside analysis by derivatization (with 4-nitrobenzoyl chloride) liquid chromatography was published by Nachtman et al. (1976), using normal phase column, and non-polar solvent systems of varying compositions. The separated compounds were glycosides and aglycones of the A, B and C cardenolide series. The reported minimum quantifiable amounts of digoxin and digitoxin were 11.0 ng per milliliter and 11.1 ng per milliliter, respectively.

Separation of digoxin and its metabolites using Sephadex LH-20 column chromatography was reported by Gault et al. (1976).

Cobb (1976) reported normal phase HPLC separations of (1) digitoxigenin, gitoxigenin and digoxigenin; (2) digitoxin, gitoxin digoxin, diginatin and gitaloxin (Cardenolides series A, B, C, D and E, respectively); (3) digoxigenin, digoxigenin mono-digitoxoside, digoxigenin bis-digitoxoside and digoxin; and (4) β-acetyl digoxin, α-acetyl digoxin and digoxin. Quantitation was carried out by using sulphamethoxazole as an internal standard and a UV-detector set at 265 nm. and 234 nm.

A comparison of reversed-phase and partition high-performance liquid chromatography of some digitalis glycosides was published by Ernie and Frei (1977). They used 3.7% and 8.0% methanol in methylene chloride saturated with water (for normal phase HPLC) and 37% acetonitrile in water (for reverse phase HPLC) as solvent systems. The minimum quantifiable

amount at the detection wavelength of 220 nm. was reported to be within the range of 10 to 100 ng.

Gfeller et al. (1977) reported a procedure for post column derivatization in high performance liquid chromatography using the air segmentation principle. They described separations of digoxin, digitoxigenin, lanatoside C and desacetyllanatoside C on a reverse phase column. Minimum detection limits of 0.5 ng (for desacetyllanatoside C, at a signal to noise ratio of 4:1) were reported. The above workers have also indicated that their fluorometric procedure is at least a hundred times more sensitive than UV detection without derivatization.

In a study of the kinetics of digoxin stability in aqueous solution, Sternson and Shaffer (1978) have used reverse phase HPLC with UV detection at 225 nm and a solvent system of methanol/water (55/45) for the separation of digoxin and its degradation products. The genin and the monodigitoxoside, however, were not completely separated. The practical detection limit for digoxin was reported to be 25 ng.

Fugii et al. (1980) have described the separation of digitalis glycosides by micro high-performance liquid chromatography (MHPLC). The above authors have reported the resolution of: (1) a mixture of digitoxin, gitoxin, digoxin, lanatoside A and lanatoside B; (2) digitoxin and its metabolites; (3) gitoxin and its metabolites and (4) digoxin and its metabolites using a reverse-phase column (16.5 cm x 0.5 mm i.d.), UV detection at 220 nm and solvent system consisting of acetonitrile/methanol/water and various mixtures of methanol and water. Separation times were within 30 to 45 minutes.

In a study of digoxin degradation in acidic dissolution medium, Sonobe et al. (1980) have used a reverse-phase column (5 μm, 15 cm x 4 mm i.d.),

a solvent system of 25% acetonitrile and UV detection at 220 nm for the HPLC separation of digoxin and its degradation products. The detection limit for digoxin was reported to be 50 ng.

(B) Analysis of Samples in Biological Fluids

(a) Methods

The introduction of the bioassay of digitalis answered the need for a preparation of known potency for clinical use. This technique was refined in its ultimate form by Friedman et al. (1947) in an embryonic duck heart preparation.

The earliest approaches to the analysis of cardiac glycosides from biological fluids involved colorimetric determinations after separation by paper chromatography (Brown et al., 1957; Ashley et al., 1958). In the past decade, the extensive use of radiolabeled ^{14}C and ^{3}H-cardiac glycosides has yielded basic information as well as data of clinical importance (Doherty, 1961). Metabolic products have been generally identified by comparison with R_f values of authentic compounds after separation by such techniques as paper, column and TLC (Doherty and Perkins, 1962; Marcus et al.,1966).

Most of the experimental studies on the metabolism of cardiac glycosides have been carried out with labelled compounds using TLC (Griffin et al., 1971; Kolenda et al., 1971; Stohs et al., 1971; Voigtlander, 1972; and Beerman, 1972). Faber (1977) reported quantitation of cardiac glycosides with high-performance thin-layer chromatography utilizing programmed multiple development with high-performance micro-thin-layer material. The author claims that this method is better than previous TLC techniques.

The undesirability of using radioactive tracers routinely in human subjects coupled with the low resolution of the above mentioned chromatographic techniques necessitated the development of alternative methods of analysis of metabolites of cardiac glycosides. The need for accurate characterizations of the pharmacokinetic parameters of digoxin has also given additional impetus for the search for better analytical techniques.

The current analytical methods for the analysis of digoxin and digitoxin (the two widely used cardenolides) in plasma are: (1) inhibition of ^{86}Rb transport by the red blood cell (Lowenstein and Corill, 1966); (2) enzymatic isotope displacement (Brooker and Jellife, 1969); and (3) radioimmunoassay (Oliver et al., 1968; Smith et al., 1969). The non-selectivity of the above methods, however, has rendered them unfit for the quantitation of metabolic products.

Watson et al. (1973) reported a gas chromatographic-mass spectroscopic analysis of dihydrodigoxin - a metabolite of digoxin in man. After extraction from urine and plasma, dihydrodigoxin and digoxin were derivatized with heptafluorobutyric anhydride to form the bisheptafluorobutyrate esters. The derivatives could then be used for identification by gas-chromatography - mass-spectrometry or quantitated at the sub-nanogram level by gas chromatography with electron capture detection.

Boguslaski and Schwartz (1975) described a column radioimmunoassay method for the determination of digitoxin in which they used a column of immobilized antibody which acted as both reaction chamber and separation device. They reported a sensitivity of about 150 pg..

Sun and Spiehler (1976) compared radioimmunoassay (RIA) and enzyme immunoassay for determination of digoxin and suggested that the latter method could give comparable results.

Loo et al. (1977) used normal phase HPLC (Lichrosorb Si60) for the separation of digoxin and its main metabolites (Digoxigenin and the mono- and bis-digitoxosides) utilizing a four-component non-polar solvent system. The reported digoxigenin quantitation was carried out by radioimmunoassay in which the detection limit was indicated to be 0.15 ng per milliliter. This two-step HPLC-RIA combination of separation and detection of digoxin and its metabolites from biological fluids, appears to be the final analytic state of the art for the present time.

An emergency (fast) procedure for digoxin radioassay was reported by Chen et al. (1978) in which they claim that the conditions for antigen-antibody interaction and for separation of bound and free fractions have been optimized.

Comparisons (Bergdahl et al., 1979; Kubasik et al., 1979; and evaluations of various kits for plasma digoxin radioimmunoassay (RIA) and improvements of RIA methodology (O'Leary et al., 1979; Halpern and Bordens, 1979; Weiler and Zenik, 1979) have recently been reported.

(b) Rationale for Measurement of Serum Digoxin and Digitoxin

The justification for the analysis of digoxin and digitoxin in biological fluids is derived from a consideration of the following:

(i) Both inotropic and toxic effects of cardiac glycosides are known to be dose-related phenomena. A large number of studies have shown increasing serum digitalis concentrations with increasing dosage (Smith and Haber, 1973) so that at least a statistical correlation should exist between plasma levels and clinical state.

(ii) A number of investigators have documented a relatively constant ratio of digoxin concentration in serum or plasma to that in the myocardium, both in experimental animal studies and human subjects (Doherty et al., 1967; Gulner et al., 1974; Hartel et al., 1976).

(iii) Evidence continues to accumulate indicating that $(Na^+ + K^+)$-ATP-ase is involved in at least some of the actions of cardiac glycosides (Schwartz, 1976). This plasma membrane enzyme transport system is influenced by cardiac glycosides only when these agents are present at the outer cell surface (Caldwell and Keynes, 1959; Hoffman, 1966). Thus the presence of at least one cardiac glycoside receptor in close proximity to the extracellular compartment, provides a basis for the transition of plasma concentration to myocardial effect.

4. Classification of Analytical Methods of Digoxin and Digitoxin

(A) Colorimetric Methods

The methods for the colorimetric determination of digoxin (and other glycosides) can be divided into:

(a) methods based on the sugar moiety, in which a wide variety of reagents are used to react with the digitoxose sugar to give a coloured derivative (e.g. the ferric chloride methods of the B.P. and U.S.P.).

(b) methods based on the butenolide (cardenolide) moiety, in which reagents are reacted with the cardenolide portion resulting in a colored derivative (e.g. the picrate method of the International Pharmacopoeia).

(B) Fluorometric Methods

These methods are based upon the reaction in the steroid moiety, and are mainly dehydration type of reactions that give rise to highly conjugated products. A number of reagents have been developed for this technique.

(C) Chromatographic Methods

Paper, thin-layer and column chromatography have been widely used for the separation of various cardiac glycosides and their aglycones prior to quantitation by colorimetric or fluorometric methods. HPLC and gas-liquid chromatographic techniques have both been utilized for the separation and quantitation of cardiac glycosides.

(D) Biochemical Methods

(i) Radioimmunoassay

By virtue of its high sensitivity, this technique ranks highest in the frequency of usage for the analysis of cardiac glycosides in biological fluids. The basis of the analysis may be schematically depicted as shown in Fig. 17. Unbound digoxin* is adsorbed onto dextran-coated charcoal and the digoxin*-antibody complex in the supernatant portion is determined using a liquid scintillation counter.

(ii) Inhibition of ^{86}Rb transport (uptake) by the red blood cell

This method involves extraction of the glycoside from plasma with methylene dichloride (dichloromethane), evaporation to dryness, reconstitution with normal saline (containing 85 mg. per cent glucose), addition of ^{86}Rb and incubation with red cells at 37°C for 2 hours. The ^{86}Rb uptake is then measured in a scintillation counter. The essentials of this technique are schematically shown in Fig. 18. A sensitivity of less than 1 ng has been reported (Lowenstein and Corril, 1966).

(iii) Enzymatic isotope displacement

Serum samples are prepared by methanol extraction, Zn-Ba precipitation and silica gel column chromatography. After incubation with enzyme, the mixture is centrifuged and the unbound supernatant radioactive digoxin is counted. A sensitivity of 0.15 ng has been reported (Brooker and Jellife, 1969). The procedure is schematically presented in Fig. 19.

(iv) Double isotope - dilution derivative assay

This method involves the following steps:

(a) addition of tritium-labeled digitoxin to plasma or urine sample

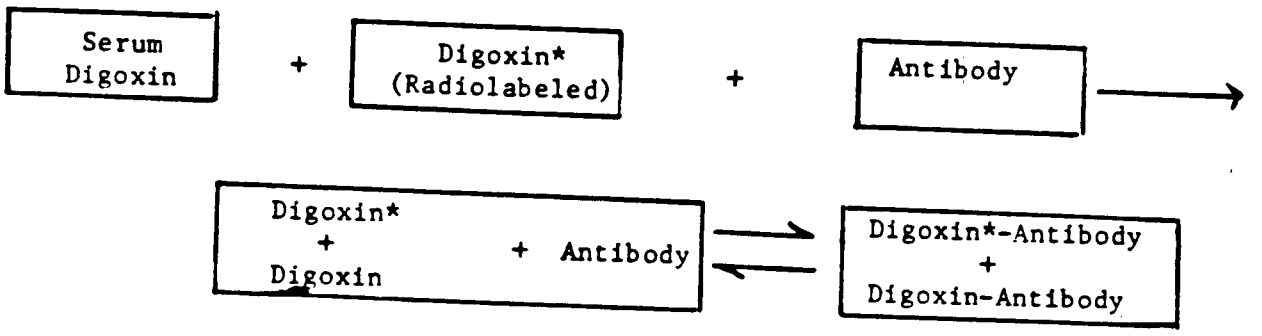


Fig. 17. Diagrammatic Representation of the Procedure for Radioimmunoassay of Digitalis Glycosides.

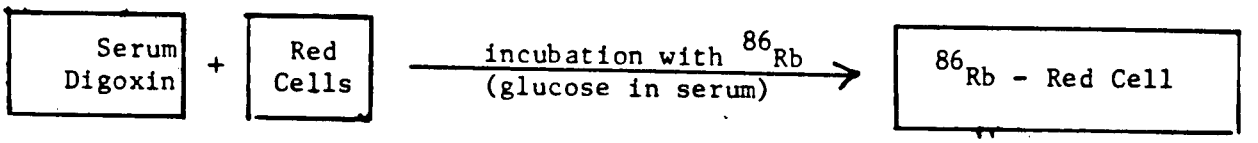


Fig. 18. Diagrammatic Representation of the Assay of Digoxin by Inhibition of ^{86}Rb Transport by the Red Blood Cell.

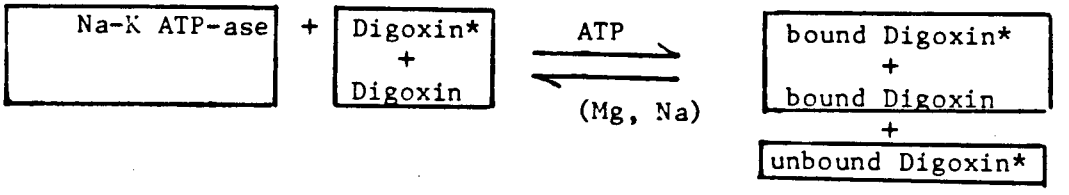


Fig. 19. Diagrammatic Representation of the Assay of Digoxin by Enzymatic Isotope Displacement.

(b) extraction and preliminary purification of digitoxin by liquid-liquid partition and paper chromatography

(c) acetylation with acetic anhydride-1-C^{14}

(d) isolation and purification of digitoxin triacetate by four different paper chromatography systems

(e) measurement of tritium and C^{14} activity by liquid scintillation spectrometry.

A sensitivity of less than 1 ng has been reported (Lucas and Peterson, 1966).

5. Limitations of the Analytical Methods

(A) Sensitivity

If 0.75 mg of digoxin is given orally (the usually recommended loading dose) as one bolus or in divided doses of 0.5 mg and 0.25 mg administered 4-6 hours apart, there would probably be digoxin levels of about 1 ng per milliliter, at the end of 24 hours, in most patients (Smith et al. (1978)). The usual therapeutic range for serum digoxin concentrations is 0.7 to 1.5 ng per milliliter (Smith et al., 1969). It is therefore evident that (1) monitoring serum digoxin levels for optimizing digoxin therapy and (2) pharmacokinetic studies of digoxin require analytic techniques that can provide a sensitivity of at least 0.5 ng per milliliter.

Minimum limits of detection of digoxin of: (1) 10 ng, by thin-layer chromatography (Jelliffe et al., 1967); (2) 25 pg., by gas-liquid chromatography - electron capture detection of the heptafluorobutyrate derivative (Kibbe and Araujo, 1973); (3) 11 ng per ml., by high-performance liquid chromatography - after derivatization with 4-nitrobenzoyl chloride (Nachtmann et al., 1976); (4) 0.2 ng per milliliter, by radioimmunoassay (Smith et al., 1969) have so far been reported in the literature.

Of the two methods that have sensitivities which are within the desirable plasma digoxin concentration range, the GLC-ECD method involves conversion of digoxin to digoxigenin-diheptafluorobutyrate and hence requires a preliminary separatory step, in the presence of metabolites.

The post-column derivatization-HPLC method of Gfeller et al. (1977) has been reported to have a sensitivity (albeit, for desacetyllanatoside C, which has a molecular weight higher than that of digoxin) of 0.5 ng.

It appears, however, that optimization of the conditions of this fluorometric method may provide comparable sensitivity for digoxin.

Radioimmunoassay is the most sensitive technique and is, therefore, the only method that is widely used for the analysis of digoxin and digitoxin in biological fluids.

(B) Selectivity

Digoxin and its metabolites are normally excreted almost entirely in the urine, with only a small percentage reported to be converted to metabolites. These include digoxigenin and its mono and bis-digitoxosides and dihydrodigoxin (Marcus et al., 1964; Luchi and Gruber, 1968; Doherty et al., 1971; Clark and Kalman, 1974). However, there are reports suggesting that digoxin metabolism can be clinically important, and in two patients, 57 per cent (Luchi and Gruber, 1968) and 60 per cent (Clark and Kalman, 1974) of ingested digoxin are believed to have been excreted as metabolites. There is also limited evidence in animals (Kolenda et al., 1971) and in man (Marcus et al., 1964; Clark and Kalman, 1974; Beerman et al., 1972) for conversion of some digoxin to water soluble metabolites such as glucuronides. Metabolism of digitoxin is known to be much more extensive (Doherty, 1973). Hence, it is again evident that monitoring serum digoxin and digitoxin levels requires an analytical method that can selectively account for the drugs and each of their metabolites.

The inherent separatory function of the chromatographic methods makes them uniquely selective. The high-resolution capability of high-performance liquid chromatography does, indeed, make it superbly suitable for selective monitoring of digoxin, digitoxin and their metabolites. Nevertheless, the coupling of this selectivity with the desirable sensitivity is yet to be seen.

On the other hand, the exclusive role of radioimmunoassay's high sensitivity appears to be tarnished by its inherent lack of selectivity. Digoxin radioimmunoassay (RIA) has been reported to show cross-reaction with digitoxin (Boink et al., 1977; Zeegers et al., 1973), Spironolactone (Boink et al., 1977; Mariss 1979) and dihydrodigoxin (Kramer et al., 1976).

A number of other investigators have also reported various factors affecting the results of digoxin radioimmunoassay. Some of these factors are: (a) quenching (Cerceo and Elloso, 1972); (b) chemiluminiscence of serum (Butler, 1971); (c) low intrinsic association constant of the antibody-antigen complex (Smith and Haber, 1973); and (d) effects of albumin on this complex (Voshall et al., 1975). Holtzman et al. (1974) have reported that there is a group of patients for whom the determination of serum digoxin concentrations (by RIA) will give an erroneously low value. In the clinical application of the RIA method, it has been indicated that there is a substantial overlap in the digoxin levels observed for theintoxicated and non-intoxicated patients (Smith et al., 1970). Several workers have suggested that some of these overlaps could be a result of the assay itself not giving the true value for the serum concentrations (Anggard et al., 1972; Fogelman et al., 1971; Burnett et al., 1973).

Klink et al. (1974) have shown that if aqueous test samples (such as urine or alcoholic solutions) and the samples used for constructing the standard curve do not contain equivalent amounts of serum and protein, the quantitative results so derived are subject to considerable error.

Boink et al. (1977) have described some factors affecting a commercial kit for radioimmunoassay of digoxin: (1) erroneously high assay values in serum of (a) pregnant women, (b) patients taking spirono-

lactone and (c) those samples that contain ethanol; (2) too low assay values if haemacel is present in the sample.

(C) Time

By virtue of their selectivity or sensitivity, the most useful methods of analysis of digoxin are chromatography and radioimmunoassay. The quantitative gas-chromatographic methods of analysis reported rely on the production of derivatives and require much time as well as laborious manipulative steps. For instance, Watson and Kalman's (1971) gas-chromatographic method of digoxin assay from plasma takes five hours. Digoxin and digitoxin radioimmunoassay have become routinely available methods because of the recent development of many commercial radioassay kits. Unfortunately, however, use of most of these kits requires two to three hours from receipt of specimen to completion of the report (Chen et al., 1978).

II. EXPERIMENTAL

1. Apparatus

(a) Beckman Model 322 Gradient Liquid Chromatograph equipped with:

- modular dual pump system (Model 100A and 110A pumps)
- microprocessor system controller (Model 420)
- dynamically stirred gradient mixing chamber
- Model 210 sample injection valve (20 µl loop)
- Model 100-10 variable wavelength UV detector
- Shimadzu Chromatopac electronic data processor (Model C-R1A)
- Ultrasphere ODS (C-18) reverse-phase column (25 cm x 46 mm i.d.) with a particle size of 5 µm.

(b) Waters Liquid Chromatograph equipped with:

- Model 6000 solvent delivery system
- Model 450 variable wavelength detector
- U6K injector
- Ominiscribe recorder (Model B-5000)
- Spherisorb ODS column (25 cm x 3.2 mm i.d.) with a particle size of 10 µm.

(c) Altex High-performance Liquid Chromatograph equipped with:

- Model 110 pump
- 20 µl loop injection valve
- Model 153 UV detector
- Ultrasphere ODS (C-18) reverse-phase column (25 cm x 4.6 mm i.d.)

with a particle size of 5 μm

- Omniscribe Model B-5000 dual pen recorder

(d) Post-column Fluorogenic Derivatization Set-up consisting of the following:

- Technicon peristaltic pump
- Solvaflex pump tubes (Technicon color codes, orange/green and orange/blue)
- Solvaflex delivery tube (1 mm i.d.)
- Acidflex pump tubes (Technicon color code, white)
- Acidflex delivery tube (1 mm i.d.)
- Technicon D2 connectors
- Modified Technicon C4 debubbler with side tube of 1 mm i.d.
- Mixing coils (120 x 0.1 cm i.d. and 240 x 0.1 cm i.d.)
- Cooling coil (180 x 0.1 cm i.d.)
- Reaction coil (1000 x 0.1 cm i.d.)
- Haake Thermoregulator (Type E51)
- Waters Model 420 fluorescence detector

(e) Accumet Model 220 pH Meter (Fisher)

(f) Thelco oven (GCA/Precision Scientific)

(g) Lab-Line oven (Lab-Line Instruments Inc.)

(h) Flash Evaporator (Buchler Instruments)

(i) Cahn Electrobalance (Ventron Instruments Corp.)

(j) Spectrophotometers:

- Beckman Models 24 and 25
- Beckman IR-10

(k) Mass spectrometer (MAT-111)

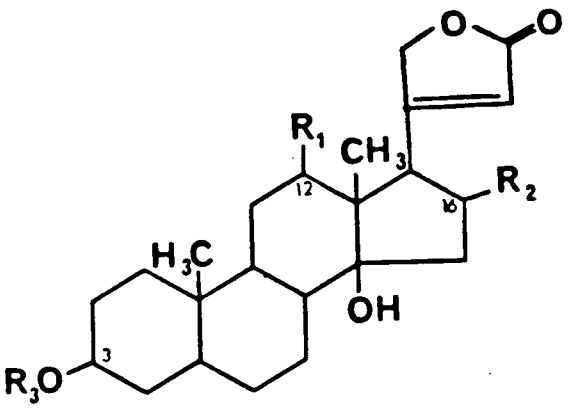
2. Materials

(a) Cardiac glycosides and aglycones:

- Digoxin
- Digitoxin
- Digoxigenin
- Digoxigenin monodigitoxoside
- Digoxigenin bisdigitoxoside
- Digitoxigenin
- Digitoxigenin monodigitoxoside
- Digitoxigenin bisdigitoxoside
- α-acetyldigoxin
- β-acetyldigoxin
- Gitoxin
- Dihydrodigoxigenin

All of the above items were of Analytical Grade and were obtained from Boehringer Mannheim Corp., Mannheim, G.F.R. Digoxin and digitoxin were identified by infrared spectrophotometry and USP XX methods. The chemical structures of the glycosides and aglycones are shown in Table VII. The chemical structure of dihydrodigoxigenin in comparison with that of digoxin is presented in Fig. 20.

TABLE VII. CHEMICAL STRUCTURES OF SOME CARDIAC GLYCOSIDES OF THE CARDENOLIDE SERIES

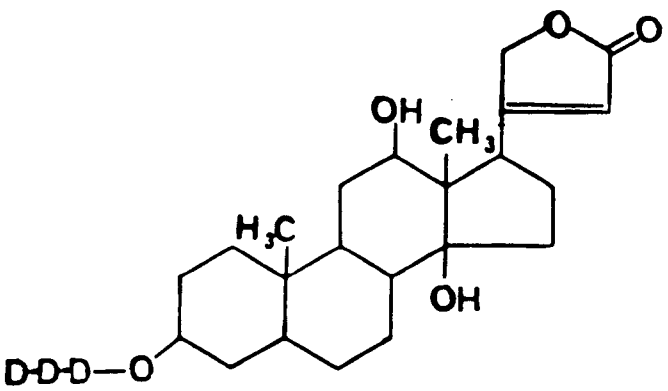


(Basic Cardenolide Structure of Cardiac glycosides)

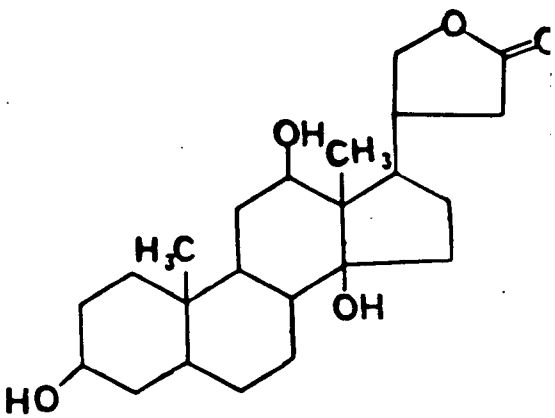
Cardenolide Series	R_1	R_2	R_3	Compound
A	H	H	H	Digitoxigenin
	H	H	D	Digitoxigenin monodigitoxoside
	H	H	D-D	Digitoxigenin bisdigitoxoside
	H	H	D-D-D	Digitoxin
B	H	OH	D-D-D	Gitoxin
C	OH	H	H	Digoxigenin
	OH	H	D	Digoxigenin monodigitoxoside
	OH	H	D-D	Digoxigenin bisdigitoxoside
	OH	H	D-D-D	Digoxin
	OH	H	D-D-D / AC	α- Acetyldigoxin / β- Acetyldigoxin

D= DIGITOXOSE

A= ACETYL



DIGOXIN (D=digitoxose)



DIHYDRODIGOXIGENIN

Fig. 20. Chemical Structuresof Digoxin and Dihydrodigoxigenin.

(b) 17α-ethynylestradiol,Sigma Chemical Co., Analytical Grade

(c) 17α-methyltestosterone, Sigma Chemical Co., Analytical Grade

(d) Hydrocortisone, Sel-Win Chemicals Ltd., Analytical Grade

(e) Ascorbic acid, British Drug Houses (Canada) Ltd., Analytical Grade

(f) Hydrogen peroxide, 30%, British Drug Houses (Canada) Ltd., Analytical Grade

(g) Hydrochloric acid, American Scientific and Chemical Co., Reagent Grade

(h) Brij^{R} 35, Atlas Chemical Industries Canada Ltd.

(i) Ethanol, Stanchem (Canada) Ltd., Reagent Grade

(j) n-propyl alcohol, Caledon Laboratories Ltd., Reagent Grade

(k) Chloroform, Caledon Laboratories Ltd., Reagent Grade

(l) Sulfuric acid, American Scientific and Chemical Co., Reagent Grade

(m) Phosphorus pentoxide, British Drug Houses (Canada) Ltd., Reagent Grade

(n) Ferric Chloride, The Nichols Chemical Co. Ltd., Reagent Grade

(o) Sodium Carbonate, Anachemia Chemicals Ltd., Reagent Grade

(p) Formamide, Mallinckrodt Chemical Works, Reagent Grade

(q) Chromatographic Siliceous Earth (Celite 545), Supelco, Inc., Analytical Grade

(r) Benzene, Caledon Laboratories Ltd., Reagent Grade

(s) Picric acid, Matheson Coleman and Bell Co., Reagent Grade

(t) Whatman No. 1 filter paper, W. and R. Balston Ltd.

(u) HPLC solvents:

- Methanol
- Dichloromethane
- Isopropanol
- Water

The above solvents were of HPLC Grade and were purchased from Fisher Scientific Co., Pittsburgh, PA., U.S.A.

(v) HPLC solvent systems:

- water/methanol/isopropanol/dichloromethane: 47/40/9/4
- " " " " : 45/37/12/6
- " " " " : 43/35/15/7
- " " " " : 51/42/5/2

- " " " " : 49/41/7/3
- " " " " : 41/34/17/8
- " " " " : 38/32/20/10
- " " " " : 46/39/10/5

- " " " " : 45/38/11/6
- " " " " : 48/40/8/4
- " " " " : 52/43/3/2

- " " " " : 51/43/4/2

(w) Equine estrogens:

- Estrone
- Equilin
- Equilenin
- 17α-estradiol
- 17β-estradiol
- 17α-dihydroequilin
- 17β-dihydroequilin
- 17α-dihydroequilenin
- 17β-dihydroequilenin

The above estrogens were of Analytical Grade and were obtained from Division of Ayerst McKenna and Harrison Ltd., Montreal, Quebec. Their chemical structures are given in Fig. 21.

(x) Dosage Forms:

- Lanoxin^{R} tablets, 0.125 mg
- Lanoxin^{R} tablets, 0.25 mg
- Lanoxin^{R} injection, 0.05 mg/ml
- Lanoxin^{R} injection, 0.25 mg/ml
- Lanoxin^{R} Elixir, 0.05 mg/ml

The above dosage forms were purchased from Wellcome Medical Division, Burroughs Wellcome Inc.

- Natigoxin^{R} tablets, 0.25 mg, Compagne Pharmacentique Vita (LTEE)
- Digitoxin^{R} tablets,0.1 mg, Wyeth Ltd. (Canada)
- Crystodigin^{R} injection, 0.2 mg/ml, Eli Lilly and Company

(y) Digitalis purpurea leaves, obtained from Dr. A. Goodeve's private garden in the Vancouver area.

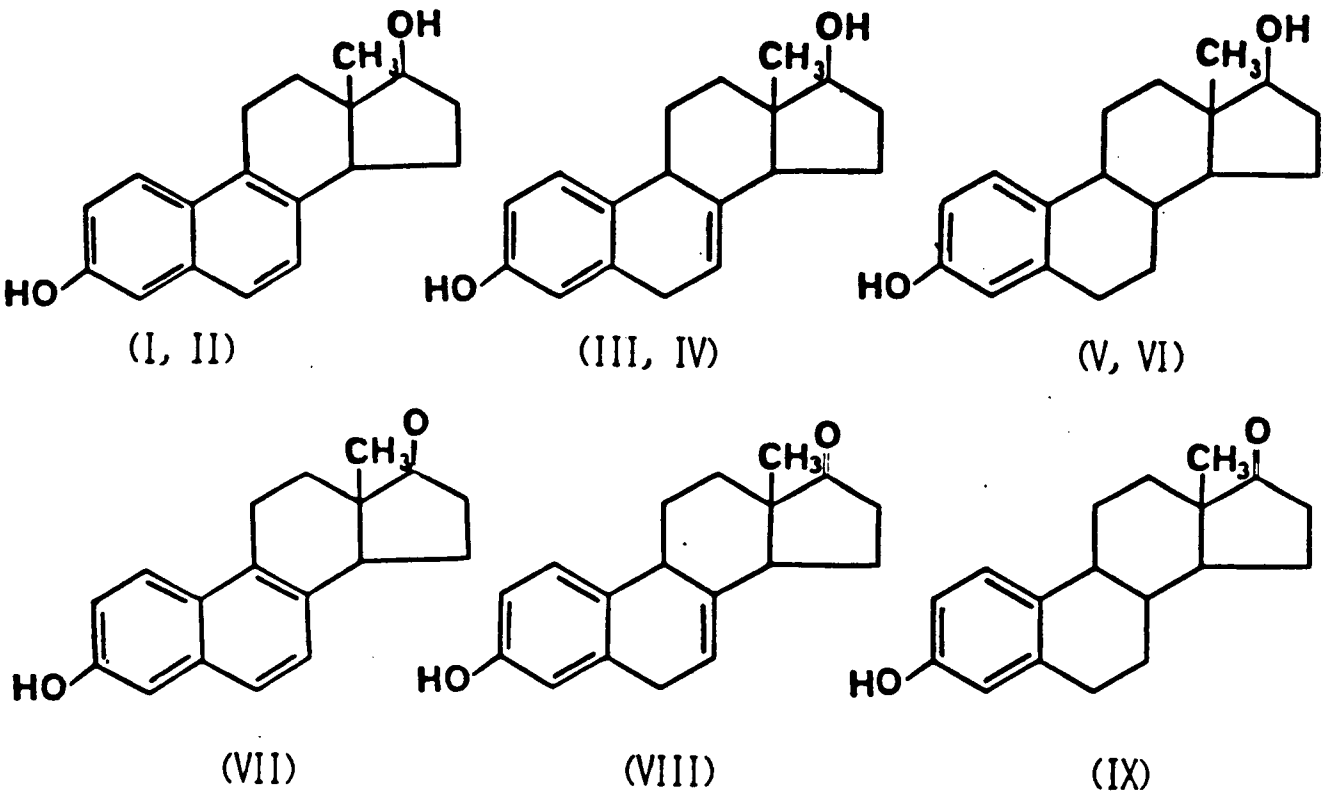


Fig. 21. Chemical Structures of Equine Estrogens. I, II = 17β- and α-dihydroequilenin; III, IV = 17β- and α-dihydroequilin; V, VI = 17β- and α-estradiol; VII = equilenin; VIII = equilin; IX = estrone.

3. Preparation of HPLC Solvent Systems

Freshly prepare 500 ml of the HPLC solvent systems by mixing accurately measured proportions (as shown in the respective chromatograms) of water, methanol, isopropanol and dichloromethane.

4. Equilibration of the HPLC Column

Equilibrate the HPLC column by pumping the freshly prepared solvent system for about one hour. Monitor the stability of the baseline using the appropriate detector and recorder.

5. Determination of Retention Time

Inject a sample of a solution of each compound into the Liquid Chromatograph and record the retention time. Repeat the injection three times and calculate the average retention time.

6. Separation of Digoxin, Digitoxin and their Metabolites or Degradation Products and Impurities

(A) Isocratic Elution

Equilibrate the column with the solvent system. Dissolve a mixture of the compounds in the eluting solvent system and inject a sample into the Liquid Chromatograph which has been adjusted previously to conditions of flow rate of 1.2 ml/min; UV detection at 220 nm, range of 0.02; and a chart speed of 0.5 cm/min. Record the chromatogram.

(B) Solvent Switchover Elution

Dissolve a mixture of the compounds in the initial solvent system (water/methanol/isopropanol/dichloromethane: 49/41/7/3) and inject a sample into the Liquid Chromatograph which has previously been adjusted to conditions of: flow rate of 1.2 ml/min; UV detection at 220 nm, range of 0.1; and a chart speed of 0.5 cm/min. Switchover to a second solvent system (water/methanol/isopropanol/dichloromethane: 41/38/17/8) at a point in time corresponding to the peak of digoxigenin bisdigitoxoside. Record the chromatogram. At the end of the chromatographic run, re-equilibrate the column using the initial solvent system.

(C) Gradient Elution

Equilibrate the column with the initial solvent system (water/methanol/isopropanol/dichloromethane: 49/41/7/3). Dissolve the mixture of the compounds in the initial solvent system and inject a sample into the Gradient Liquid Chromatograph which has been adjusted to the following conditions: flow rate of 1.2 ml/min; UV detection at 220 nm, range of 0.1; a chart speed of 0.5 cm/min; and a linear gradient of 0 to 100% of the second solvent system (water/methanol/isopropanol/dichloromethane: 38/32/20/10) programmed between the chromatographic times of 2.5 and 3 minutes. Record the chromatogram. Re-equilibrate the column using the initial solvent system.

The chemical structures of the glycosides and aglycones that were employed in this study are presented in Table VII.

7. Separation of Digoxin and its Metabolites after Fluorogenic Post-Column Derivatization using the Air-Segmentation Principle with 100% Fluid Recovery

(A) Preparation of Hydrogen Peroxide Solution

Add 1 ml of hydrogen peroxide solution (30% v/v) to 200 ml of water and mix.

(B) Preparation of Dehydroascorbic Acid Solution

Weigh 100 mg of ascorbic acid and dissolve in 200 ml of water. Add 5 ml of the hydrogen peroxide solution dropwise with continuous stirring.

(C) Preparation of Brij^{R} 35 Solution

Weigh 1 g of Brij^{R} 35 and add to 99 g of water and mix.

(D) Dual Detector Monitoring of the Separation of Digoxin and its Metabolites before and after Fluorogenic Derivatization.

Position solvaflex orange-green and orange-blue and acidflex white pump tubes on the Technicon pump to deliver air, dehydroascorbic acid solution and hydrochloric acid, respectively. Segment the stream of hydrochloric acid by connecting the acid and air delivery tubes with a miniaturized D_2 connector (with side arms of 1 mm i.d.). Connect the tubes delivering the air-segmented hydrochloric acid and the HPLC column effluent that has passed through a UV detector, using a miniaturized D_2 connector and pass the mixture through a mixing coil (120 x 0.1 cm i.d.). Connect the delivery tube leading out of the first mixing coil to the dehydroscorbic acid solution delivery tube using a miniaturized D_2 connector and shunt the mixture into a second mixing coil (240 x 0.1 cm i.d.). Pass the segmented and mixed solution

through a reaction coil (1000 x 0.1 cm i.d.; 55°C), cooling coil (180 x 0.1 cm i.d.), debubbling, set-up, fluorescent detector before finally directing it into a waste receptacle. Assemble the debubbling set-up for 100% fluid recovery as follows: Using acidflex tubing connect the vertical exit of the debubbler to the tapering end (1 mm i.d.) of a glass tube (84 x 1.1 cm i.d.). Position the glass tube as high as possible so that when it is three-quarters full with the circulating fluid there will be sufficient hydrostatic pressure for the fluid entering the debubbler to pass through the horizontal exit into the fluorescence detector,while the air segments escape through the vertical exit as bubbles. Optimize the debubbling process to achieve 100% fluid recovery by careful up and down adjustments of the position of the waste receptacle. Adjust the flow rate of the fluid coming out of the fluorescence detector to about 1 ml/min. (A schematic diagram of the post-column fluorogenic derivatization system is presented in Fig. 22.)

(E) HPLC Procedure and Conditions

Equilibrate the column with the solvent system (water/methanol/isopropanol/dichloromethane: 50/41/6/3). Dissolve a mixture of dihydrodigoxigenin, digoxin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside in the eluting solvent system. Inject a sample of the solution into the Liquid Chromatograph which has previously been adjusted to the following conditions: flow rate of 0.4 ml/min; fluorescent detection at $\lambda_{exc.}$ of 360 nm and $\lambda_{em.}$ (cut off) of 460 nm; UV detection at 254 nm; and a chart speed of 0.5 cm/min. Record the chromatogram.

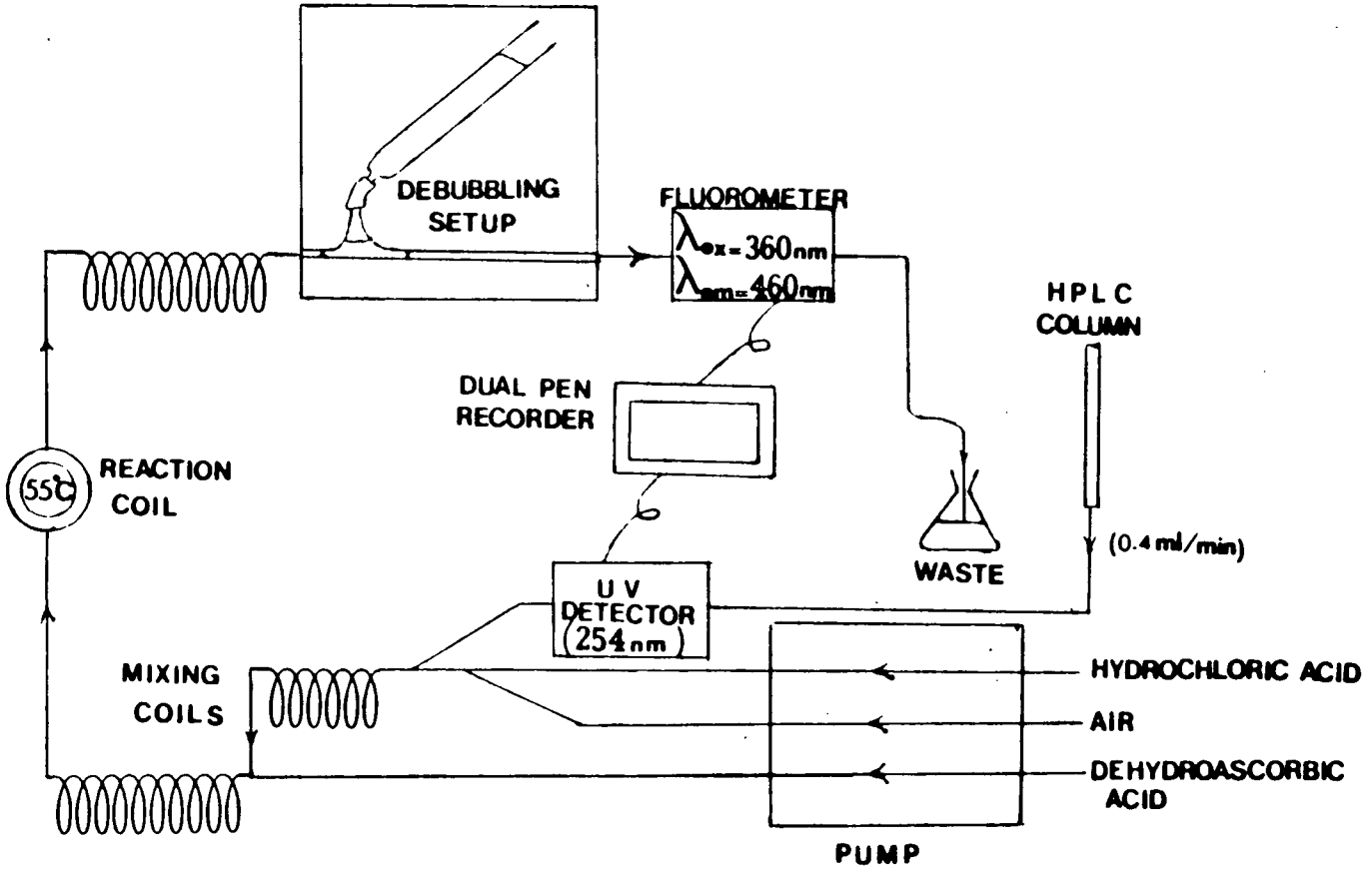


Fig. 22. Schematic Diagram of the Post-Column Fluorogenic Derivatization System using the Air-Sementation Principle and 100% Fluid Recovery Set-up.

8. Separation of Nine Equine Estrogens as Evidence of Selectivity

Equilibrate the column with the solvent system. Dissolve a mixture of the equine estrogens in 50% ethanol. Inject a sample of the solution into the Liquid Chromatograph which has previously been adjusted to the following conditions: flow rate of 1.2 ml/min; UV detection at 220 nm; and chart speed of 0.5 cm/min. Record the chromatogram.

9. Isolation of Digitoxin from Digitalis purpurea Leaf

(A) Extraction

Macerate 10 g of the dried leaf in 50 ml of 20% v/v ethanol for 48 hours in a stoppered conical flask. Add 50 ml of 20% ethanol, stopper the flask and agitate for four hours using a mechanical shaker. Filter the slurry through a funnel plugged with cotton wool. Wash the residue with additional portions of 20% ethanol until 200 ml of filtrate has been collected. Add 20 ml of 0.35 M sodium hydroxide solution, stir and set aside for 15 minutes to ensure de-acetylation of any acetyl-digitoxin that may be present. Adjust the pH of the solution to about 6.5 with 0.35 M hydrochloric acid solution. Extract the liquor with four, 20 ml portions of dichloromethane and dry each extract by passing it through the same 10 g of anhydrous sodium sulfate, packed into a filter funnel that is fitted with a plug of glasswool. Evaporate the dichloromethane extract to dryness and dissolve the residue in 20 ml of methanol.

(B) HPLC Procedure and Conditions

Equilibrate the column with the solvent system (water/methanol/isopropanol/dichloromethane: 45/38/11/6) and inject a sample of the

methanolic solution into the Liquid Chromatograph which has been previously adjusted to the following conditions: flow rate of 1.1 ml/min; UV detection at 220 nm and a chart speed of 0.5 cm/min. Record the chromatogram.

10. Analysis of Digoxin in its Dosage Forms

(A) Infrared spectrum of Digoxin

Prepare a KBr pellet and record the spectrum on Beckman IR-10 spectrophotometer.

The IR spectrum obtained was compared with a Sadtler reference spectrum and found to have the same characteristic absorption bands (Fig. 23).

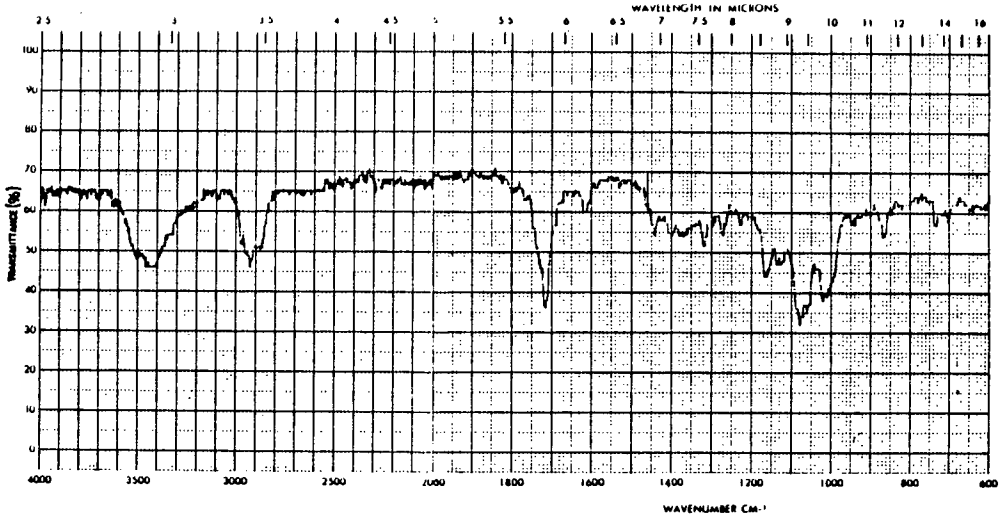
(B) Spectral Characteristics of Digoxin

Using a Cahn electrobalance weigh accurately 10.0 mg of digoxin and transfer into a 100 ml volumetric flask with the aid of about 50 ml of boiling methanol. Dissolve, cool to room temperature and make to volume with methanol. Dilute 10 ml of the above solution to 100 ml with 35% methanol. Record the spectrum on a Beckman Model 24 spectrophotometer using 35% methanol as the blank.

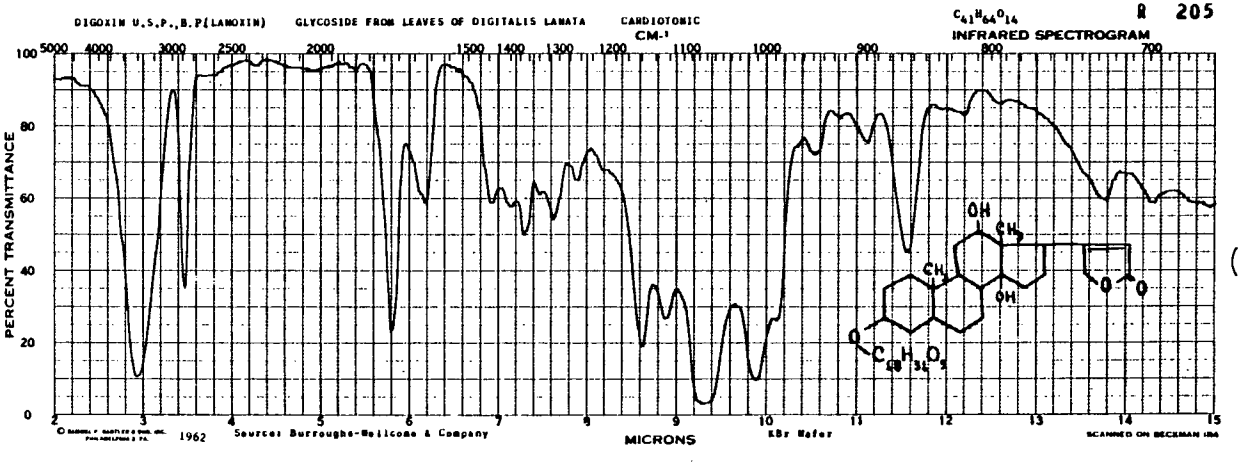
The spectral absorbance curve that was obtained is shown in Fig. 24. The maximum absorption of radiant energy was found to occur at 220 nm.

(C) HPLC Procedure and Conditions for the Analysis of Tablets and Injection

Equilibrate the column with the solvent system (water/methanol/isopropanol/dichloromethane: 47/40/9/4). Inject a 20 µl sample into the Liquid Chromatograph which has been previously adjusted to the following conditions: flow rate of 1.2 ml/min; UV detection at 220 nm, range of 0.02; and a chart speed of 0.5 cm/min. Record the chromatogram.



(a)



(b)

Fig. 28. (a) The Infrared Spectra (KBr) of Digoxin as obtained with the Beckman IR-10; and (b) Sadtler Reference IR Spectra.

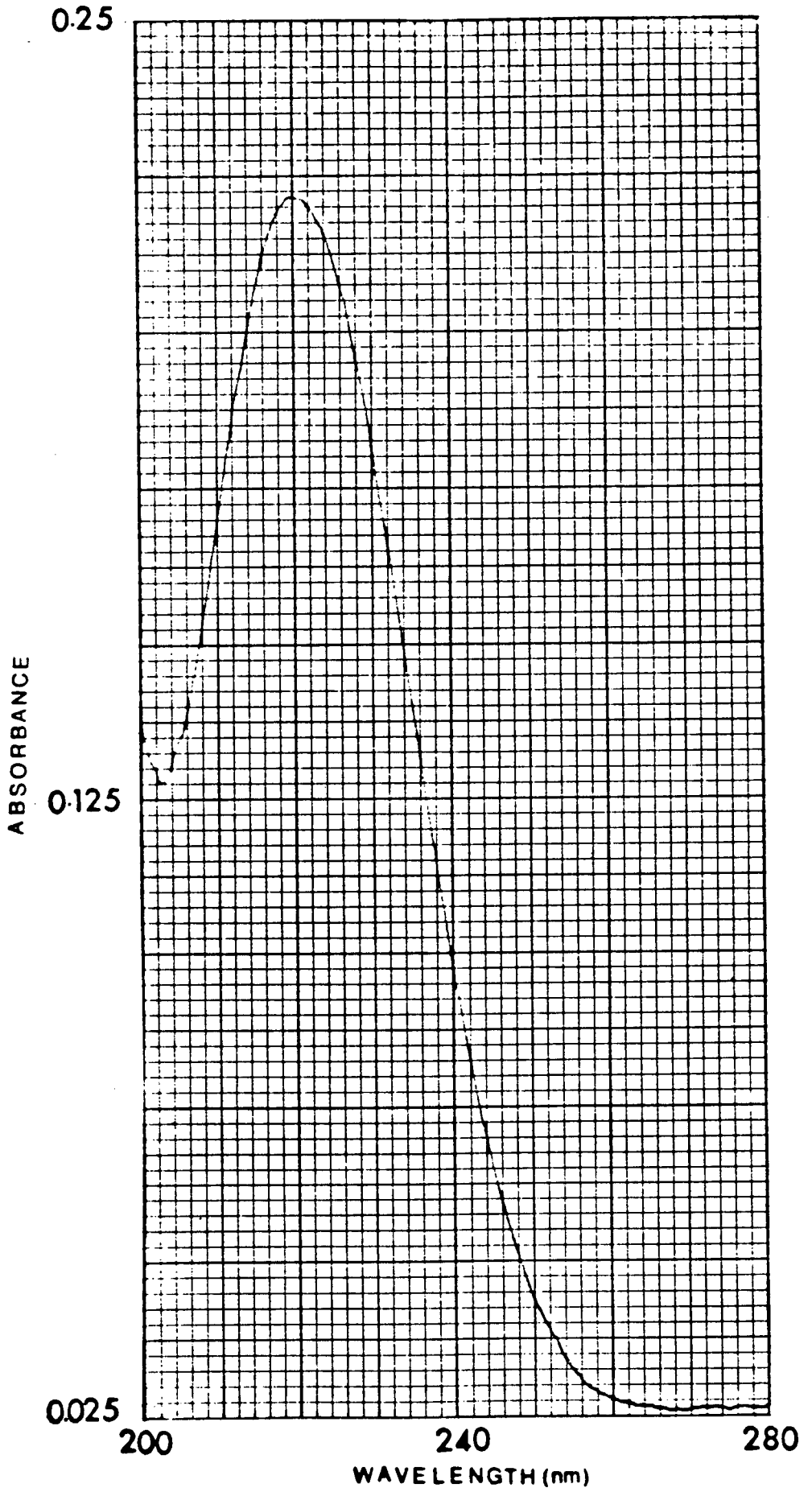


Fig. 24. A Spectral Absorbance Curve for Digoxin in 35% Methanol.

(D) HPLC Procedure and Conditions for the Analysis of Elixir

Proceed as in (C) using in this case a solvent system of water/methanol/isopropanol/dichloromethane: 51/42/5/2.

(E) Preparation of Internal Standard Solutions

Weigh accurately 100.0 mg of 17α-ethynylestradiol and transfer into a 100 ml volumetric flask with the aid of about 50 ml of methanol. Dissolve, make to volume with methanol and mix. Similarly, prepare a solution of 100.0 mg of 17β-dihydroequilin in 100 ml of methanol.

(F) Preparation of Standard Solutions of Digoxin

Accurately weigh, using a Cahn electrobalance, 20.0 mg of digoxin and transfer into a 100 ml volumetric flask with the aid of about 80 ml of boiling methanol. Dissolve, cool to room temperature and make to volume with methanol. Transfer aliquots of 0.5, 1.0, 2.0, 5.0, 10.0 and 12.5 ml of the above stock solution to 100 ml volumetric flasks. To each flask, add 2.5 ml of the internal standard solution and an amount of methanol sufficient to bring the volume to 35 ml. Dilute the solution in each flask to 100 ml with distilled water and mix. The six solutions will, therefore, have digoxin concentrations of 1, 2, 4, 10, 20 and 25 ng/µl while the concentration of the internal standard in each solution will be 25 ng/µl.

(G) Preparation of Calibration Curves

Inject a 20 µl sample of each standard solution into the Liquid Chromatograph and obtain area values for digoxin and the internal standard. Make six determinations for each solution.

The calibration curves that were obtained using 17α-ethynylestradiol and 17β-dihydroequilin as internal standards are presented in Figs. 25, 26, and 27 and indicate linear relationships with lines passing through the origin.

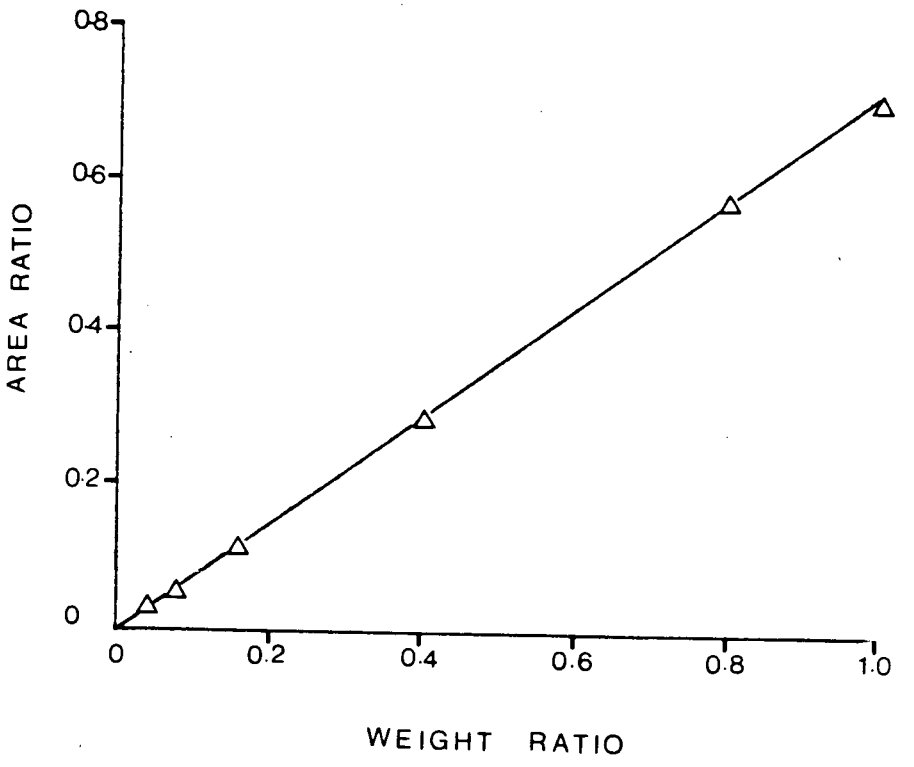


Fig. 25. A Calibration Curve for Digoxin in 35% Methanol as obtained with a Solvent System of water/methanol/isopropanol/dichloromethane: 47/40/9/4. Area and weight ratios are in terms of digoxin/internal standard (17α-ethynylestradiol). The least squares line of best fit is: $y = 0.7137x - 0.0026$. The coefficient of determination (r^2) = 0.9976.

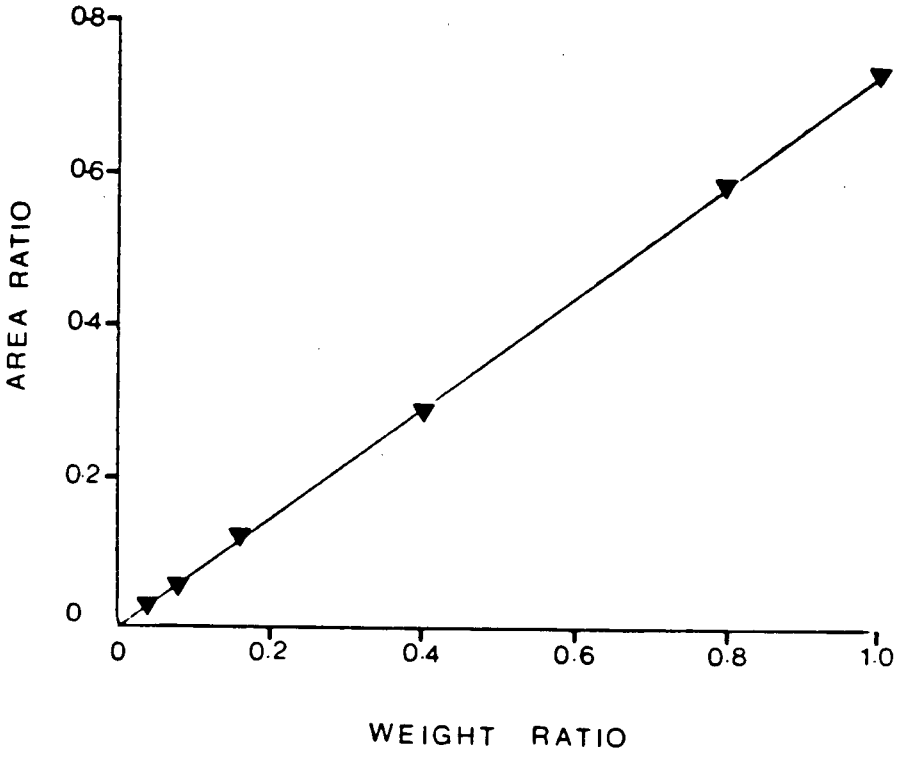


Fig. 26. A Calibration Curve for Digoxin in 35% Methanol as obtained with a Solvent System of water/methanol/isopropanol/dichloromethane: 51/42/5/2. Area and weight ratios are in terms of digoxin/internal standard (17α-ethynylestradiol). The least squares line of best fit is defined by the equation, $y = 0.7199x + 0.0049$. The coefficient of determination (r^2) = 0.9924.

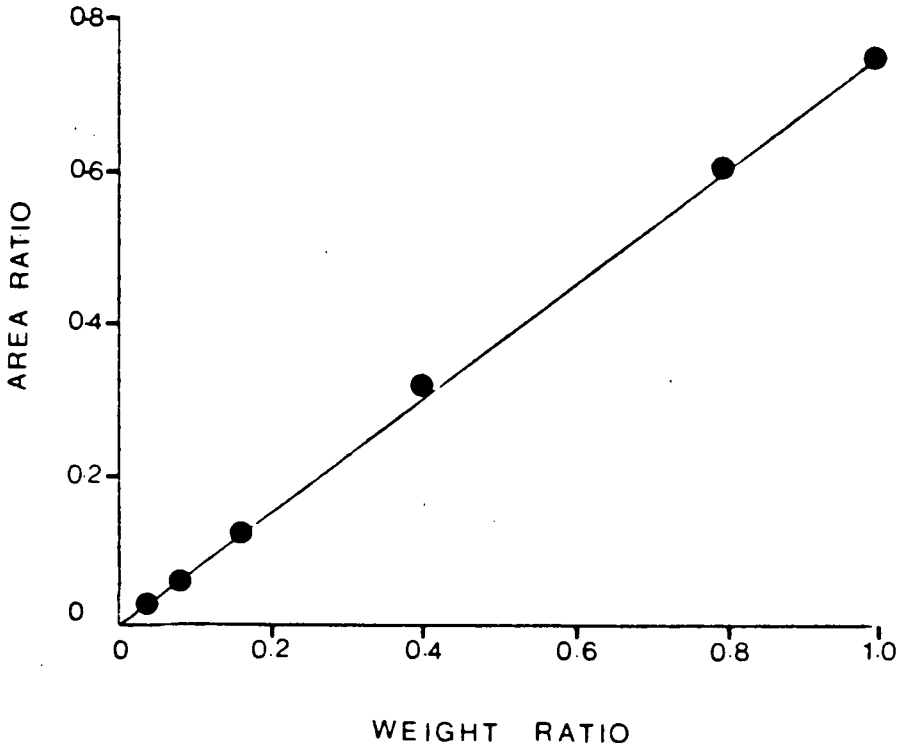


Fig. 27. A Calibration Curve for Digoxin in 35% Methanol as obtained with a Solvent System of water/methanol/isopropanol/dichloromethane: 51/42/5/2. Area and weight ratios are in terms of digoxin/internal standard (17β-dihydroequilin). The least squares line of best fit is defined by the equation, $y = 0.7368x + 0.0092$. The coefficient of determination (r^2) = 0.9972.

(H) Sample Preparation of Digoxin Dosage Forms

(a) Composite Tablet Assay

Weigh not less than 30 digoxin tablets that are selected at random, and triturate to a fine powder. Accurately weigh an amount of the powdered tablet material equivalent to 1.25 mg of digoxin and quantitatively transfer into a 100 ml volumetric flask. Add 10 ml of distilled water and swirl the flask for 2-3 minutes. Add 32.5 ml of methanol and mechanically shake the mixture for about 15 minutes. Filter the suspension using a No. 1 Whatman filter paper and wash the residue with three, 5 ml portions of distilled water. Collect the filtrate and washings in a 100 ml volumetric flask. Add a 2.5 ml aliquot of 17α-ethynylestradiol internal standard solution, dilute the mixture with distilled water to a volume of 100 ml and mix.

(b) Single Tablet Assay

Place one tablet in a 100 ml volumetric flask and proceed as directed in the Composite Tablet Assay above, beginning with "add 10 ml of distilled water and swirl the flask for 2-3 minutes".

(c) Injectable Formulation Assay

Empty the contents of 20 randomly selected ampules into a 50 ml conical flask and mix. Transfer a 2 ml aliquot of the above solution into a 10 ml volumetric flask (for the 0.05 mg/ml injection) or to a 50 ml volumetric flask (for the 0.25 mg/ml injection). Add 0.25 ml or 1.25 ml aliquots of 17α-ethynylestradiol internal standard solution to the 10 ml or 50 ml volumetric flasks, respectively. Add 2.8 ml of methanol into the 10 ml volumetric flask or 14.0 ml of methanol into the 50 ml volumetric flask. Dilute the solution to volume with distilled water and mix.

(d) Elixir Assay

Transfer a 20 ml aliquot of the elixir into a 100 ml volumetric flask. Add a 2.5 ml aliquot of the internal standard solution (17α-ethynyl-estradiol or 17β-dihydroequilin) and 20 ml of methanol. Dilute the solution to 100 ml with distilled water and mix.

(I) Quantitation

After ascertaining the linearity of the relationship of area ratios versus weight ratios, quantitation of digoxin was carried out by an internal standard single-point automatic calibration method using a Shimadzu Chromatopac C-R1A data processor. Response factors were determined as follows:

> Transfer a 5 ml aliquot of the digoxin stock solution into a 100 ml volumetric flask. Add 2.5 ml of internal standard solution and 27.5 ml of methanol and dilute the solution to 100 ml with distilled water and mix. Make triplicate injections of a 20 µl sample into the Liquid Chromatograph and obtain the response factor.

The response factors of digoxin obtained by using 17α-ethynylestradiol as internal standard and solvent systems of water/methanol/isopropanol/dichloromethane: 47/40/94 and 51/42/5/2 were 1.4261 and 1.3864, respectively. The response factor obtained using 17β-dihydroequilin and the latter solvent system was 1.3652. Calculation of amount of digoxin in a sample was based on the equation:

$$W_{(S)} = \frac{A_{(S)} \cdot W_{(I.S.)} \cdot \text{Response Factor}}{A_{(I.S.)}} \qquad \text{(Eq. 15)}$$

where, $W_{(S)}$ = weight of digoxin

$A_{(S)}$ = area of digoxin

$W_{(I.S.)}$ = weight of internal standard

$A_{(I.S.)}$ = area of internal standard

Peak identity was automatically monitored by the data processor.

(J) Determination of Precision of Tablet Assay

Weigh a total of 40 digoxin tablets and triturate to a fine powder. Transfer six aliquots of accurately weighed tablet material, each equivalent to 1.25 mg of digoxin, into six 100 ml volumetric flasks. Proceed as directed in the Composite Tablet Assay, beginning with "add 10 ml of distilled water and swirl for 2-3 minutes". Make three injections for each sample.

(K) Determination of Percentage Recovery of Digoxin from Tablets

Weigh a total of 60 digoxin tablets and triturate to a fine powder. Transfer nine aliquots of accurately weighed tablet material, each equivalent to 1.25 mg of digoxin into nine 100 ml volumetric flasks. Add an accurately weighed aliquot of digoxin reference standard, equivalent to 0.625 mg, into each of six flasks. Treat each of the nine samples as directed in the Composite Tablet Assay, beginning with "add 10 ml of distilled water and swirl for 2-3 minutes". Make three injections for each sample.

11. Analysis of Digitoxin in its Dosage Forms

(A) Infrared Spectrum of Digitoxin

Prepare a KBr pellet and record the spectrum on a Beckman IR-10 spectrometer.

The IR spectrum obtained was compared with a Sadtler reference spectrum and found to have the same characteristic absorption bands (Fig. 28).

(B) Spectral Characteristics of Digitoxin

Weigh accurately, using a Cahn electrobalance, 10.0 mg of digitoxin and transfer into a 100 ml volumetric flask, with the aid of about 50 ml of boiling methanol. Dissolve, cool to room temperature and make to volume with methanol. Dilute 10 ml of the above solution to 100 ml with 35% methanol. Record the spectrum on a Beckman Model 24 spectrophotometer using 35% methanol as the blank.

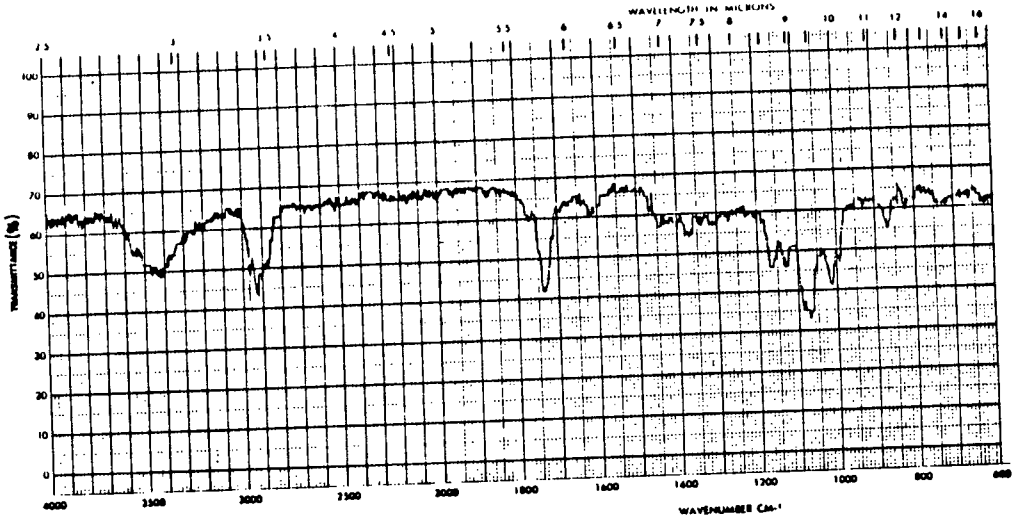
A spectral absorbance curve that was obtained is shown in Fig. 29. The maximum absorption of radiant energy was found to occur at a wavelength of 220 nm.

(C) HPLC Procedure and Conditions for the Analysis of Tablets and Injection

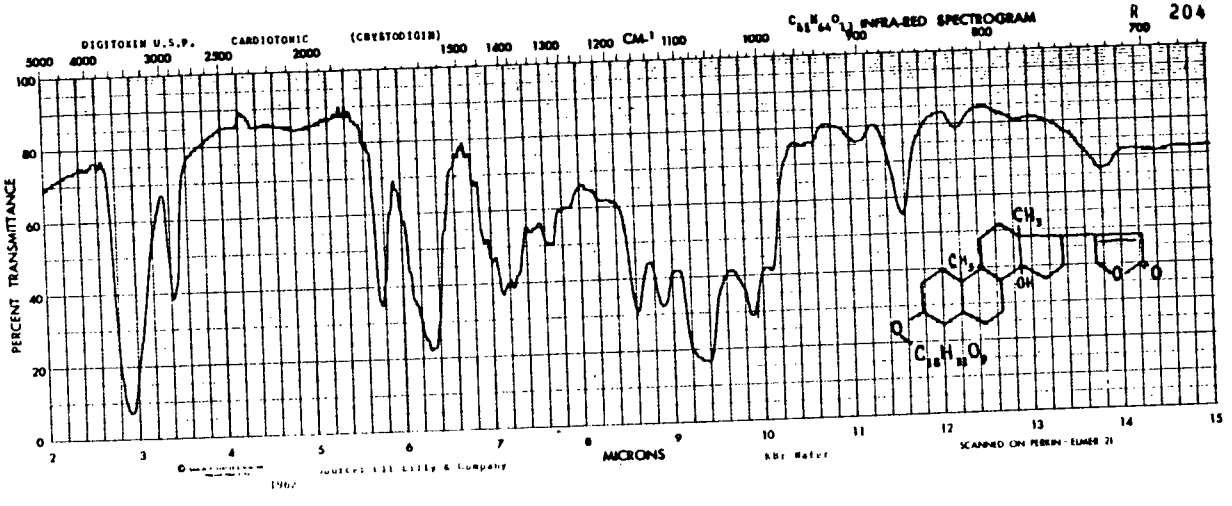
Equilibrate the column with solvent system, water/methanol/isopropanol/dichloromethane: 45/38/11/6. Inject a 20 µl sample into a Liquid Chromatograph which has been previously adjusted to the following conditions: flow rate of 1.1 ml/min; UV detection at 220 nm, range of 0.02; and a chart speed of 0.5 cm/min. Record the chromatogram.

(D) Preparation of Internal Standard Solution

Accurately weigh 100.0 mg of 17α-methyltestosterone and transfer into a 100 ml volumetric flask with the aid of about 50 ml of methanol. Dissolve, make to volume with methanol and mix.



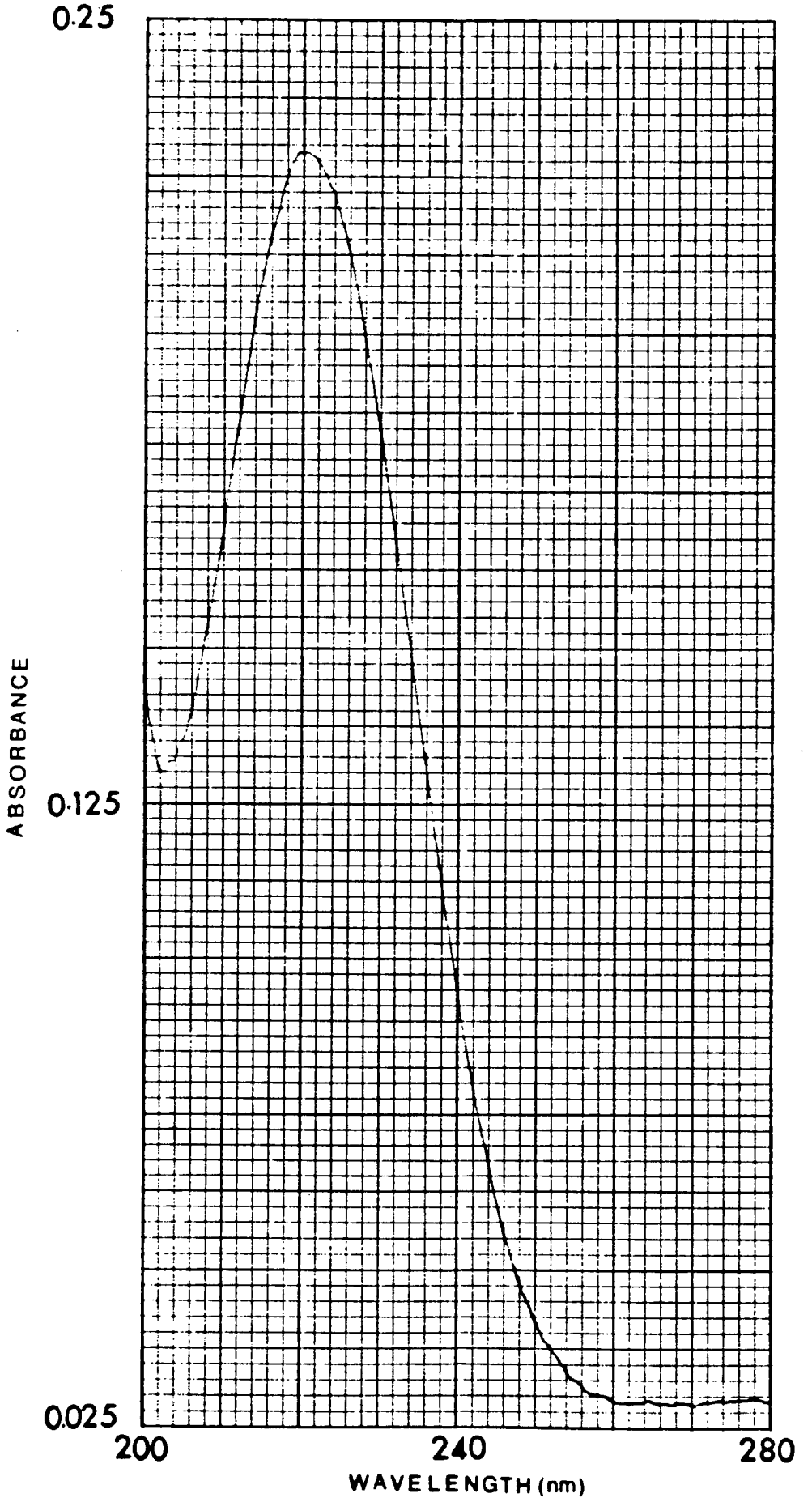
(a)



(b)

Fig. 28.(a) The Infrared Spectra (KBr) of Digitoxin as obtained with the Beckman IR-10; and (b) Sadtler reference IR spectra.

Fig. 29. A Spectral-Absorbance Curve for Digitoxin in 35% Methanol.


0.25
0.125
0.025
ABSORBANCE
200
240
280
WAVELENGTH (nm)

(E) Preparation of Standard Solutions of Digitoxin

Weigh accurately, using a Cahn electrobalance, 10.0 mg of digitoxin and transfer into a 100 ml volumetric flask with the aid of about 60 ml of boiling methanol. Dissolve, cool to room temperature, make to volume with methanol and mix. Transfer aliquots of 1.0, 2.0, 5.0, 10.0, 20.0 and 30.0 ml of the above solution to 100 ml volumetric flasks. To each flask add 1.0 ml of internal standard solution and an amount of methanol sufficient to bring the volume to 35 ml. Dilute the solution in each flask to 100 ml with distilled water and mix.

(The standard solutions will, therefore, have digitoxin concentrations of 1, 2, 5, 10, 20 and 30 ng/ml while the concentration of the internal standard in each solution will be 10 ng/µl.)

(F) Preparation of a Calibration Curve

Inject a 20 µl sample of each standard solution into the Liquid Chromatograph and obtain area values for digitoxin and the internal standard. Make six determinations for each solution.

The calibration curve obtained using 17α-methyltestosterone as internal standard indicates a linear relationship with the line passing through the origin (Fig. 30).

(G) Sample Preparation of Digitoxin Formulations

(a) Composite Tablet Assay

Weigh not less than 40 digitoxin tablets that were selected at random, and triturate to a fine powder. Weigh accurately an amount of powdered tablet material equivalent to 1.0 mg of digitoxin and quantitatively transfer into a 100 ml volumetric flask. Add 10 ml

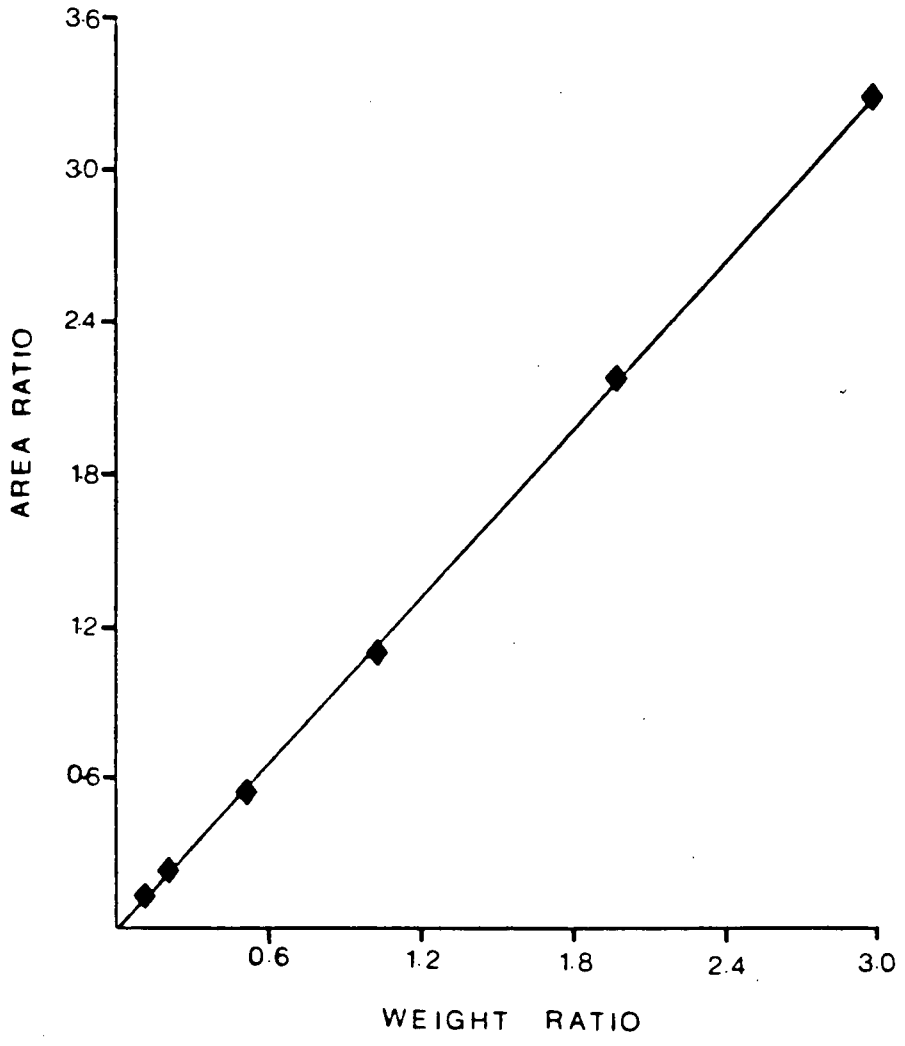


Fig. 30. A Calibration Curve for Digitoxin in 35% Methanol as obtained with a Solvent System of water/methanol/isopropanol/dichloromethane: 45/38/11/6. Area and weight ratios are in terms of digitoxin/internal standard (17α-methyltestosterone). The least squares line of best fit is defined by the equation $y = 1.0848x + 0.0042$; and the coefficient of determination (r^2) is 0.9957.

of distilled water and swirl the flask for 2-3 minutes. Add 34 ml of methanol and mechanically shake the mixture for about 15 minutes. Filter the suspension using a No. 1 Whatman filter paper and wash the residue with three 5 ml portions of distilled water. Collect the filtrate and washings in a 100 ml volumetric flask. Add 1 ml of 17α-methyltestosterone internal standard solution, dilute the mixture with distilled water to a volume of 100 ml and mix.

(b) Single Tablet Assay

Place one tablet in a 10 ml volumetric flask. Add 1 ml of distilled water and swirl the flask for 2-3 minutes. Add 3.4 ml of methanol and mechanically shake the mixture for about 15 minutes. Filter the suspension using a No. 1 Whatman filter paper and wash the residue with three 1 ml. portions of distilled water. Collect the filtrate and washings in a 10 ml of volumetric flask. Add a 0.1 ml aliquot of 17α-methyltestosterone internal standard solution, dilute the mixture with distilled water to a volume of 10 ml and mix.

(c) Injectable Formulation Assay

Transfer a 1 ml aliquot of injection into a 10 ml volumetric flask. Add a 0.1 ml aliquot of 17α-methyltestosterone internal standard solution and 3.3 ml of methanol. Dilute the solution to volume with distilled water and mix.

(H) Quantitation

Since the calibration curve (Fig. 30) indicates linearity with the line passing through the origin, quantitation was carried out by an internal standard single-point automatic calibration method using a Shimadzu Chromatopac C-R1A data processor. The response factor was determined as follows:

Transfer a 10 ml aliquot of the digitoxin stock solution into a 100 ml volumetric flask. Add 1 ml of internal standard solution and 25 ml of methanol and dilute the solution to 100 ml with distilled water and mix. Make triplicate injections of a 20 µl sample into the Liquid Chromatograph and obtain the response factor.

The response factor was found to be 0.9348 and determination of the amount of digitoxin was on the basis of Equation 15. Peak identity was automatically monitored by the data processor.

(I) Determination of Precision of Tablet Assay

Weigh a total of seventy tablets and triturate to a fine powder. Transfer six aliquots of accurately weighed tablet material, each equivalent to 1.0 mg of digitoxin, into six 100 ml volumetric flasks. Proceed as directed in the digitoxin Composite Tablet Assay, beginning with "add 10 ml of distilled water and swirl for 2-3 minutes". Make three injections for each sample.

(J) Determination of Percentage Recovery of Digitoxin from Tablets

Weigh a total of 80 tablets and triturate to a fine powder. Transfer seven aliquots of accurately weighed tablet material, each equivalent to 1.0 mg of digitoxin, into seven 100 ml volumetric flasks. Add an accurately weighed aliquot of digitoxin reference standard, equivalent to 0.5 mg, into each of four flasks. Treat each of the seven samples as directed in the digitoxin Composite Tablet Assay, beginning with "add 10 ml of distilled water and swirl for 2-3 minutes". Make three injections for each sample.

12. Comparison of the Analysis of Digoxin and Digitoxin Dosage Forms by HPLC and USP XX Methods

(A) Brands of Digoxin and Digitoxin Tablets used:

(a) Lanoxin^{R} Tablets (Brand A)

(b) Natigoxin^{R} Tablets (Brand B)

(c) Digoxin Tablets (B.D.H.) (Brand C)

(d) Purodigin^{R} Tablets (Digitoxin)

(B) Dosage Forms and Strengths of Digoxin and Digitoxin used:

(a) Lanoxin^{R} Tablets 0.125 mg

(b) Lanoxin^{R} Tablets 0.25 mg

(c) Lanoxin^{R} Injection 0.05 mg/ml

(d) Lanoxin^{R} Injection 0.25 mg/ml

(e) Lanoxin^{R} Elixir 0.05 mg/ml

(f) Natigoxin^{R} Tablets 0.25 mg

(g) Digoxin (B.D.H.) Tablets 0.25 mg

(h) Purodigin^{R} Tablets 0.1 mg

(i) Crystodigin^{R} Injection 0.2 mg/ml (Digitoxin)

The HPLC and USP assay procedures are presented in the form of flow charts in order that the respective number of steps and times of analysis can be better visualized.

(C) Sample Preparation of Digoxin Dosage Forms for HPLC Analysis

The procedure for sample preparation of digoxin tablets (in composite tablets and single tablet assay is presented in the flow chart shown in Fig. 31. A more detailed procedure is given in Section 10(H): (a) and (b) of this Chapter. The procedures for the injection and elixir are given in Figs. 32 and 33; and the details of the sample preparations are presented in Section 10 (H): (c) and (d).

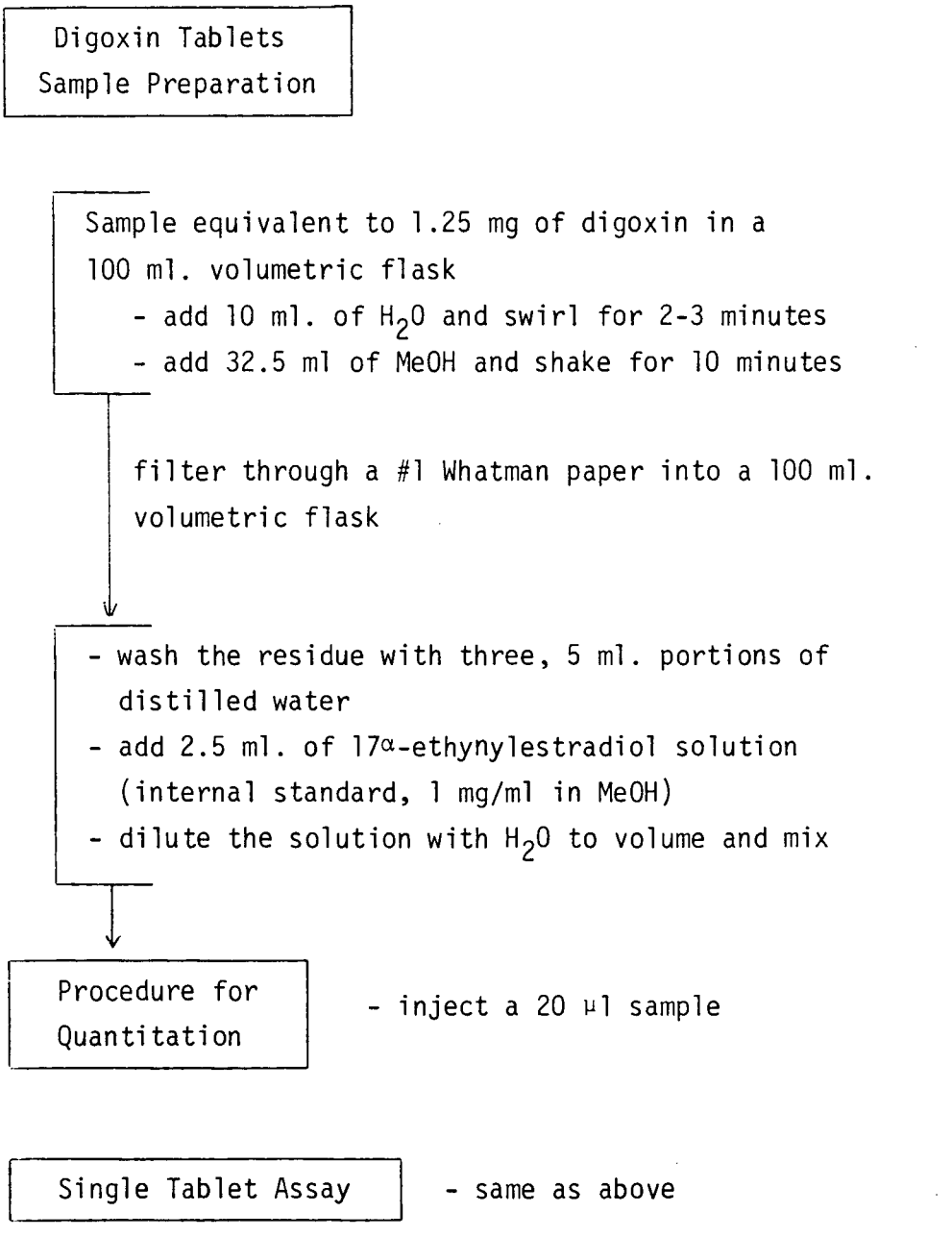


Fig. 31. Flow Chart Presentation of the HPLC Assay of Digoxin Tablets

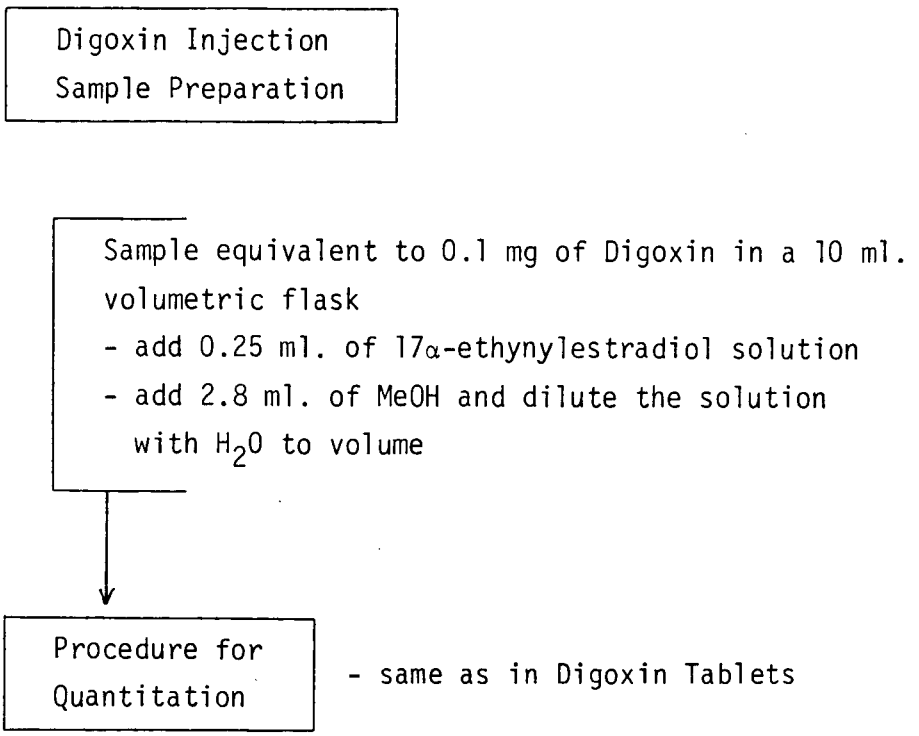


Fig. 32. Flow Chart Presentation of the HPLC Assay of Digoxin Tablets.

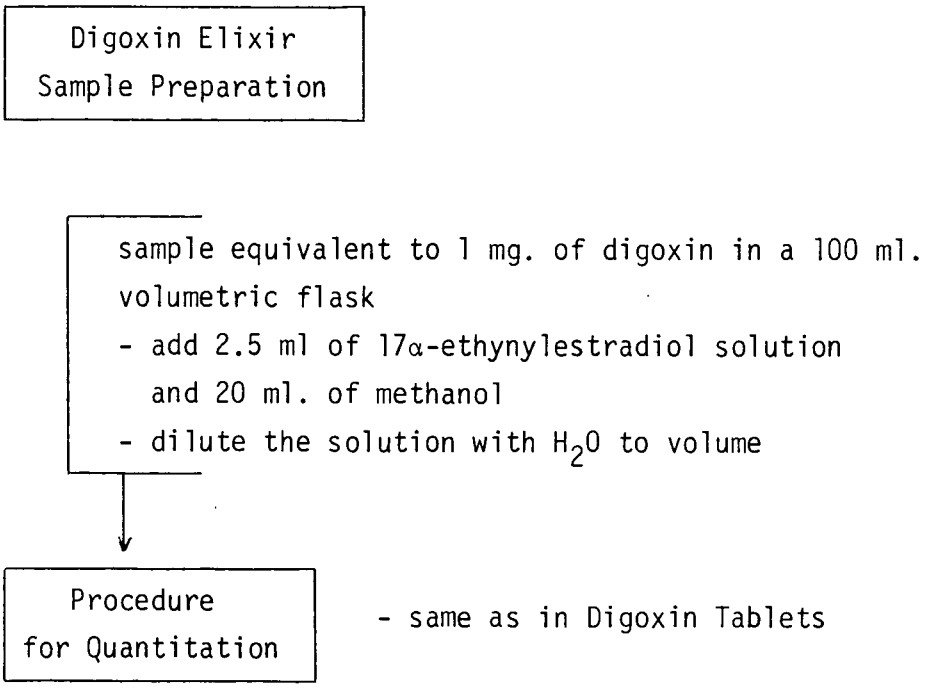


Fig. 33. Flow Chart Presentation of the HPLC Assay of Digoxin Elixir.

(D) HPLC Procedure for Quantitation of Digoxin

This is indicated in the flow charts shown in Figs. 31, 32 and 33; and the details have been described in Section 10 (I) of this Chapter.

(E) Sample Preparation of Digoxin Dosage Forms for Analysis by USP Methods

The procedure for sample preparation of digoxin tablets (in composite tablets and single tablet assay) is shown in the flow chart presented in Fig. 34. The flow charts depicting procedures of sample preparation for the injection and elixir are shown in Figs. 36 and 37.

(F) USP Procedure for Quantitation of Digoxin

The USP quantitation procedure is presented in Fig. 35.

(G) Sample Preparation of Digitoxin Dosage Forms for HPLC Analysis

The proceudre for sample preparation of digitoxin tablets (in composite tablets and single tablet assay) is shown in Fig. 38. The detailed procedure is given in Section 11 (G): (a) and (b) of this Chapter. The procedure for the injection is given in Fig. 39 and the details of the sample preparation are presented in Section 11 (G): (c).

(H) HPLC Procedure for Quantitation of Digitoxin

This is indicated in the flow charts shown in Figs. 38 and 39 and the details have been described in Section 11 (H) of this Chapter.

(I) Sample Preparation of Digitoxin Dosage Forms for Analysis by USP Methods

The procedure for sample preparation of digitoxin tablets (in

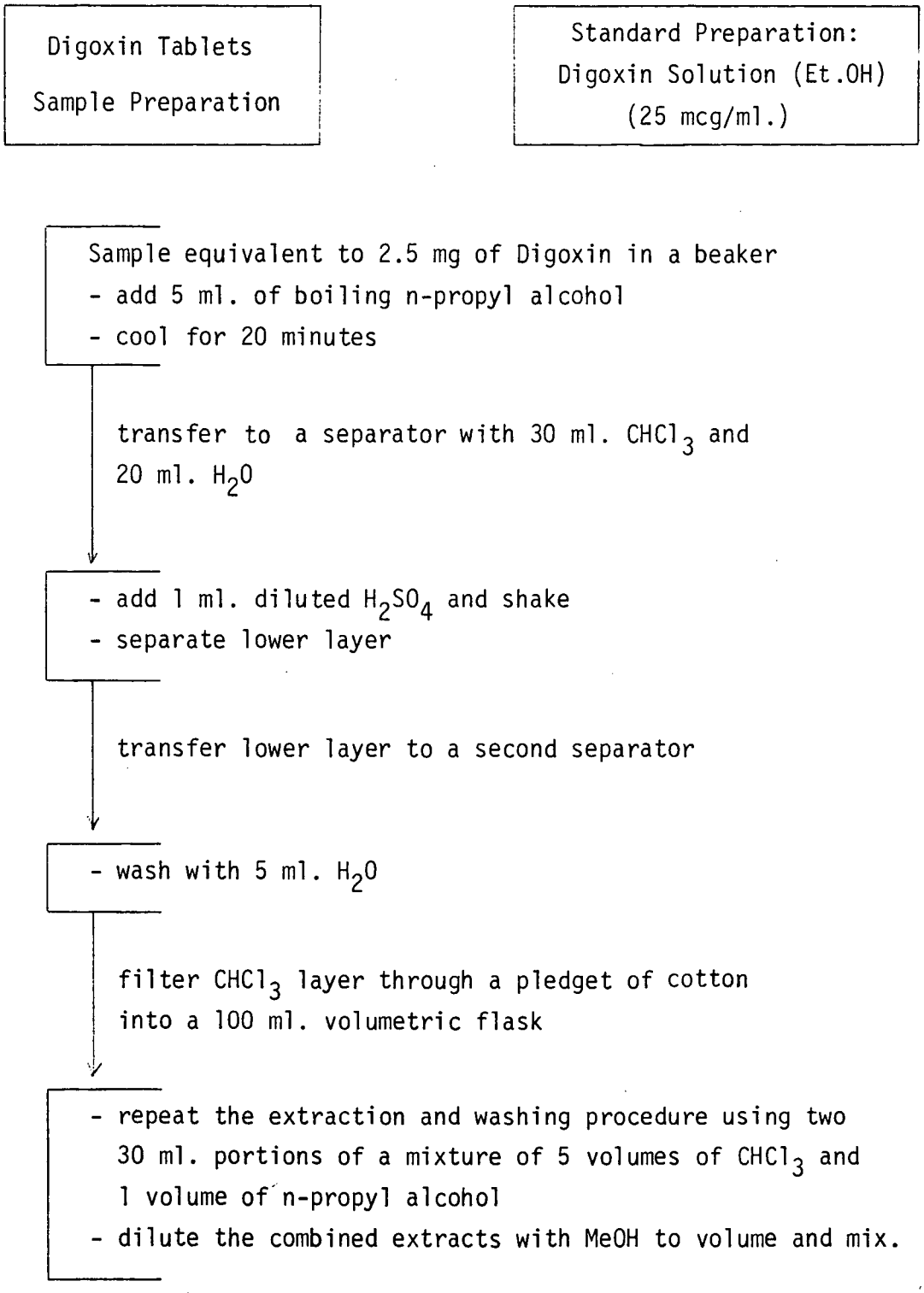


Fig. 34. Flow Chart Presentation of the USP Procedure for Sample Preparation of Digoxin Tablets.

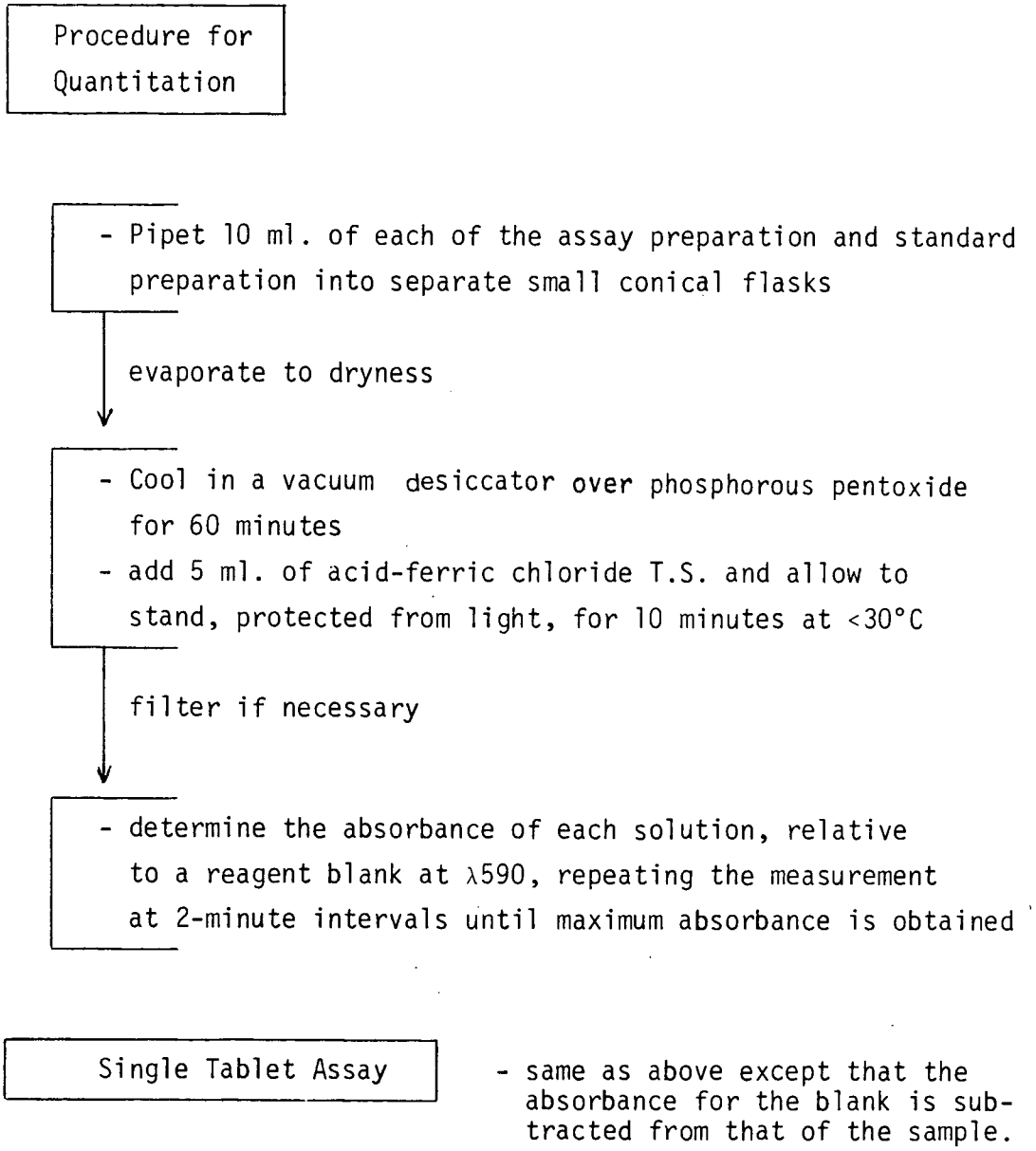


Fig. 35. Flow Chart Presentation of the USP Procedure for the Quantitation of Digoxin in Tablets.

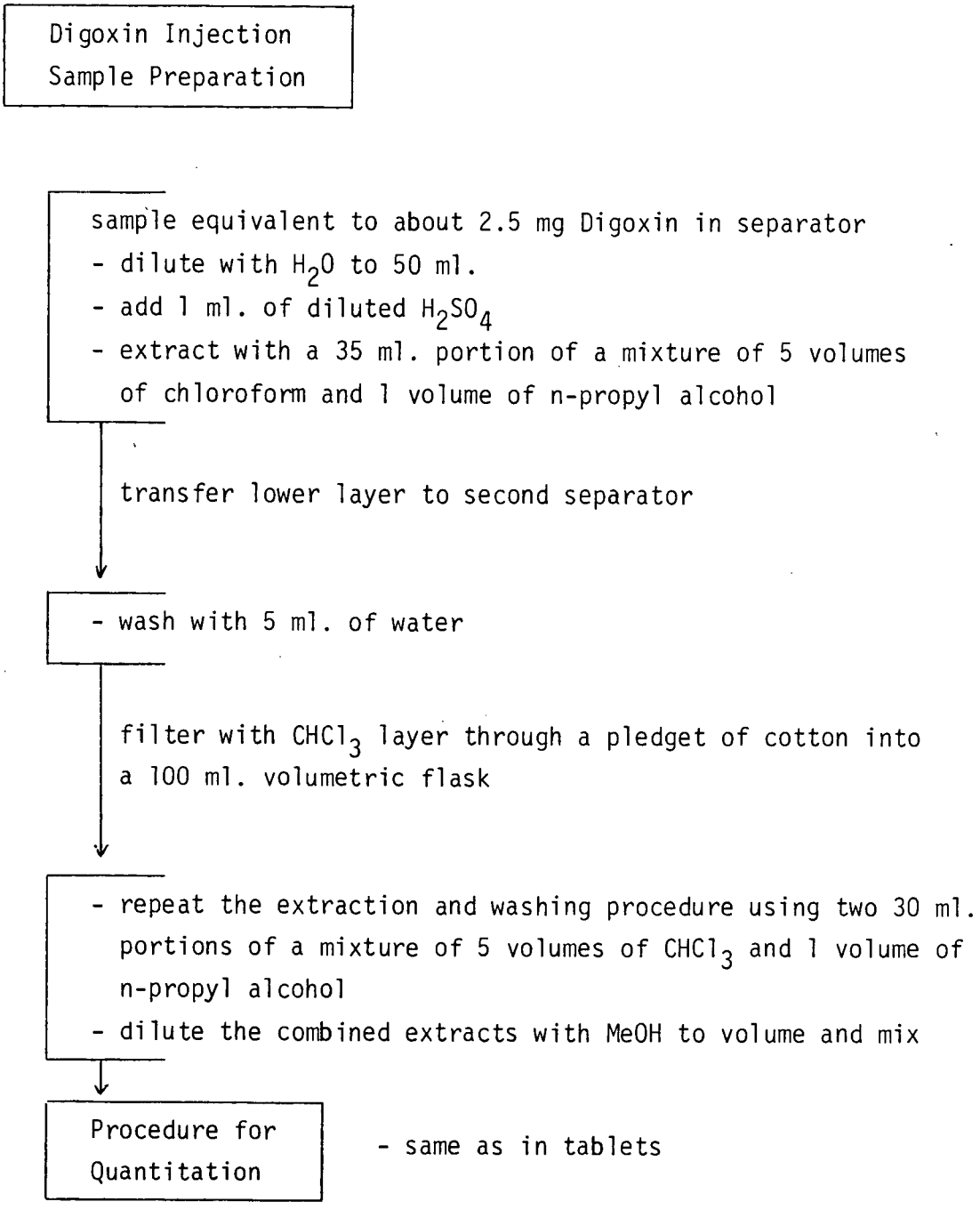


Fig. 36. Flow Chart Presentation of the USP Assay of Digoxin Injection.

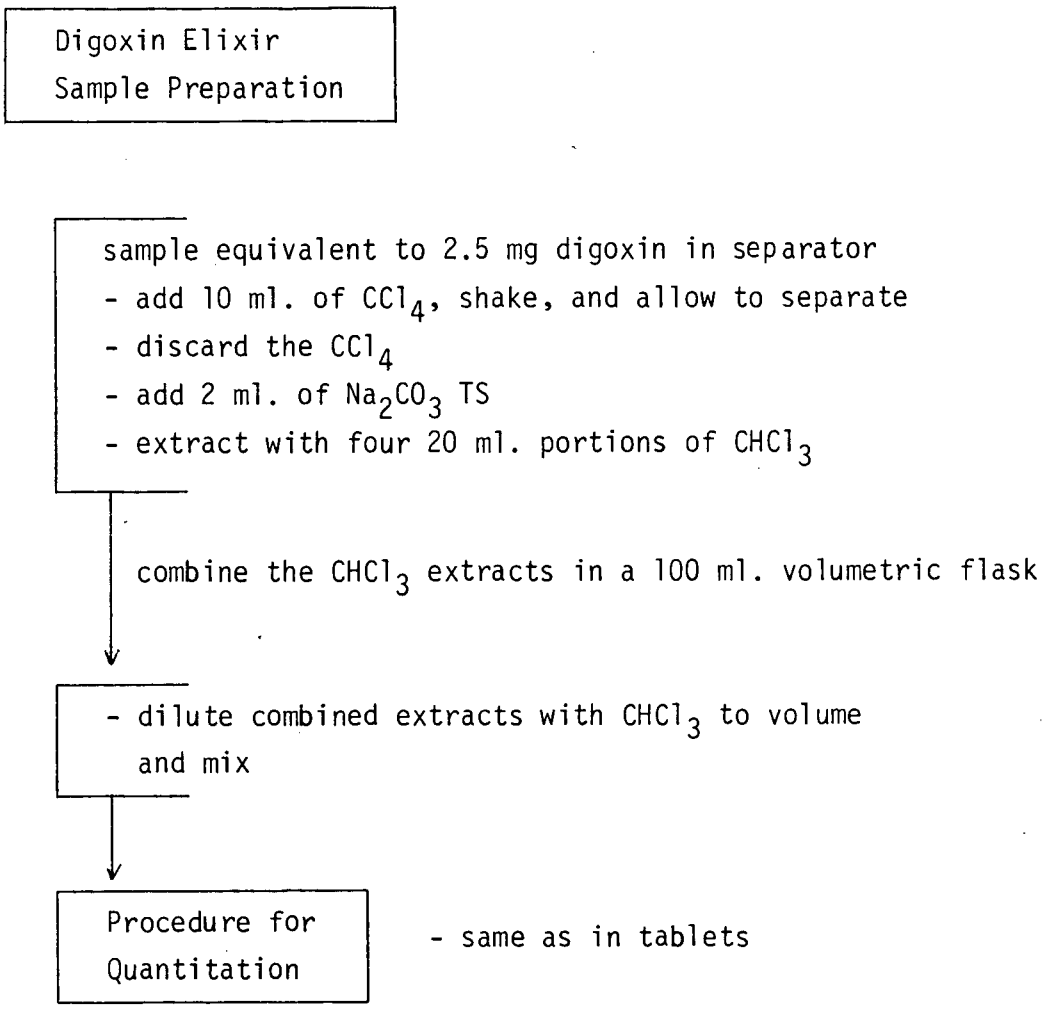


Fig. 37. Flow Chart Presentation of the USP Assay of Digoxin Elixir.

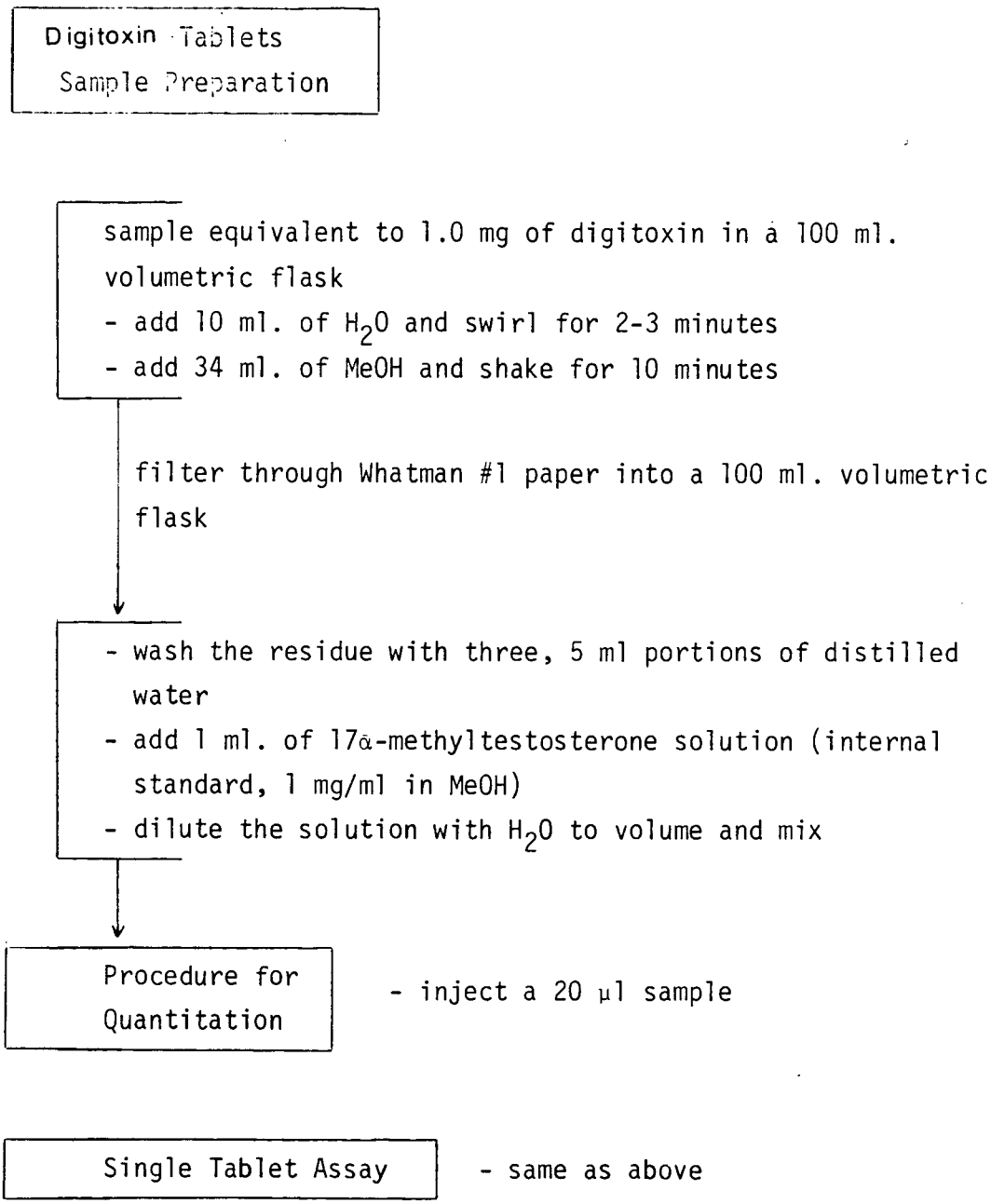


Fig. 38. Flow Chart Presentation of the HPLC Assay of Digitoxin Tablets.

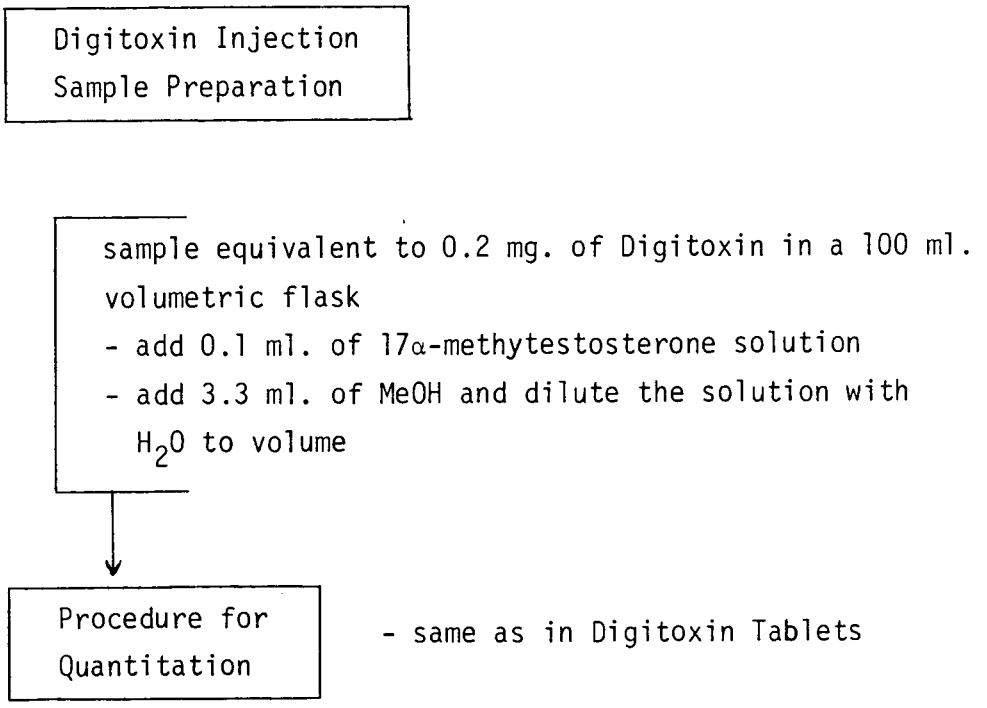


Fig. 39. Flow Chart Presentation of the HPLC Assay of Digitoxin Injection.

composite tablets and single tablet assay) and injection are shown in the flow charts presented in Figs. 40, 42 and 44.

(J) USP Procedure for Quantitation of Digitoxin

The USP quantitation procedures for the analysis of composite tablets, single tablets and injection, are presented in Figs. 41, 43 and 44, respectively.

(K) Determination of Precision of the USP Method for Digoxin Tablet Assay

Weigh a total of 70 tablets and triturate to a fine powder. Transfer six aliquots of accurately weighed tablet material, each equivalent to 2.5 mg of digoxin, into six 50 ml beakers. Proceed as directed in the flow charts shown in Figs. 34 and 35. Make three determinations for each sample.

(L) Determination of Precision of the USP Method for Digitoxin Tablet Assay

Weigh a total of 70 tablets and triturate to a fine powder. Transfer three aliquots of accurately weighed tablet material, equivalent to 2.0 mg of digitoxin, into three 100 ml beakers. Proceed as directed in the flow charts shown in Figs. 40 and 41. Make three determinations for each sample.

(M) Determination of Percentage Recovery of Digoxin from Tablets using the USP Method

Weigh a total of 100 tablets and triturate to a fine powder. Transfer nine aliquots of accurately weighed tablet material, each

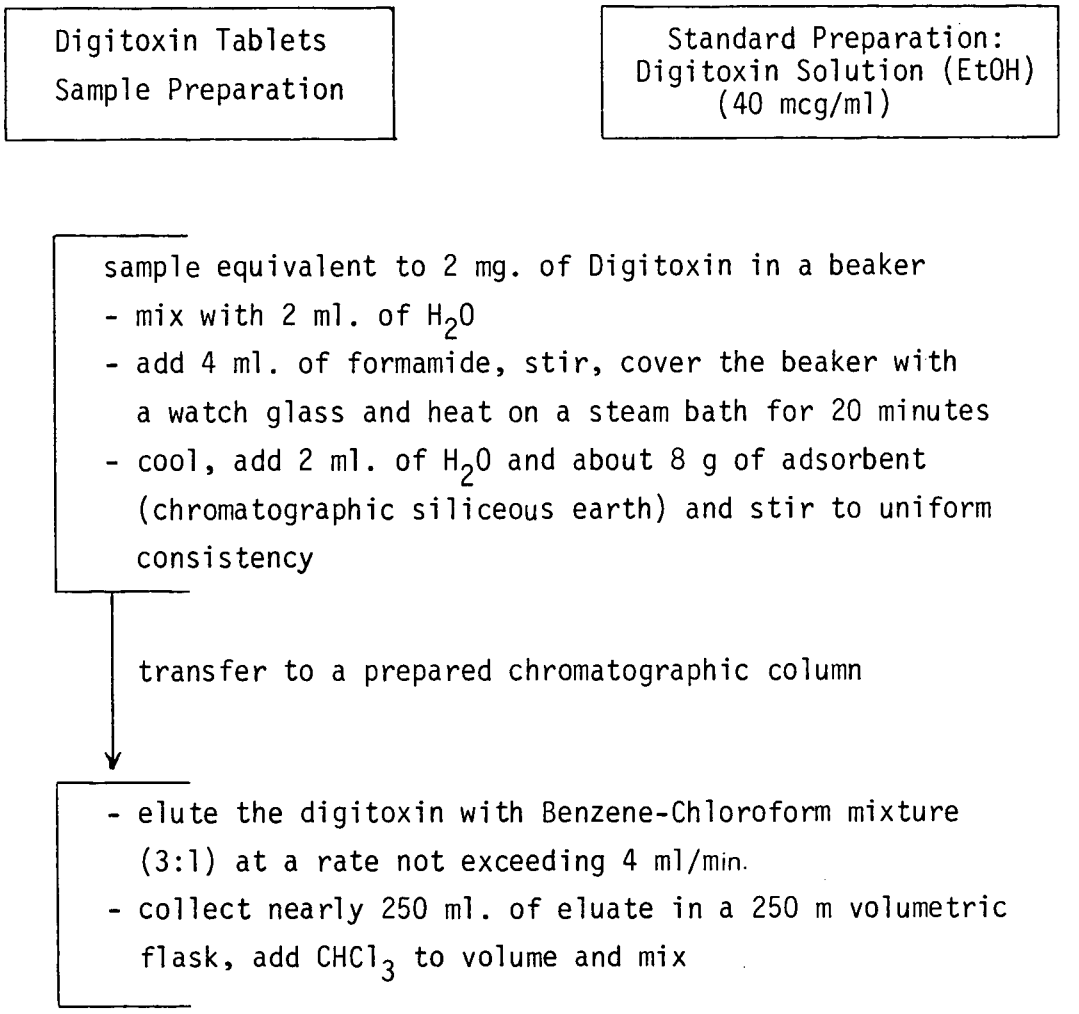


Fig. 40. Flow Chart Presentation of the USP Procedure for Sample Preparation of Digitoxin Composite Tablets.

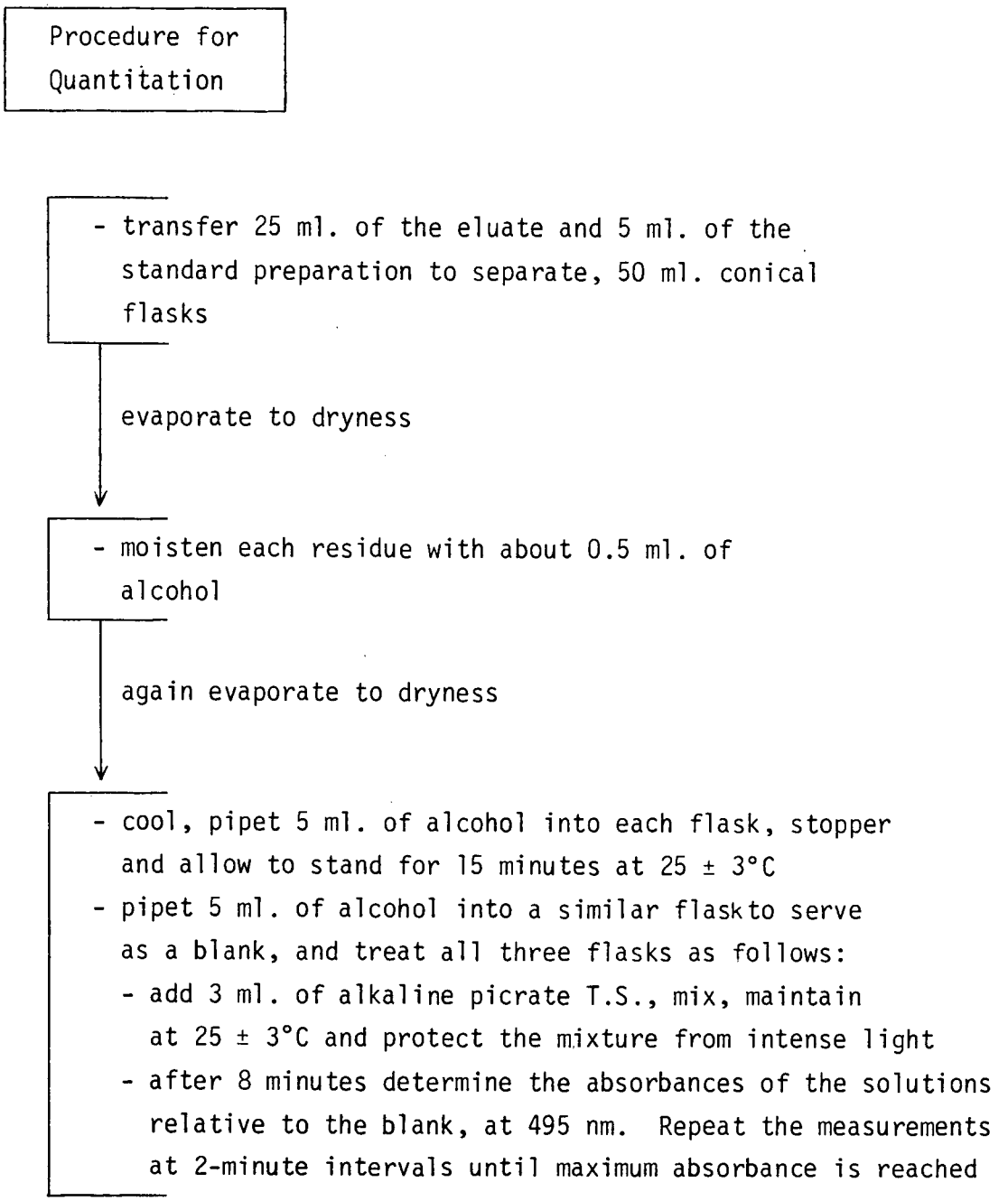


Fig. 41. Flow Chart Presentation of the USP Procedure for the Quantitation of Digitoxin Composite Tablets.

Sample Preparation for Digitoxin Single Tablet Assay

Standard Preparation: Digitoxin Solution (EtOH) (5 mcg/ml)

- place 1 tablet in a beaker
- add 5 to 10 drops of water & allow to disintegrate
- add 5 ml. of acetonitrile, heat on a steam bath for 5 minutes and cool

↓

transfer the solution to a separator with 30 ml of $CHCl_3$ and 20 ml. of H_2O

↓

- shake the separator
- separate the $CHCl_3$ layer, wash in a second separator containing 5 ml. of $NaHCO_3$ solution

↓

filter through a pledget of cotton into a suitable container

↓

- repeat the extraction and washing, using two 30 ml. portions of $CHCl_3$
- evaporate to dryness
- dissolve the residue in 80% alcohol by shaking for 20 minutes to obtain a solution having a concentration of about 5 mcg/ml

Fig. 42. Flow Chart Presentation of the USP Procedure for Sample Preparation in Digitoxin Single Tablet Assay.

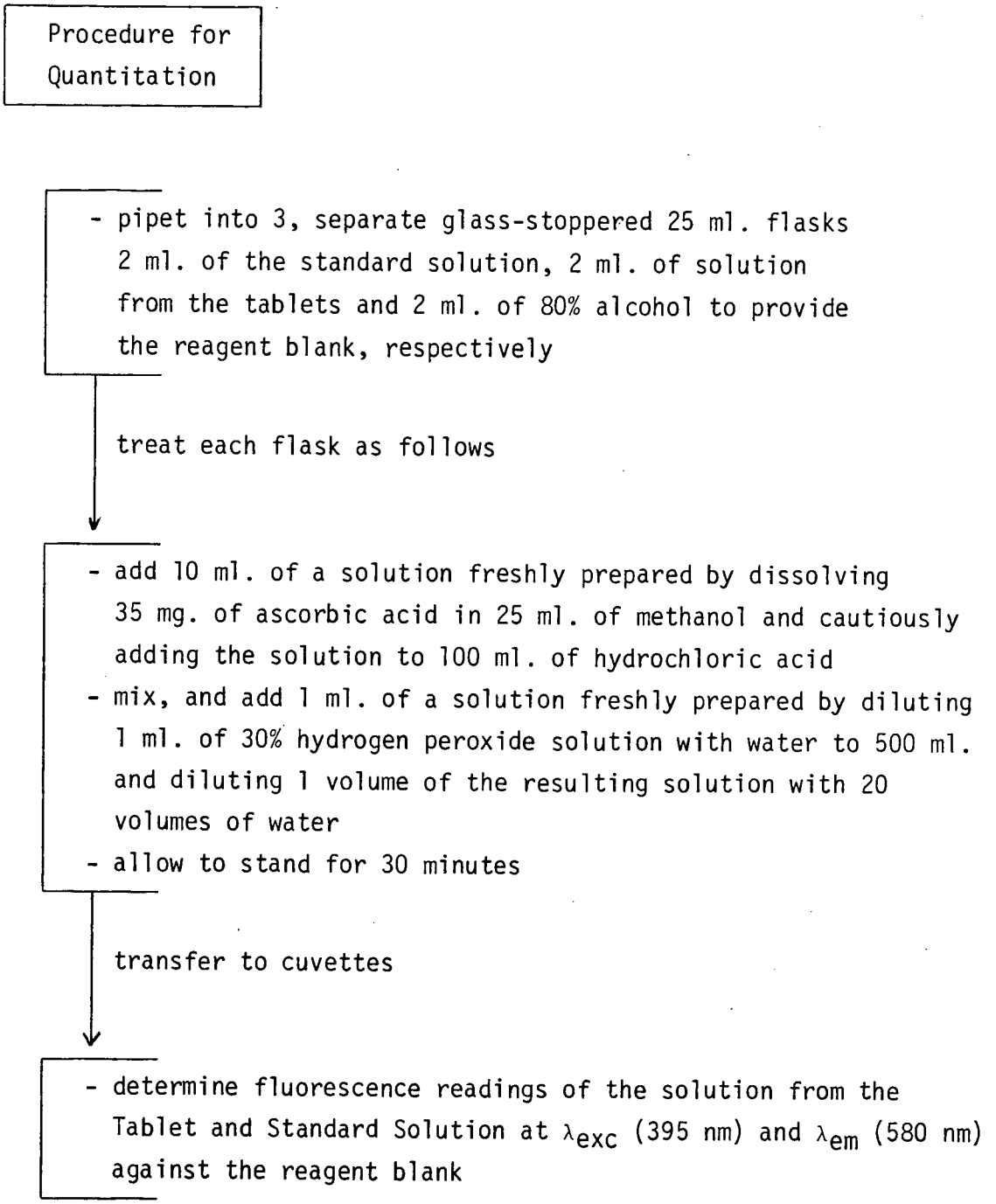


Fig. 43. Flow Chart Presentation of the USP Procedure for the Quantitation of Digitoxin in Single Tablets.

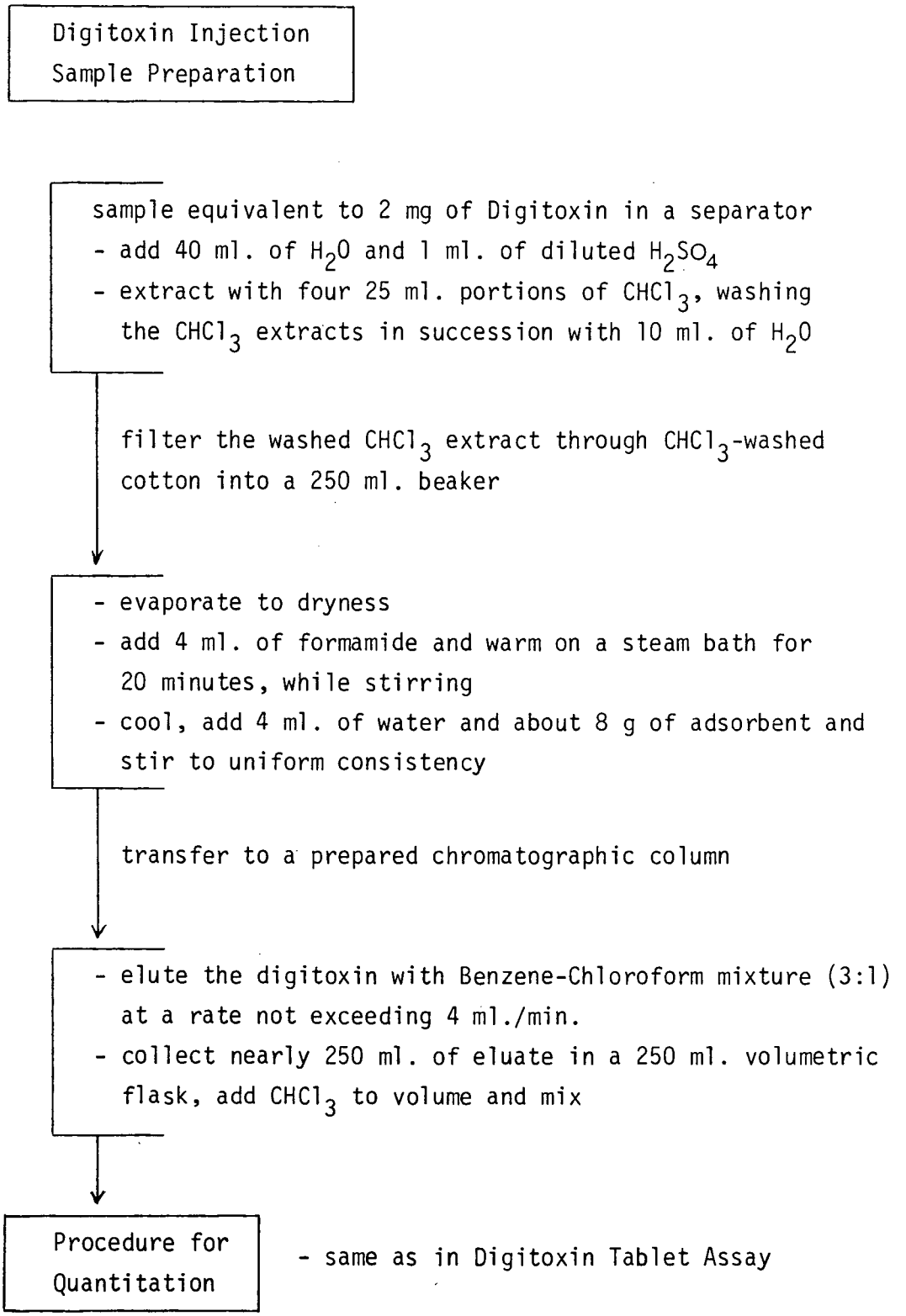


Fig. 44. Flow Chart Presentation of the USP Assay of Digitoxin Injection.

equivalent to 2.5 mg of digoxin, into nine 50 ml beakers. Add an accurately weighed aliquot of digoxin reference standard, equivalent to 1.25 mg, into each of six beakers. Treat each of the nine samples as directed in the digoxin tablet assay shown in Figs. 34 and 35. Make three determinations for each sample.

(N) Determination of Percentage Recovery of Digitoxin from Tablets using the USP Method

Weigh a total of 150 tablets and triturate to a fine powder. Transfer seven aliquots of accurately weighed tablet material, each equivalent to 2.0 mg of digitoxin, into seven 100 ml beakers. Add an accurately weighed amount of digitoxin reference standard, equivalent to 1.0 mg into each of four beakers. Treat each of the seven samples as directed in the digitoxin tablet assay shown in Figs. 40 and 41. Make three determinations for each sample.

13. Stability Monitoring of Digoxin and Digitoxin in their Respective Dosage Forms

(A) Brands of Digoxin and Digitoxin Tablets Used:

(a) Lanoxin^{R} Tablets (digoxin)

(b) Natigoxin^{R} Tablets (digoxin)

(c) Purodigin^{R} Tablets (digitoxin)

(B) Dosage Forms and Strengths of Digoxin and Digitoxin Used:

(a) Lanoxin Tablets 0.125 mg

(b) Lanoxin Tablets 0.25 mg

(c) Lanoxin Injection 0.05 mg/ml

(d) Lanoxin Elixir 0.05 mg/ml

(e) Natigoxin Tablets 0.25 mg

(f) Purodigin Tablets 0.1 mg

(C) Conditions of Storage

Triplicate samples of the same batch of drug substances and dosage forms were stored in their original containers at ambient conditions; 60°C and 70.4% Relative Humidity; and 80°C and 37.1% Relative Humidity, respectively. Concentrations of aqueous sulfuric acid and the corresponding relative humidity values are given in Table VIII.

(D) HPLC Procedure and Conditions

(a) For analysis of Digoxin Tablets and Injection

Proceed as directed in Section 10 (C) of this Chapter.

(b) For Analysis of Digoxin Elixir

Equilibrate the column with solvent system, water/methanol/isopropanol/dichloromethane: 51/43/5/1. Inject the sample, using a Waters U6K injector, into a Beckman Liquid Chromatograph which has been previously adjusted to the following conditions: flow rate of 1.2 ml per minute, UV detection at 220 nm, range of 0.02; attenuation of 6; and a chart speed of 0.5 cm per minute. Record the chromatogram.

(c) For Analysis of Digitoxin Tablets

Proceed as directed in Section 11 (G): a of this Chapter.

(E) Preparation of Internal Standard Solutions

(a) For Analysis of Digoxin Tablets and Injection

Proceed as directed in Section 10 (E) of this Chapter.

(b) For Analysis of Digoxin Elixir

Accurately weigh 100.0 mg of hydrocortisone and transfer into a

Table VIII. Relative Humidity Values Obtained with Aqueous Sulfuric Acid Solutions[a]

Density of H_2SO_4 Solution	% H_2SO_4 in Aq. Solution	Relative Humidity (%)	Vapour Pressure at 20°C (mm Hg)
1.25	75.0	70.4	12.2
1.40	81.0	37.1	6.5

[a] Handbook of Chemistry and Physics, 57th ed., E46.

100 ml volumetric flask with the aid of about 50 ml of methanol. Dissolve, make to volume with methanol and mix.

(c) For Analysis of Digitoxin Tablets

Proceed as directed in Section 11 (D) of this Chapter.

(F) Preparation of Standard Solutions of Digoxigenin, Digoxigenin monodigitoxoside, Digoxigenin bisdigitoxoside and Digoxin

(a) For Analysis of Digoxin and its Degradation Products in Tablets and Injection

Weigh accurately, using a Cahn Electrobalance, 10.0 mg of digoxin and transfer into a 50 ml volumetric flask with the aid of about 40 ml of boiling methanol. Dissolve, cool to room temperature, make to volume with methanol, and mix. Transfer aliquots of 2.5, 5.0, 10.0, 15.0, 20.0 and 25.0 ml of the above stock solution to six 100 ml volumetric flasks. To each flask and 2.5 ml of 17α-ethynylestradiol internal standard solution add an amount of methanol sufficient to bring the volume to 35 ml. Dilute the solution in each flask to 100 ml with distilled water and mix. Similarly prepare stock and standard solutions of digoxigenin, digoxigenin monodigitoxoside and digoxigenin bisdigitoxoside. The six standard solutions prepared for each of the four compounds will, therefore, have concentrations of 5, 10, 20, 30, 40 and 50 ng/μl.

(b) For Analysis of Digoxin and its Degradation Products in the Elixir Dosage Form

Proceed as in (a) adding 2 ml of hydrocortisone internal standard solution instead of 2.5 ml of 17α-ethynylestradiol solution.

(G) Preparation of Standard Solutions of Digitoxigenin, Digitoxigenin monodigitoxoside, Digitoxigenin bisdigitoxoside and Digitoxin.

Proceed as directed in Section (F), (a) adding 1 ml of 17α-methyltestosterone internal standard solution instead of 2.5 ml of 17α-ethynylestradiol solution.

(H) Preparation of Calibration Curves

Inject a 20 µl sample of each standard solution of the eight compounds (Digoxin, Digitoxin and their Degradation Products) into a Liquid Chromatograph and obtain area values of the compound and the corresponding internal standard. Make six determinations for each solution. The calibration curves (Area ratio versus Weight ratio) obtained for digoxin and its degradation products, using 17α-ethynylestradiol as the internal standard are shown in Fig. 45. The calibration curves of digoxin and its degradation products using hydrocortisone as the internal standard are given in Fig. 46. The calibration curves obtained for digitoxin and its degradation products are shown in Fig. 47.

(I) Sample Preparation

(a) Digoxin Tablets

Proceed as directed in Section 10 (H): (a) of this Chapter.

(b) Digoxin Injection

Proceed as directed in Section 10 (H): (c) of this Chapter.

(c) Digoxin Elixir

Transfer a 10 ml aliquot of the elixir into a separatory funnel and add 2 ml of sodium carbonate T.S. Extract with four, 15 ml portions of dichloromethane and filter each extract through the same No. 1 Whatman filter paper plugged with cotton wool that has been previously washed with dichloromethane. After filtration of the fourth extract, wash the filter paper and plug of cotton

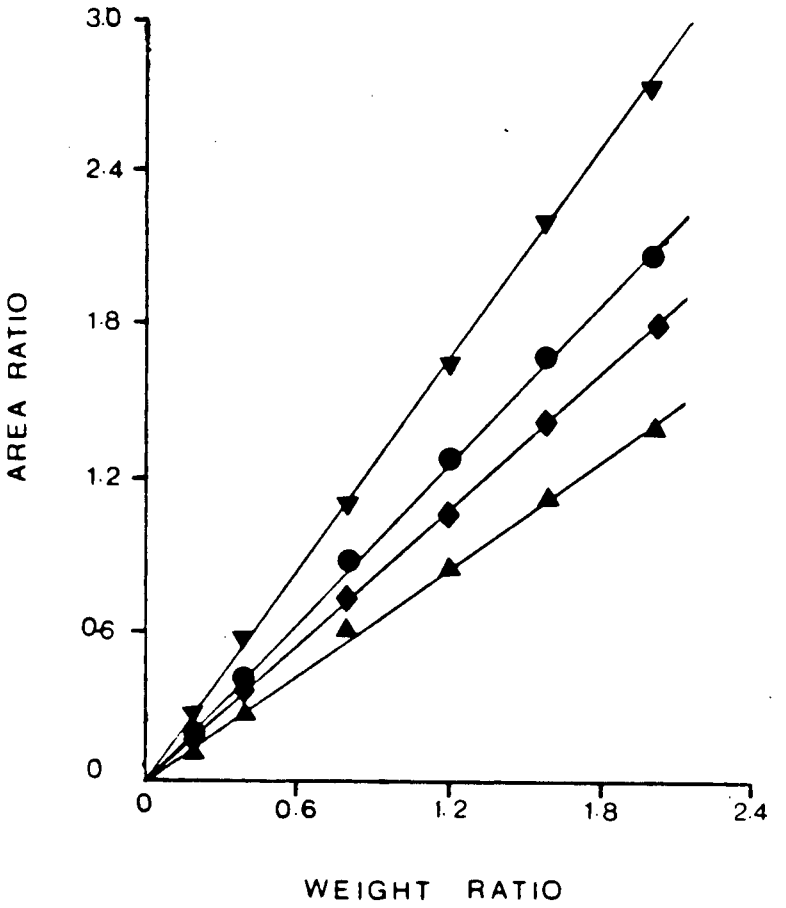


Fig. 45. Calibration Curves for Digoxigenin (▼), Digoxigenin monodigitoxoside (●), Digoxigenin bisdigitoxoside (◆) and Digoxin (▲) in 35% Methanol as obtained with a Solvent System of water/methanol/isopropanol/dichloromethane: 47/40/9/4. Area and weight ratios are in terms of compound/internal standard (17α-ethynyl-estradiol). The respective least squares lines of best fit are defined by the equations: (▼) $y = 1.3770x + 0.0117$, $r^2 = 0.9937$); $y = 1.0614x + 0.0088$ (●), ($r^2 = 0.9955$); $y = 0.8910x + 0.0603$,(◆) $r^2 = 0.9974$; and $y = 0.7049x + 0.01401$ (▲), $r^2 = 0.9933$.

Fig. 46. Calibration Curves for Digoxigenin (▲), Digoxigenin monodigitoxoside (●), Digoxigenin bisdigitoxoside (▼) and Digoxin (◆) in 35% Methanol as obtained with a Solvent System of water/methanol/isopropanol/ dichloromethane: 51/43/5/1. Area and weight ratios are in terms of compound/internal standard (hydrocortisone). The respective least squares lines of best fit are defined by the equations: $y = 2.1709x + 0.0010$ (▲), $r^2 = 0.9974$; $y = 1.8442x + 0.0105$ (●), $r^2 = 0.9999$; $y = 1.6065x + 0.0084$ (▼), $r^2 = 0.9967$; and $y = 1.4356x + 0.0034$ (◆), $r^2 = 0.9919$.

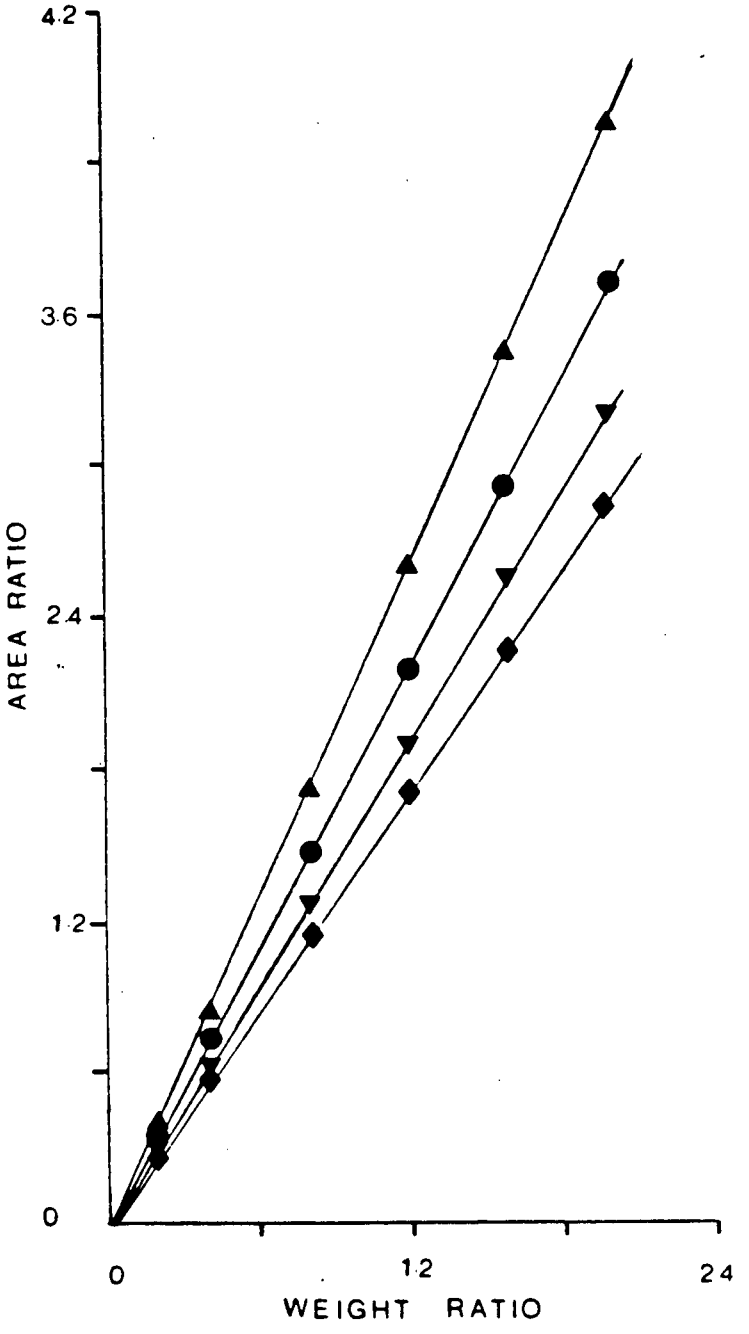
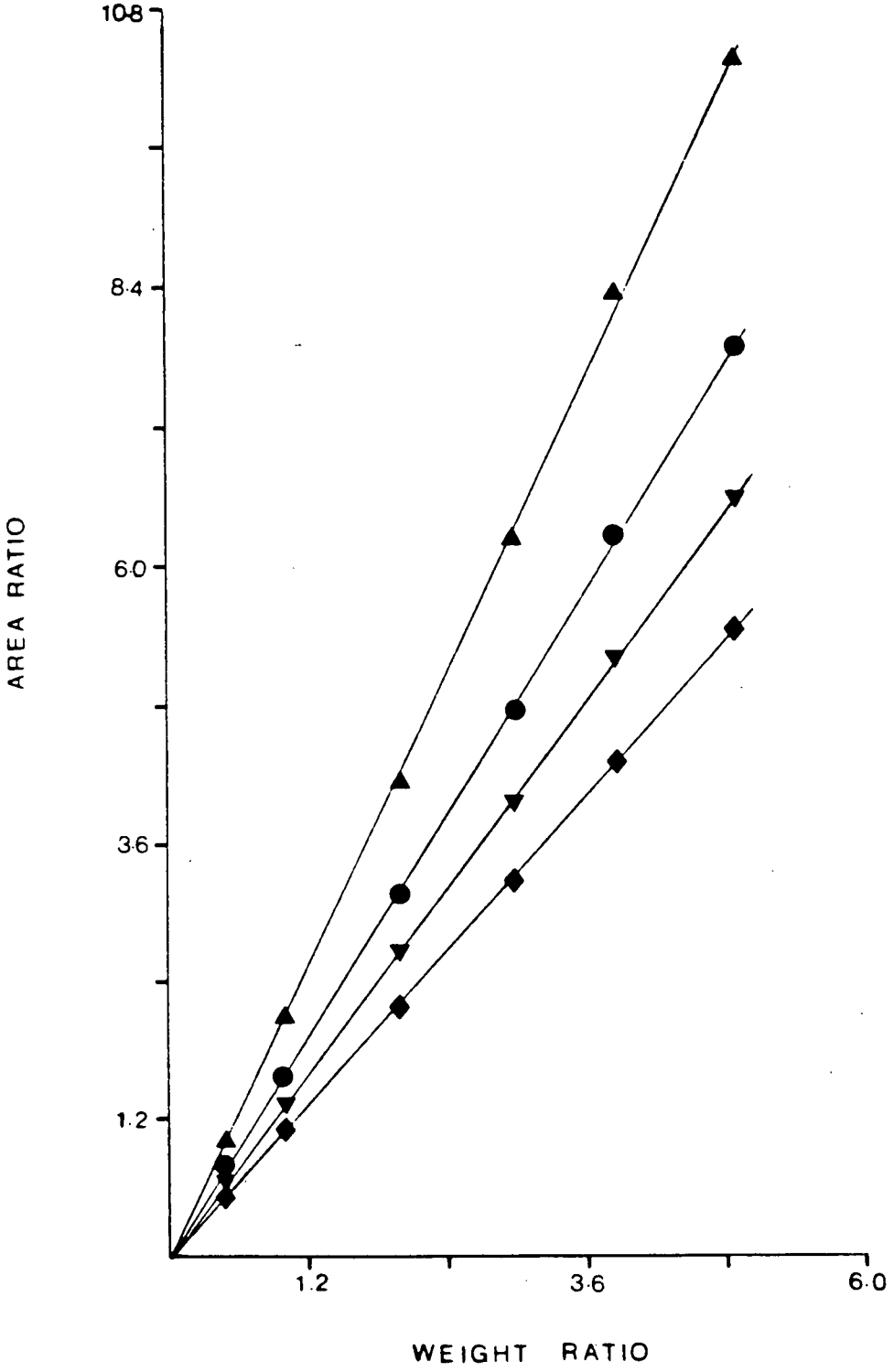

4·2
3·6
2·4
1·2
0
AREA RATIO
0
1·2
2·4
WEIGHT RATIO

Fig. 47. Calibration Curves for Digitoxigenin (▲), Digitoxigenin monodigitoxoside (●), Digitoxigenin bisdigitoxoside (▼) and Digitoxin (◆) in 35% Methanol as obtained with a Solvent System of water/methanol/isopropanol/ dichloromethane: 45/38/11/6. Area and weight ratios are in terms of compound/internal standard (17α-methyltestosterone). The respective least squares lines of best fit are defined by the equations: $y = 2.0758 + 0.0042$ (▲), $r^2 = 0.9936$; $y = 1.5759x + 0.0060$ (●), $r^2 = 0.9968$; $y = 1.3093x + 0.0074$ (▼), $r^2 = 0.9972$; and $y = 1.0793x - 0.0969$ (◆), $r^2 = 0.9942$.


10·8
8·4
6·0
3·6
1·2
AREA RATIO
1·2
3·6
6·0
WEIGHT RATIO

wool with two, 5 ml portions of dichloromethane. Collect the extract and washings in a 100 ml round-bottom flask, evaporate to dryness using a rotary evaporator at low heat (about 40°C) and cool. Add 12.5 ml of methanol, 12.5 ml of distilled water and swirl for about five minutes. Transfer the solution to a 50 ml volumetric flask. Rinse the round-bottom flask with two, 5 ml portions of distilled water and add the washings into the volumetric flask. Add 1 ml of hydrocortisone internal standard solution, dilute to volume with distilled water and mix.

(d) Digitoxin Tablets

Proceed as directed in Section 11 (G): (a) of this Chapter.

(J) Quantitation

Quantitation of digoxin, digitoxin and their degradation products was carried out by an internal standard single-point automatic calibration method using a Shimadzu Chromatopac C-R1A data processor. Response factors were determined as follows:

(a) For Analysis of Digoxin Tablets and Injection:

Transfer a 5 ml aliquot of each of the stock solutions of digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and digoxin into a 100 ml volumetric flask. Add 2.5 ml of 17α-ethynylestradiol internal standard solution and 27.5 ml of methanol. Dilute the solution with distilled water and mix. Make triplicate injections of a 20 µl sample into a Liquid Chromatograph and obtain the response factors.

(b) For Analysis of Digoxin Elixir

Proceed as in (a) using 2 ml of hydrocortisone internal standard solution instead of 2.5 ml of 17α-ethynylestradiol solution.

(c) For Analysis of Digitoxin Tablets

Proceed as in (a) using stock solutions of digitoxigenin, digitoxigenin monodigitoxoside, digitoxigenin bisdigitoxoside and digitoxin instead of the digoxin series; and adding 1 ml of 17α-methyltestosterone internal standard solution.

The response factors that were obtained are presented in Table 9 and determination of the amount of each compound was based on Equation 15.

(K) Determination of pH

Cool the sample to room temperature and measure the pH using a pH meter that has been calibrated previously using a buffer solution of pH 7.0.

Table IX. Response Factors obtained for the Analysis of Digoxin, Digitoxin and their potential Degradation Products using; 17α-ethynylestradiol (a), Hydrocortisone (b), and 17α-methyltestosterone (c) as Internal Standards

Series	Compound	Response Factor		
		(a)	(b)	(c)
1	Digoxigenin	0.7176	0.4469	
2	Digoxigenin monodigitoxoside	0.9403	0.5862	
3	Digoxigenin bisdigitoxoside	1.1365	0.7084	
4	Digoxin	1.3890	0.8656	
5	Digitoxigenin			0.4864
6	Digitoxigenin monodigitoxoside			0.6351
7	Digitoxigenin bisdigitoxoside			0.7702
8	Digitoxin			0.9385

III. RESULTS AND DISCUSSION

1. Evolution of the Basic HPLC Solvent System for the Analysis of Cardiac Glycosides

Digoxin, digitoxin and their metabolites are non-ionic, of medium to low polarity, sparingly soluble in water and have molecular weights of less than 1000. Therefore, as shown in the general guide for HPLC mode selection (Table IV), a reverse-phase column (Spherisorb ODS, 25 cm x 3.2 mm, particle size 10 μm) was selected for the initial study. On the basis of their relatively high polarity characteristics, water and methanol were chosen for the preparation of the preliminary solvent system.

In an effort to obtain separation of the digoxin series in a reasonable period of time various mixtures of methanol and water were tried. A solvent system of methanol/water: 60/40 resulted in a 7 minute chromatogram (Fig. 48) in which the compounds eluted in the order of digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and digoxin. It was evident that digoxigenin (which was not available at this stage) if separated, would elute before digoxigenin monodigitoxoside whose retention time was quite close to the solvent front. Therefore, it was necessary to push the first peak a little bit further from the solvent front in order to make room for digoxigenin. This was accomplished with a solvent system of water/methanol: 60/40, as shown in Fig. 49. The total chromatographic time in this case was about 23 minutes. This solvent system, however, was found to be unfit for the separation of digitoxin and its metabolites, because of the extremely long chromatographic time.

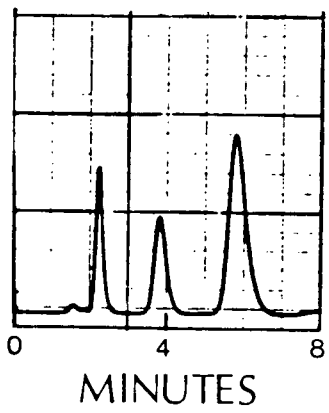


Fig. 48. A Chromatogram for the HPLC Separation of a Standard Mixture of Digoxigenin monodigitoxoside (peak 1), Digoxigenin bis-digitoxoside (peak 2) and Digoxin (peak 3). HPLC conditions: Spherisorb ODS column; Solvent system, water/methanol: 40/60, flow rate = 1.5 ml/min; UV detection at 254 nm, range = 0.2; Chart speed = 0.5 cm/min; the compounds were dissolved in methanol.

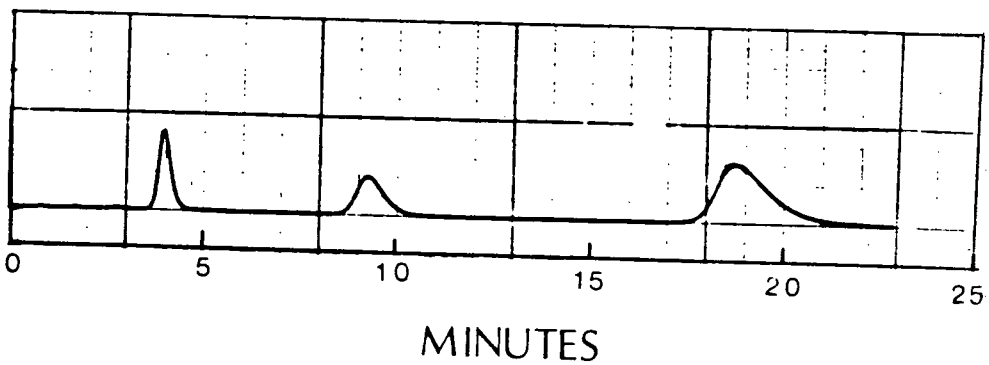


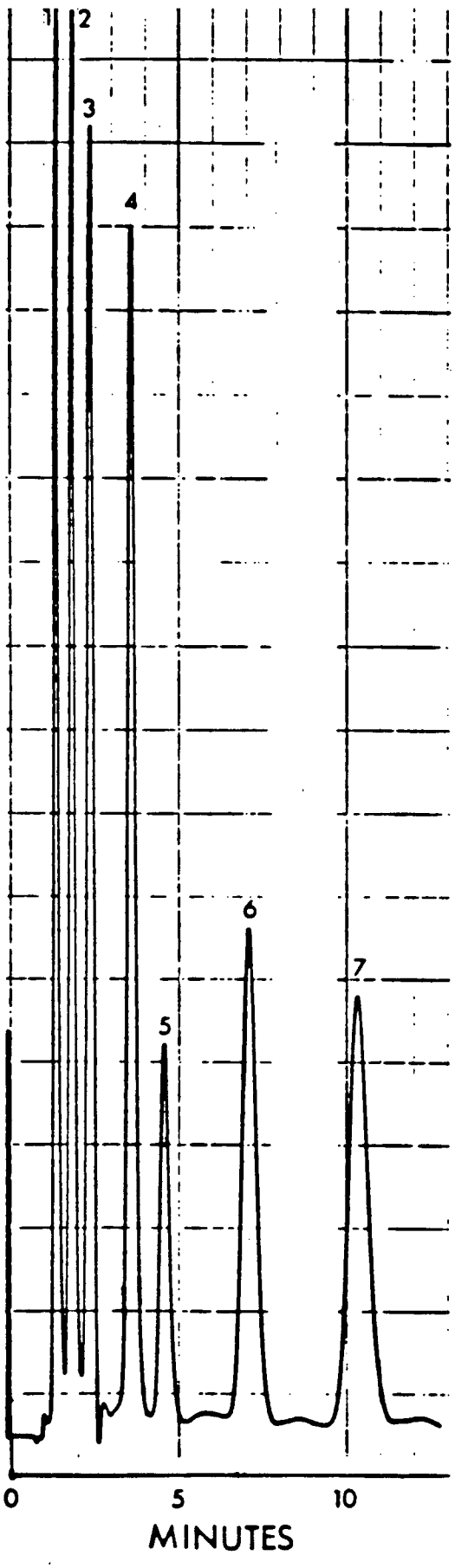
Fig. 49. A Chromatogram for the HPLC Separation of a Standard Mixture of Digoxigenin monodigitoxoside (peak 1). Digoxigenin bis-digitoxoside (peak 2) and Digoxin (peak 3) as obtained with a solvent system of methanol/water: 40/60. Other HPLC conditions were the same as in Fig. 48.

In order to optimize the capacity factor (retention) of both the digoxin and digitoxin series of compounds, while maintaining the sequence of elution, a variety of relatively non-polar solvents was introduced for the preparation of tertiary or quaternary solvent systems. The chromatogram shown in Fig. 50(a) was obtained with a solvent system of water/methanol/isopropanol/dichloromethane: 46/39/10/5. This solvent system was basically found to be most convenient for the isocratic resolution of digoxin, digitoxin and their metabolites in one chromatogram. The sharpness of the peaks and short chromatographic time indicate the high differentiating capability and strength of the solvent system. The 12 minute chromatogram (Fig. 50(a)) shows separation of a standard mixture of seven compounds with an elution order of digoxigenin monodigitoxoside, digoxigenin bis-digitoxoside, digoxin, digitoxigenin, digitoxigenin monodigitoxoside, digitoxogenin bisdigitoxoside and digitoxin, as monitored by a UV detector set at a wavelength of 220 nm. The chromatogram in Fig. 50(b) was obtained under the same conditions except that UV detection was carried out at a wavelength of 254 nm.

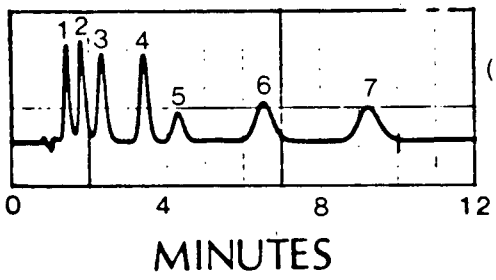
The presence of a relatively small amount of dichloromethane in the solvent system contributes to absorption of radiant energy at a wavelength of 220 nm and therefore raises the baseline. However, the presence of a considerably large amount of water minimizes the absorbance effect of dichloromethane. The problem of miscibility of water and dichloromethane is resolved by the presence of methanol and isopropanol. Moreover, since there is sufficient differential absorbance contributed by digoxin and the other cardenolides, at the wavelength of 220 nm, it has been consistently shown that no problem arises from the presence of dichloromethane. The advantages of this wavelength (220 nm) in terms of increased sensitivity can be observed from a comparison of Fig. 50 (a) and (b).

The unique quaternary solvent system consisting of water, methanol,

Fig. 50. A Chromatogram for the Isocratic HPLC Separation of a Standard mixture of Digoxin, Digitoxin and their Metabolites. Sequence of elution: 1 = digoxigenin monodigitoxoside; 2 = digoxigenin bisdigitoxoside; 3 = digoxin; 4 = digitoxigenin; 5 = digitoxigenin monodigitoxoside; 6 = digitoxigenin bisdigitoxoside; 7 = digitoxin. HPLC conditions: Spherisorb column; solvent system, water/methanol/isopropanol/dichloromethane: 46/39/10/5, flow rate = 1.5 ml/min; Chart speed = 0.5 cm/min; UV detection at 220 nm (a) and 254 nm (b), range = 0.02. The compounds were dissolved in the eluting solvent system.



(a)



(b)

isopropanol and dichloromethane, as described above, was employed as the basic mobile phase for all HPLC separations reported in this investigation. Optimization of the selectivity factor for particular HPLC separations of any of the compounds used in this study was accomplished by various alterations of the relative proportion of the four components of the solvent system.

2. Development of HPLC Systems for the Separation of Digoxin, Digitoxin, their respective Metabolites or Degradation Products and related Compounds

(A) Separations by Isocratic Elution

It is generally recognized that digoxin can be a metabolic product of digitoxin (Kramer et al., 1976). It has also been observed that both digoxin and digitoxin can undergo degradation to produce the respective genins and their mono-and bisdigitoxosides (Kuhlman et al., 1973). Hence a method that would separate digoxin, digitoxin and their metabolites, in one chromatogram, would be desirable.

HPLC separations have been reported for mixtures of a wide variety of digitalis glycosides. The only paper (Castle, 1975) that has addressed itself to the problem of separating digoxin, digitoxin and their metabolites, reports gradient elution. However, since analysis of digoxin and digitoxin requires high sensitivity detector settings, the baseline fluctuations that may be associated with gradient elution can introduce difficulties in quantitation. Therefore the need for an isocratic HPLC system that would separate these compounds in one chromatogram is quite apparent. For purposes of attaining greater sensitivity, the isocratic system should

be compatible with aqueous media so that it may be used along with the post-column fluorogenic derivatization process. If such an isocratic system is possible,the chances of its being selective enough to allow the analysis of digoxin or digitoxin in the presence of its metabolites, degradation products, impurities, endogenous steroids and drugs commonly prescribed for cardiac patients will, indeed, be high. It is within this content that the following isocratic separations were developed.

The isocratic separation of digoxin, digitoxin and their metabolites is presented in Fig. 51, as obtained with a solvent system of water/methanol/isopropanol/dichloromethane: 47/40/9/4. The 27-minute chromatogram indicates elution of the compounds in the order of digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside, digoxin, digitoxigenin, digitoxigenin monodigitoxoside, digitoxigenin bisdigitoxoside and digitoxin. Provided that the necessary sensitivity is achieved, this solvent system can be useful for monitoring the levels of digitoxin, digoxin and their metabolites in biological specimens. Separation by HPLC for subsequent quantitation by radioimmunoassay is one possibility.

A slightly altered solvent system (water/methanol/isopropanol/dichloromethane: 45/37/12/6) offers a chromatographic time of about 13 minutes (Fig. 52) in which all components except digoxigenin and digoxigenin monodigitoxoside have baseline resolution. It can be observed that this solvent system may be applicable for the analysis of digitoxin and its metabolites including digoxin.

In order to optimize time, capacity factor and resolution of the separation of the digitoxin series, further alterations of the proportion of the mobile phase components were tried. A more non-polar solvent system of water/methanol/isopropanol/dichloromethane: 43/35/15/7 resulted in the chromatogram shown in Fig. 53. This chromatogram resolves all of

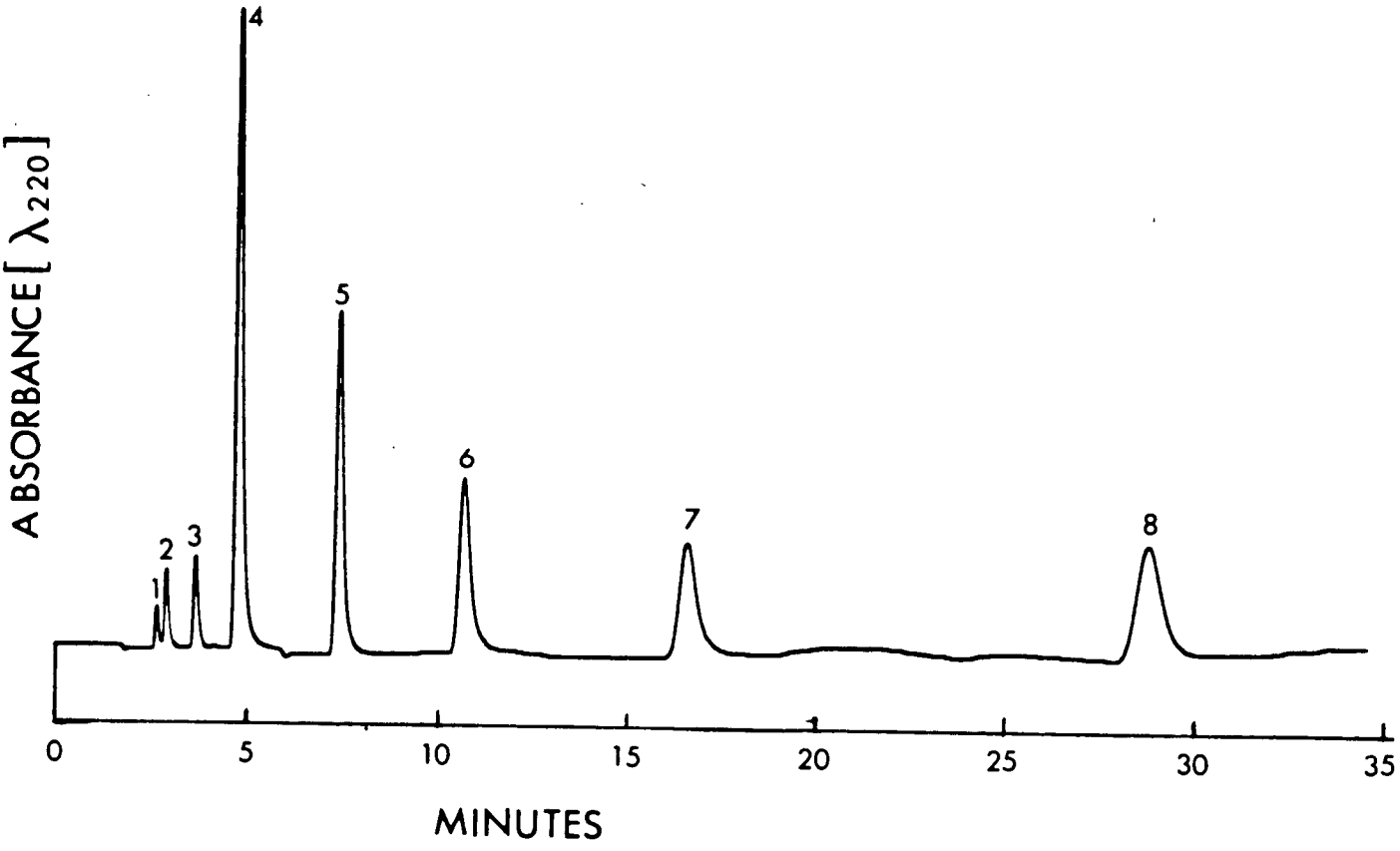


Fig. 51. A Chromatogram for the Isocratic HPLC Separation of a Standard Mixture of Digoxin, Digitoxin and their Metabolites. Sequence of elution: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = digitoxigenin; 6 = digitoxigenin monodigitoxoside; 7 = digitoxigenin bisdigitoxoside; 8 = digitoxin. HPLC conditions: Ultrasphere ODS column; solvent system, water/methanol/isopropanol/dichloromethane: 47/40/9/4; flow rate = 1.2 ml/min; UV detection at 220 nm, range = 0.02, attenuation = 6; Chart speed = 0.5 cm/min. The compounds were dissolved in the eluting solvent system.

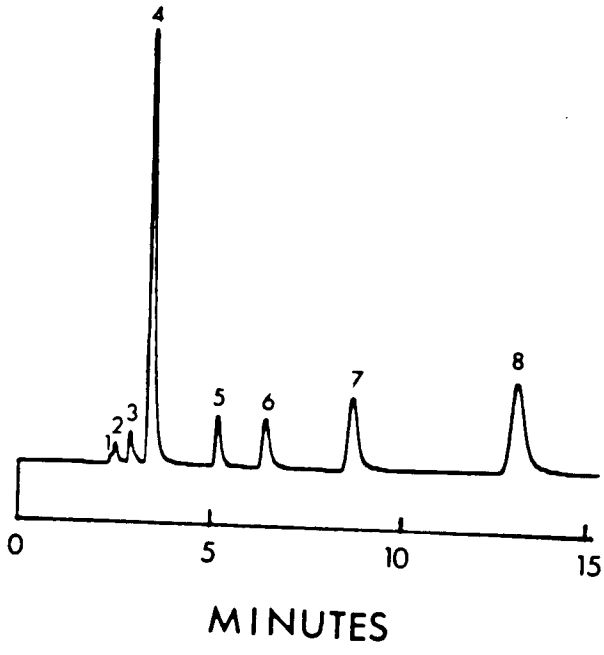


Fig. 52. A Chromatogram for the Isocratic HPLC Separation of a Standard Mixture of Digoxin, Digitoxin and their Metabolites obtained with a Solvent System of water/methanol/isopropanol/dichloromethane: 45/37/12/6. Other HPLC conditions: same as in Fig. 51.

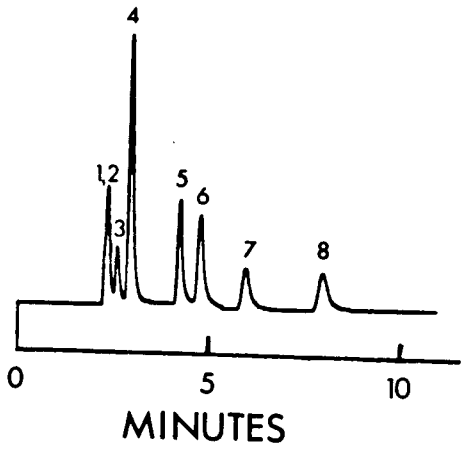


Fig. 53. A Chromatogram for the Isocratic HPLC Separation of a Standard Mixture of the Digitoxin Series from the Digoxin Series. Sequence of elution: 1,2 = digoxigenin and digoxigenin monodigitoxoside, 3 = digoxigenin bisdigitoxoside, 4 = digoxin, 5 = digitoxigenin, 6 = digitoxigenin monodigitoxoside, 7 = digoxigenin bisdigitoxoside and 8 = digitoxin. HPLC conditions: same as in Fig. 51 except that the solvent system is water/methanol/isopropanol/ dichloromethane: 43/35/15/7.

the digitoxin series of compounds and digoxin in about eight minutes while the peaks of the digoxin metabolites are clustered together at the initial portion of the chromatogram with the digoxigenin and digoxigenin monodigitoxoside peaks co-eluting. This chromatogram (Fig. 53) represents the fastest separation of the digitoxin series and is only limited by the resolution of digitoxigenin and digitoxigenin monodigitoxoside. The HPLC system used for this separation appears to be useful for a fast simultaneous analysis of digitoxin and its metabolites including digoxin. Since the sequence of elution is kept constant in all separations a more non-polar solvent system will be able to isolate digitoxin in a much shorter period of time. Such a system would be useful in situations where the primary concern is the isolation and quantitation of digitoxin.

In situations where the compounds of interest are digoxin and its metabolites, the initial portion of the chromtogram shown in Fig. 51 can be expanded in such a way that baseline separation of these compounds can be achieved. The possibility of such a chromatogram was investigated by varying the proportions of the mobile phase components in order to obtain a relatively more polar solvent system with sufficient differentiating capacity. A solvent system of water/methanol/isopropanol/dichloromethane: 51/42/5/2 resulted in a baseline separation of digoxigenin, digoxigenin monodigitoxoside, digoxigenin bisdigitoxoside and digoxin in a chromatographic time of about nine minutes (Fig. 54). This isocratic system is faster than the gradient system reported (Castle, 1975) for the resolution of digoxin and its metabolites.

Gitoxin is known to be a common impurity of digoxin, and the USP monograph on digoxin includes a test for gitoxin. Therefore, the possibility of its separation from the digoxin series was investigated. It was

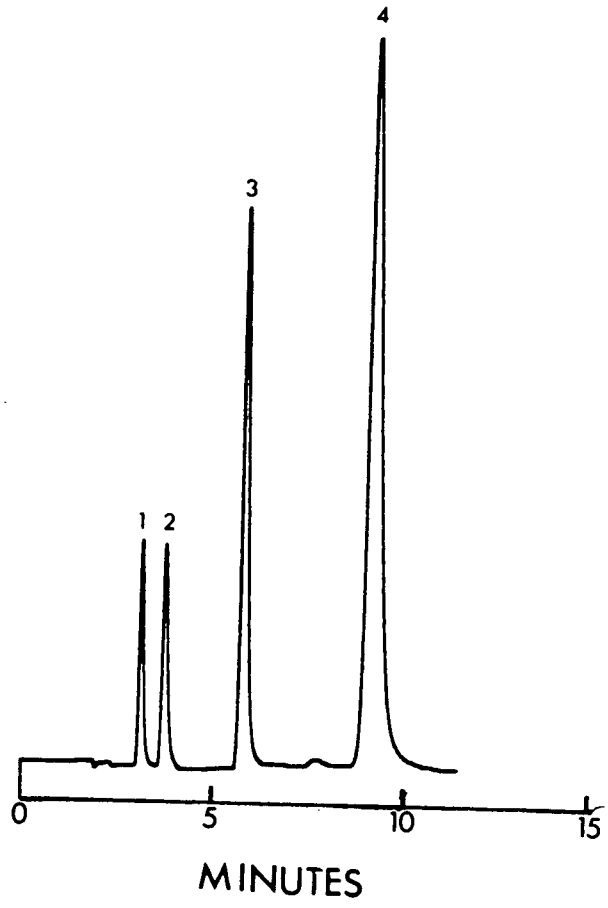


Fig. 54. A Chromatogram for the Isocratic HPLC Separation of a Standard Mixture of Digoxigenin (peak 1); Digoxigenin monodigitoxoside (peak 2); Digoxigenin bisdigitoxoside (peak 3) and Digoxin (peak 4) as obtained with a Solvent System of water/methanol/isopropanol/dichloromethane: 51/42/5/2. Other HPLC conditions: same as in Fig. 51.

found that the solvent system, water/methanol/isopropanol/dichloromethane: 47/40/9/4 elutes gitoxin at a chromatographic time of about 12 minutes, separating it from digoxin, digitoxin and their respective metabolites (Fig. 55).

Previous attempts (Cobb, 1976) to separate α and β-acetyldigoxin have not been fully successful. The possibility of their resolution by any of the systems described above was, therefore, studied. The complete separation of α and β-acetyldigoxin as well as digoxin and its metabolites was found to be possible (Fig. 56) using the solvent system water/methanol/isopropanol/dichloromethane: 51/42/5/2. This system, which is identical to that used for the resolution of the digoxin series (Fig. 54) elutes α and β-acetyldigoxin in a chromatographic time of about 14 and 20 minutes, respectively.

(B) Separation by Gradient Elution

Even though it was possible to resolve all of the components of a standard mixture of digoxin, digitoxin and their metabolites by isocratic elution, the possibility of a reduced chromatographic time with gradient elution was investigated. After a number of attempts to obtain an optimum gradient system, a solvent system of water/methanol/isopropanol/dichloromethane: 49/41/7/3 was found to be most convenient for the initial portion of the chromatogram (Fig. 57). A linear gradient of 0 to 100% of a second solvent system (water/methanol/isopropanol/dichloromethane: 38/32/20/10) obtained between the chromatographic times of 2.5 to 3 minutes and maintained till the end of the run, resulted in a complete separation of all components in about 13 minutes (Fig. 57). It can be observed from the chromatogram that this method is much faster than the gradient separation (in 21.5 minutes) that was previously reported (Castle, 1975). In order

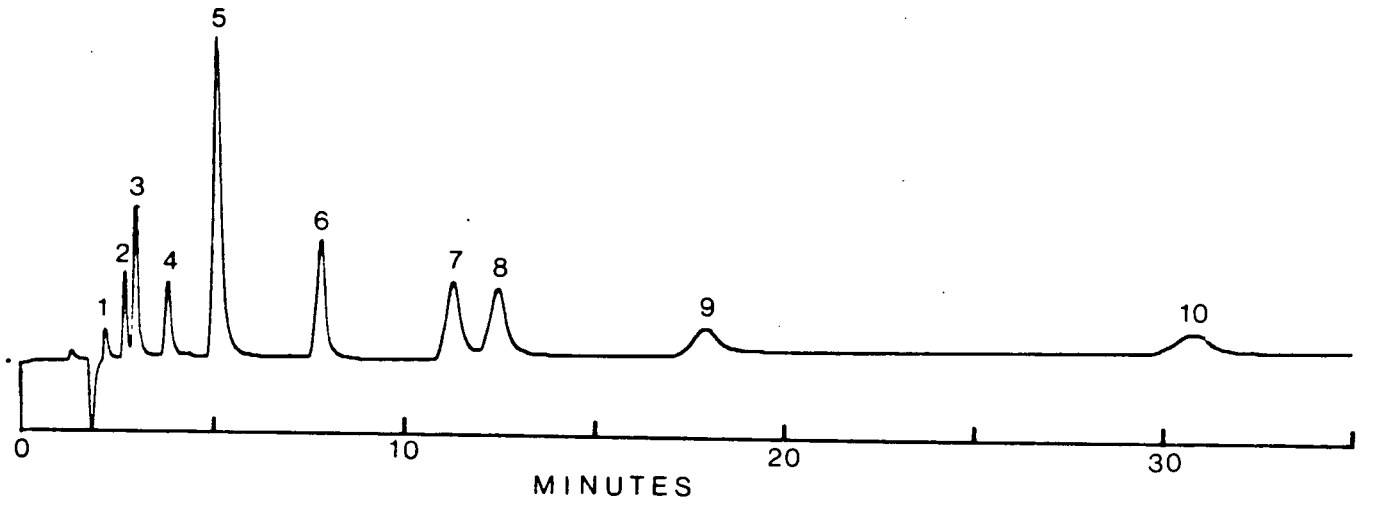


Fig. 55. A Chromatogram for the Isocratic HPLC Separation of a Standard Mixture of Digoxin, Digitoxin, the respective Metabolites and Gitoxin. Sequence of elution: 1 = unknown impurity; 2 = digoxigenin; 3 = digoxigenin monodigitoxoside; 4 = digoxigenin bisdigitoxoside; 5 = digoxin; 6 = digitoxigenin; 7 = digitoxigenin monodigitoxoside; 8 = gitoxin; 9 = digitoxigenin bisdigitoxoside and 10 = digitoxin. HPLC conditions: same as in Fig. 51.

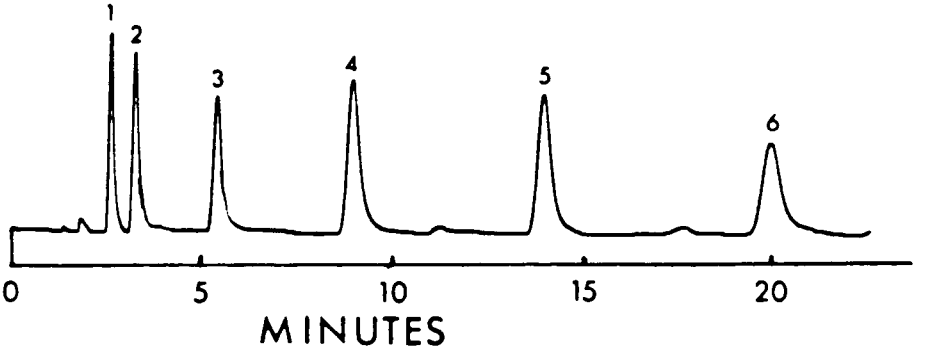


Fig. 56. A Chromatogram for the Isocratic HPLC Separation of α and β-acetyldigoxin from Digoxin and its Metabolites. Sequence of elution: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin; 5 = α-acetyldigoxin; and 6 = β-acetyldigoxin. HPLC conditions: same as in Fig. 54.

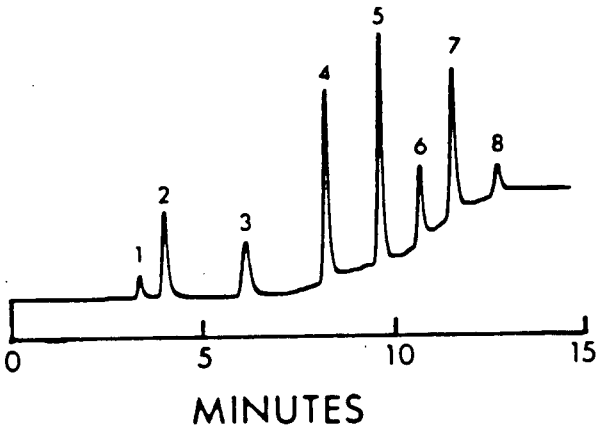


Fig. 57. A Chromatogram for the HPLC Separation of a Mixture of Digoxin, Digitoxin and their Metabolites by Gradient Elution. Sequence of elution: same as in Fig. 53. HPLC conditions: same as in Fig. 53 with the following exceptions - A solvent system of water/methanol/isopropanol/dichloromethane: 49/41/7/3 is used as the initial eluting solvent and a linear gradient from 0 to 100% of a second solvent system (water/methanol/isopropanol/dichloromethane: 38/32/20/10) obtained at the chromatographic time of 2.5 to 3 minutes is maintained until the end of the run. Range = 0.1; attenuation = 9; column re-equilibration takes about 15 minutes.

to minimize the baseline fluctuations that are associated with gradient elution, low detector settings and a relatively high sample load were employed. As discussed before, the constraints of low detector sensitivity may preclude the use of gradient elution for the analysis of cardiac glycosides. Nevertheless, the fast gradient separation can be useful for qual itative HPLC work with these compounds.

(C) Separation using a Solvent Switch-over System

The initial use of a solvent system that would be able to resolve all of the digoxin series at the earlier part of the chromatogram, with a subsequent change-over to a relatively less polar solvent that would rapidly elute the digitoxin series in the same chromatogram, while maintaining baseline resolution was tried. A switch-over from an initial solvent system of water/methanol/isopropanol/dichloromethane: 49/41/7/3 to a second solvent system with a proportion of 41/34/17/8 at a chromatographic time that corresponds with the peak of digoxigenin bisdigitoxoside resulted in a 16-minute chromatogram that shows complete separation of all the compounds (Fig. 58). It can be seen that this chromatogram closely approximates the separation obtained by gradient elution. The solvent switch-over method, therefore, can be useful for qualitative HPLC work in situations where the liquid chromatograph does not have gradient capability.

As indicated earlier, changes in the proportion of the solvents employed in the four-component solvent system have been shown to bring about an expansion or contraction of the distances between adjacent peaks and hence resulting in the desired separations. This could be due to changes in any one or a combination of the following factors: polarity, solubility effect and diffusion effect (viscosity). It can also be

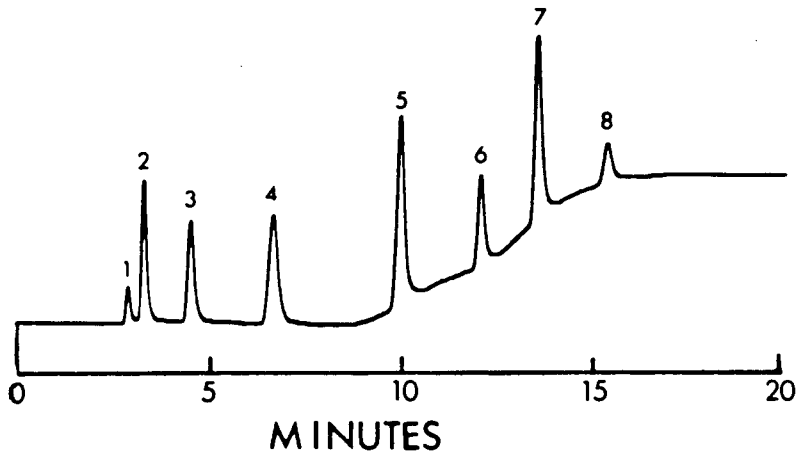


Fig. 58. A Chromatogram for the HPLC Separation of a mixture of Digoxin, Digitoxin and their Metabolites by Solvent Switchover elution. Sequence of elution and HPLC conditions: same as in Fig. 57 with the following exception: A solvent system of water/methanol/isopropanol/dichloromethane: 49/41/7/3 is used as the initial eluting solvent and a switchover to a second solvent system (water/methanol/isopropanol/dichloromethane: 41/34/17/8 is made at a point in time that corresponds to the peak of digoxigenin bisdigitoxoside.

Table X. Retention Times (tr, in minutes) and Capacity Ratio Values (K') of the Compounds Studied, under the Conditions Defined by the Corresponding Figures

Compound	Fig. 51		Fig. 52		Fig. 53		Fig. 54		Fig. 55		Fig. 56		Fig. 57		Fig. 58	
	tr	K'	tr	K'	tr	K'	tr	K'	tr	K'	tr	K'	tr	K'	tr	K'
Digoxigenin	2.63	0.31	2.40	0.20	2.32	0.16	3.15	0.57	2.78	0.35	2.60	0.30	3.37	0.68	2.95	0.47
Digoxigenin monodigitoxoside	2.87	0.43	2.51	0.25	2.32	0.16	3.76	0.88	3.04	0.46	3.30	0.65	4.00	1.00	3.36	0.68
Digoxigenin bisdigitoxoside	3.62	0.81	2.91	0.45	2.58	0.25	5.81	1.90	3.88	0.80	5.20	1.60	6.03	1.01	4.59	1.29
Digoxin	4.72	1.36	3.43	0.71	2.90	0.45	9.14	3.57	5.14	1.46	9.00	3.50	8.27	3.31	6.78	2.39
Digitoxigenin	7.29	2.64	5.21	1.60	4.15	1.07							9.73	3.86	10.11	4.05
Digitoxigenin mono-digitoxoside	10.35	4.17	6.45	2.22	4.62	1.31							10.83	4.41	12.28	5.14
Digitoxigenin bisdigitoxoside	15.93	6.96	8.72	3.36	5.75	1.87							11.70	4.85	13.81	5.90
Digitoxin	27.38	12.69	12.99	5.49	7.65	2.82							12.90	5.45	15.68	6.84
Gitoxin									12.48	5.08						
α-Acetyldigoxin											14.00	6.00				
β-Acetyldigoxin											20.43	9.21				

observed that the sequence of elution of the compounds remains the same in all cases. For purposes of comparison of the various chromatograms that have been obtained, values for retention time and capacity ratio factor (K') are presented in Table X. It can perhaps be surmised, therefore, that the versatility of this solvent system is such that it may be applicable for the separation of other cardiac glycosides and even steroids in general. Moreover, under tightly controlled conditions, the specificity of the retention times may warrant the use of these methods for confirmatory identification of any of the cardiac glycosides.

3. Development of a 100% Fluid Recovery System for the HPLC Analysis of Digoxin and its Metabolites after Fluorogenic Post-Column Derivatization using the Air-segmentation Process

The presence of dihydro derivatives of digoxin, digoxigenin and the mono- and bisdigitoxosides as metabolic products of digoxin has been reported (Gault et al., 1980). The dihydro products are formed by the reduction of the C_{20} - C_{22} double bond of the lactone ring and therefore do not absorb UV radiant energy. Hence HPLC separation of these compounds can not be monitored using a UV detector. For purposes of attaining increased sensitivity by fluorescence detection Gfeller et al. (1977) have reported a post-column fluorogenic derivatization procedure using the air-segmentation process for the HPLC analysis of some cardiac glycosides. The possibility of using this fluorogenic derivatization procedure for monitoring the HPLC separation of digoxin and its metabolites including some of the dihydro products was therefore investigated. The post-column derivatization set-up was modified for purposes of attaining 100% fluid recovery. A schematic diagram of the modified post-colum derivatization

set-up is shown in Fig. 22.

The pump tubes; the D_2 connectors; the mixing reaction and cooling coils and debubbler were the same as in the set-up described by Gfeller et al. (1977) except that: (1) the D_2 connectors and debubbler were replaced by miniaturized ones (1 mm i.d.); (2) the vertical exit of the debubbler was connected by means of an acidflex tubing, to an overhanging glass tube; and (3) the reaction chamber was maintained at 55°C. The HPLC eluate was passed through a UV detector, the reaction system, the debubbler set-up and the fluorometer before it was directed into the waste receptacle.

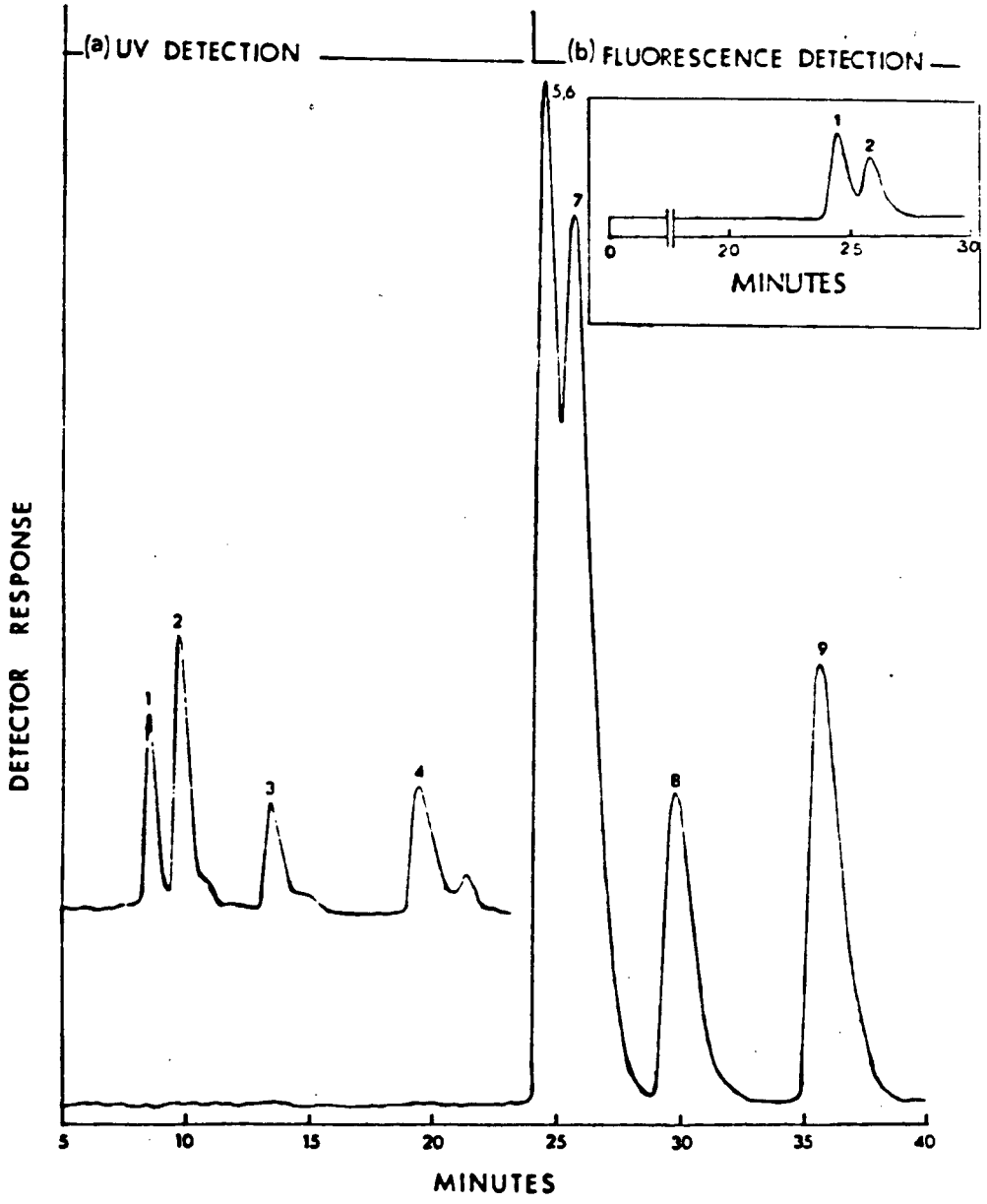
The set-up for the 100% fluid recovery system consists of an 84 cm long glass tube (1.1 cm i.d.) tapering at its lower end to 1 mm i.d. where it is connected to a modified C_4 debubbler which has side tubes of 1 mm i.d. The glass tube is positioned as high as possible so that when three-quarters full with the circulating fluid, it exerts hydrostatic pressure on the surface of the liquid in the debubbler. The air-segmented fluid entering the debubbler, therefore, passes through the horizontal exit into the fluorescence detector, whereas the segments of air escape through the vertical exit as bubbles. In order to attain 100% fluid recovery, the debubbling process is optimized by careful up and down adjustments of the position of the waste receptacle until the upward pressure (in the debubbler) exerted by the pump is equalized by the hydrostatic pressure of the fluid in the suspended glass tube. The fluid coming out of the fluorescence detector had a flow rate of about 1 ml per minute and pumping the waste fluid out of the detector was not found to be necessary.

HPLC separation of digoxin and its metabolites using UV detection and the fluorogenic derivatization procedure described above, is presented

in Fig. 59. The UV and fluorescence detector responses were obtained with a dual pen recorder after one sample injection. The initial portion of the chromatogram (Fig. 59(a)) shows the separation of digoxin (Peak 4), digoxigenin (Peak 1) and the mono- and bisdigitoxosides (Peaks 2 and 3, respectively) after UV detection at a wavelength of 254 nm. Even though the sample contains dihydrodigoxigenin, this compound does not have a peak in the UV monitored chromatogram since it does not have UV absorbance. After on-line post-column fluorogenic derivatization, however, all of the five compounds are detected by the fluorometer after a total chromatographic and derivatization period of 36 minutes. This is shown in the latter portion of the chromatogram (Fig. 59(b)) where dihydrodigoxigenin and digoxigenin elute together (Peaks 5, 6) while digoxigenin monodigitoxoside (Peak 7), digoxigenin bisdigitoxoside (Peak 8) and digoxin (Peak 9) are separated. The simultaneous elution of dihydrodigoxigenin and digoxigenin is evident in the greater magnitude of Peak 5, 6 relative to Peak 7 as compared to the relative sizes of Peaks 1 and 2. Separation of dihydrodigoxigenin (Peak 1) and digoxigenin monodigitoxoside (Peak 2) is shown in the inset of Fig. 59. It can be observed that fluorometric monitoring of digoxin and its metabolites results in a chromatogram with well defined symmetrical peaks. It has been shown that the solvent system (water/methanol/isopropanol/dichloromethane: 50/41/6/3) used for generating the chromatogram in Fig. 59 is compatible with the aqueous media of the derivatization process.

The debubbling system that was developed for 100% fluid recovery prior to fluorometric detection was found to be stable over long periods of time. Complete fluid recovery without the presence of any visible air bubbles could continuously be maintained for as long as eight hours. It is to be noted that the 100% fluid recovery system, by virtue of preventing sample loss, can be useful for purposes of collecting the relatively small

Fig. 59. A Chromatogram for the Isocratic HPLC Separation of a Mixture of Digoxin, Dihydrodigoxigenin and the other Digoxin Metabolites as obtained by Dual Detector Monitoring. Peak identities: (a) UV detection (λ254)- (1) digoxigenin, (2) digoxigenin monodigitoxoside, (3) digoxigenin bisdigitoxoside, (4) digoxin; (b) Fluorescence detection ($\lambda_{exc.}$ = 360 nm and $\lambda_{em.}$ cut off = 460) - (5,6) digoxigenin and dihydrodigoxigenin, (7) digoxigenin monodigitoxoside, (8) digoxigenin bisdigitoxoside, (9) digoxin. HPLC conditions: Ultrasphere ODS column; solvent system, water/methanol/isopropanol/dichloromethane: 50/41/6/3; Chart speed 0.5 cm/min. The compounds were dissolved in the eluting solvent. Inset: A chromatogram for the separation of dihydrodigoxigenin(1) and digoxigenin monodigitoxoside(2) under the conditions of (b).


(a) UV DETECTION
(b) FLUORESCENCE DETECTION
DETECTOR RESPONSE
1
2
3
4
5,6
7
8
9
5
10
15
20
25
30
35
40
MINUTES
1
2
0
20
25
30
MINUTES

amounts of separated dihydro metabolites of digoxin for subsequent quantitation using more sensitive methods. Using the set-up, the minimum detectable amount of digoxin (at a response to noise ratio of 2:1) was found to be 10 ng.

4. Separation of Nine Equine Estrogens using the HPLC System, as evidence of Selectivity

In the search for internal standards to be used for the HPLC analysis of cardiac glycosides, various steroids including the equine estrogens were considered. Three equine estrogens were found to satisfy the required chromatographic conditions. During the course of this study it was also observed that most of the nine equine estrogens that were tested had somewhat different retention times. An inspection of the chemical structures of these compounds (Fig. 21) indicates that they are closely related and differ only on the basis of whether or not they have (1) α-hydroxyl, β-hydroxyl or a keto group on C-17 and (2) two, one or no double bond on ring B of the steroid moiety. The structural differences among the cardenolides shown in Table VII appear to be more significant compared to the variations among the estrogens. If the HPLC system that has been developed could separate a mixture of compounds with such minor structural differences it would be an additional evidence of selectivity. The possibility of such a separation was, therefore, investigated as a test for the ability of the solvent system to differentiate related steroids.

The separation of a standard mixture of the nine equine estrogens and 17α-ethynylestradiol, obtained with a solvent system of water/methanol/isopropanol/dichloromethane: 48/40/8/4, is shown in Fig. 60. It can be observed that all components are sufficiently differentiated to warrant peak height quantitation in a chromatographic time of about 16 minutes. Equilin (peak 10) and estrone (peak 11) which are therapeutically the

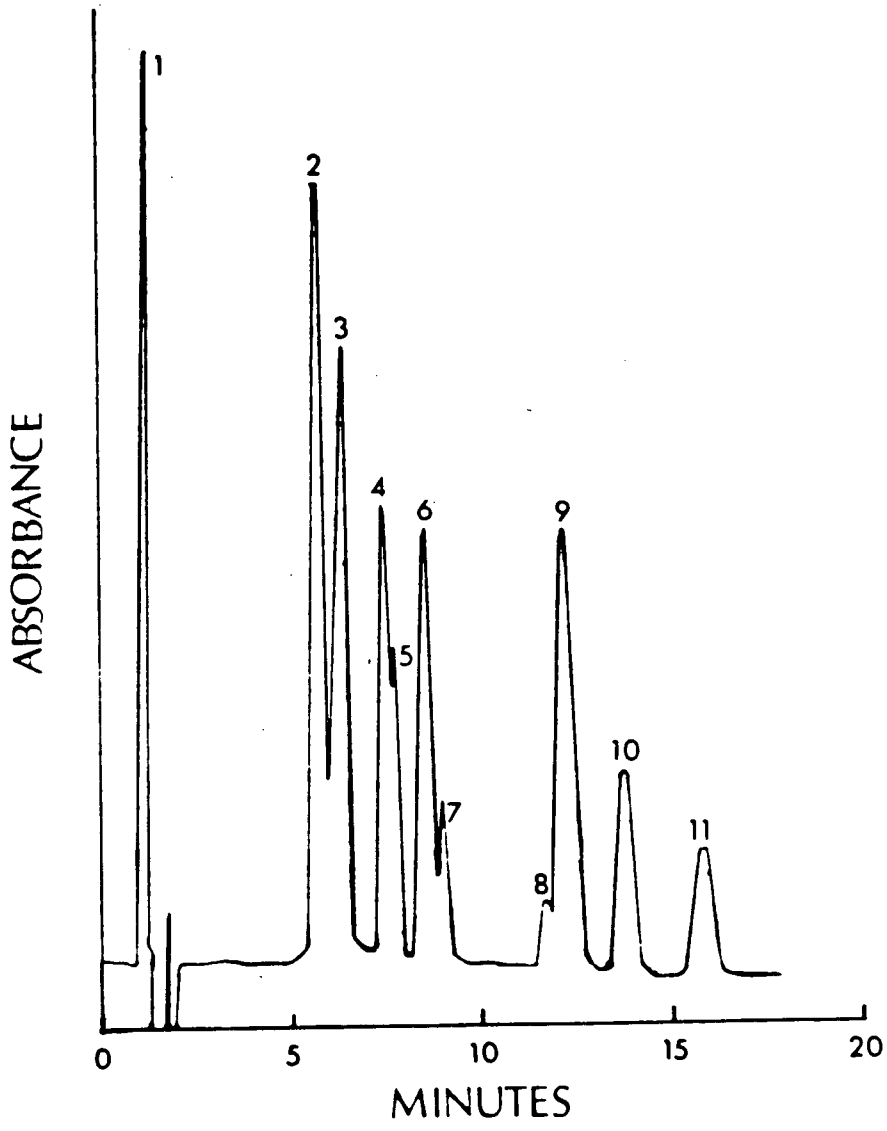


Fig. 60. A Chromatogram for the Isocratic HPLC Separation of a mixture of nine Equine Estrogens and 17α-ethynylestradiol. Sequence of elution: (1) solvent peak; (2) 17β-dihydroequilenin; (3) 17α-dihydroequilenin; (4) 17β-dihydroequilin; (5) 17α-dihydroequilin; (6) 17α-ethynylestradiol; (7) 17β-estradiol; (8) 17α-estradiol; (9) equilenin; (10) equilin; (11) estrone. HPLC conditions: Ultrasphere ODS column; solvent system, water/methanol/isopropanol/dichloromethane: 48/40/8/4; flow rate, 1.2 ml/min; UV detection at 220 nm; Chart speed, 0.5 cm/min. The compounds were dissolved in 35% methanol.

most important estrogens, are completely separated.

A 22-minute chromatogram that shows baseline separation of 17β-dihydroequilenin (peak 2), 17α-estradiol (peak 7), equilenin (peak 8), equiline (peak 9) and estrone (peak 10) as well as a fairly good separation of 17α-dihydroequilenin (peak 3) from 17β-dihydroequilin (peak 4) and 17α-dihydroequilin (peak 5) from 17β-estradiol (peak 6) is presented in Fig. 61. This chromatogram was obtained with a solvent system of water/methanol/isopropanol/dichloromethane: 49/41/7/3.

The complete baseline separation of the nine equine estrogens, in about 34 minutes (Fig. 62) was found to be possible with a more polar solvent system of water/methanol/isopropanol/dichloromethane: 52/43/3/2. Peak 1 and the negative peaks are due to solvent effect. The nine estrogens elute in the following sequence: 17β-dihydroequilenin (peak 2), 17α-dihydroequilenin (peak 3), 17β-dihydroequilin (peak 4), 17α-dihydroequilin (peak 5), 17α-estradiol (peak 6), equilenin (peak 7), 17β-estradiol (peak 8), equilin (peak 9) and estrone (peak 10). It is interesting to observe that 17β-estradiol is relatively more retained and therefore shows a reversal in its sequence of elution with respect to 17α-estradiol and equilenin. The sequence of elution of the others, however, remains the same. It is perhaps because of this extra-sensitivity to polarity changes that 17β-estradiol was a major source of problems in resolution (Johnson et al., 1975).

The HPLC separations shown in Figs. 60, 61 and 62 were obtained with solvent systems that consist of water/methanol/isopropanol/dichloromethane in the proportions of: 52/43/3/2, 49/41/7/3 and 48/40/8/4, respectively. It can therefore be observed that the selectivity and capacity ratio factors were optimized by slight changes in the relative proportions of the solvents.

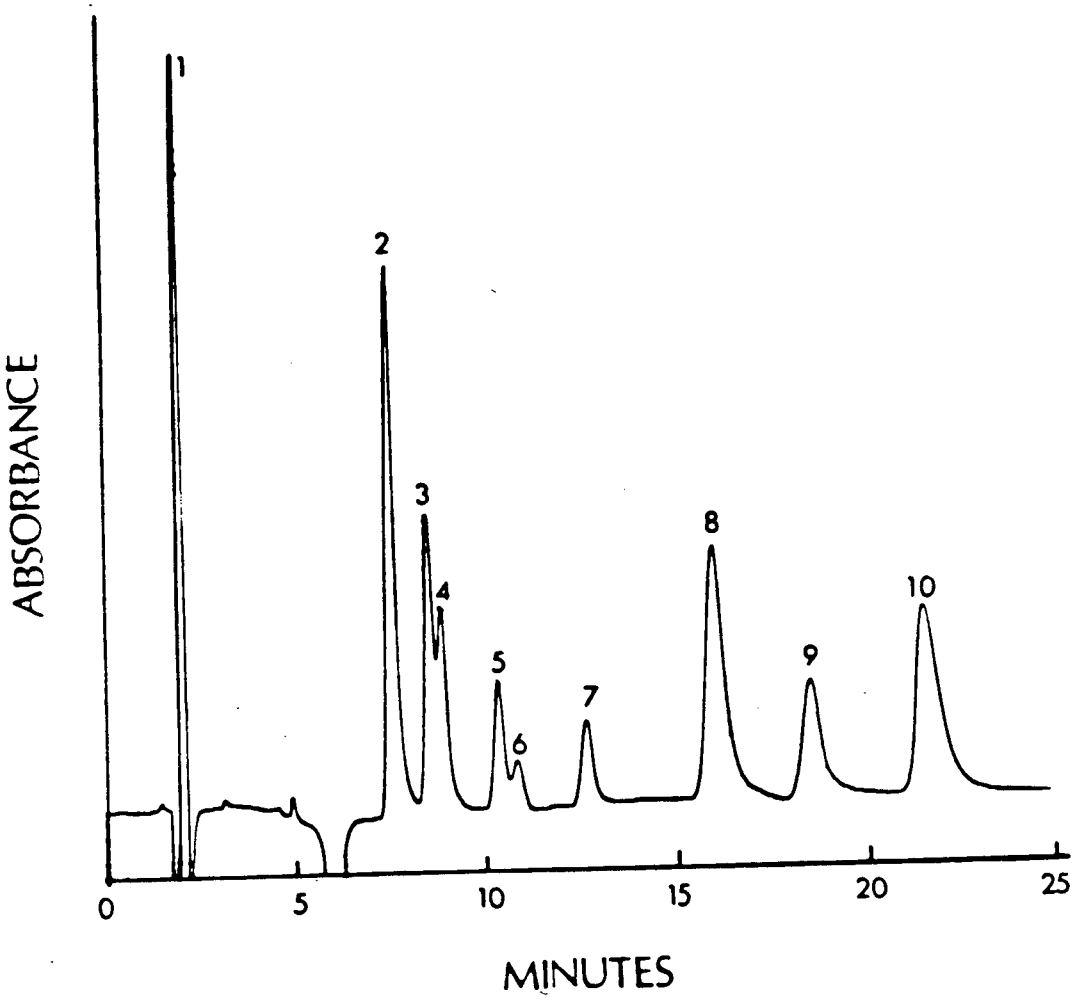


Fig. 61. A Chromatogram for the Isocratic HPLC Separation of a mixture of nine Equine Estrogens as obtained with a Solvent System of water/methanol/isopropanol/dichloromethane: 49/41/7/3. Sequence of elution: (1) solvent peak; (2) 17β-dihydroequilenin; (3) 17α-dihydroequilenin; (4) 17β-dihydroequilin; (5) 17α-dihydroequilin; (6) 17β-estradiol; (7) 17α-estradiol; (8) equilenin; (9) equilin; (10) estrone. Other HPLC conditions: same as in Fig. 60 except that the compounds were dissolved in 50% ethanol.

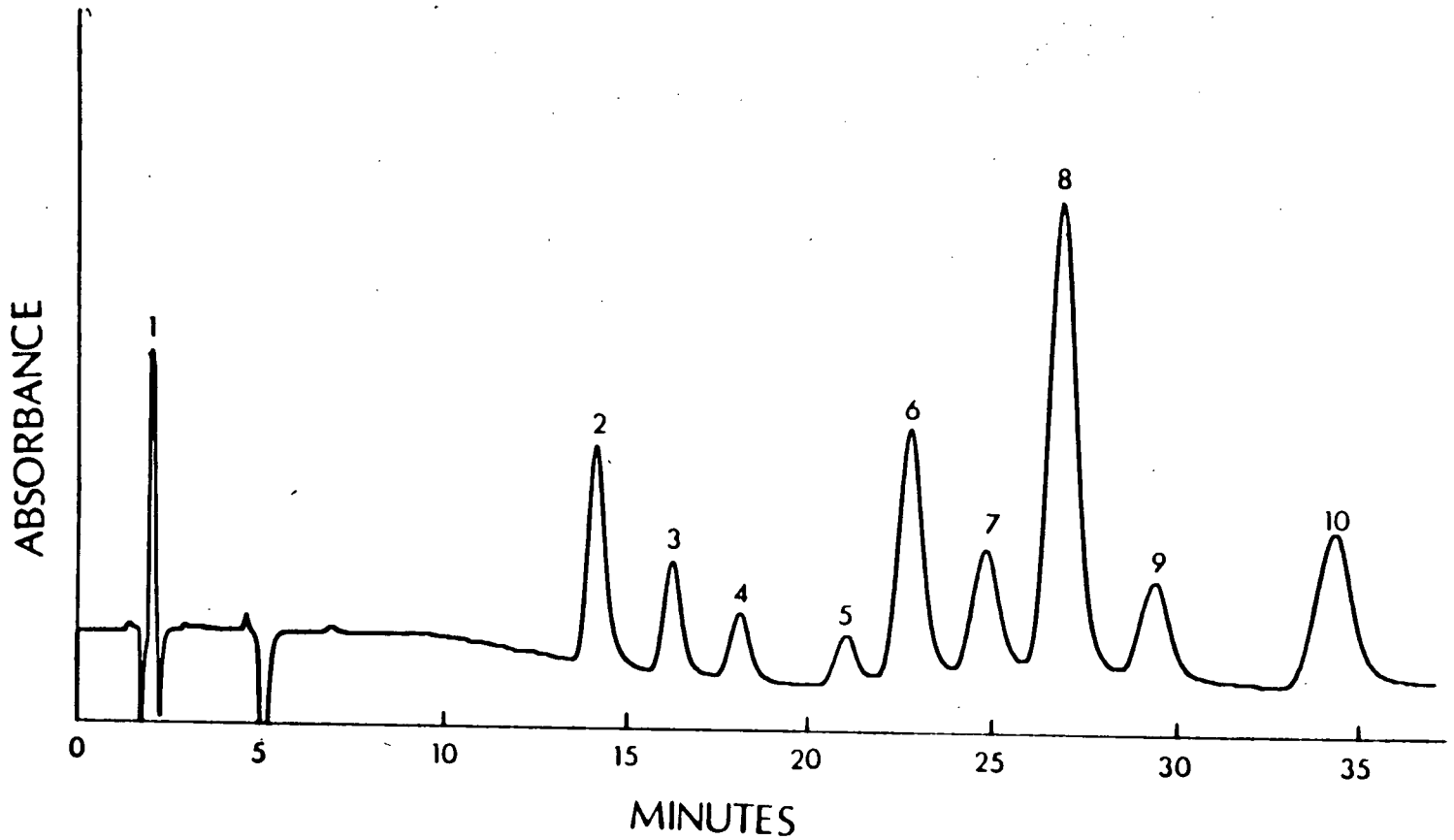


Fig. 62. A Chromatogram for Complete Isocratic HPLC Separation of a mixture of nine Equine Estrogens as obtained with a Solvent System of water/methanol/isopropanol/dichloromethane: 52/43/3/2. Sequence of elution: (1) solvent peak; (2) 17β-dihydroequilenin; (3) 17α-dihydroequilenin; (4) 17β-dihydroequilin; (5) 17α-dihydroequilin; (6) 17α-estradiol; (7) equilenin; (8) 17β-estradiol; (9) equilin; (10) estrone. Other HPLC conditions: same as in Fig. 61 except that the flow rate was 1.3 ml/min.

The current USP assay procedure for Conjugated Estrogens, and Esterified Estrogens and their respective tablet dosage forms is basically colorimetric after column chromatographic separation and a series of long and tedious extraction steps. Compared to the slow separation process and off-line detection often associated with column chromatography and the derivatization step that is inherent in GLC, HPLC appears to be the technique of choice for the analysis of conjugated and esterified estrogens. This is so because the HPLC method described above is faster and more selective than column chromatography, avoids the derivatization step of GLC and therefore has a definite advantage with respect to time and simplicity of analysis.

5. Isolation of Digitoxin from Digitalis purpurea Leaf

Digitalis purpurea leaf contains a mixture of dititoxin, gitoxin, gitaloxin, other related glycosides and plant constituents. The purified form of digitoxin and the other cardiac glycosides are obtained from plant extracts after chromatographic separation and purification processes. The feasibility of using the solvent system that has been developed, for the isolation of digitoxin from the leaf extract was, therefore, studied.

After a number of optimizations, the chromatogram shown in Fig. 63 was obtained using a solvent system of water/methanol/isopropanol/dichloromethane: 45/38/11/6. It can be observed that peak 6 (which

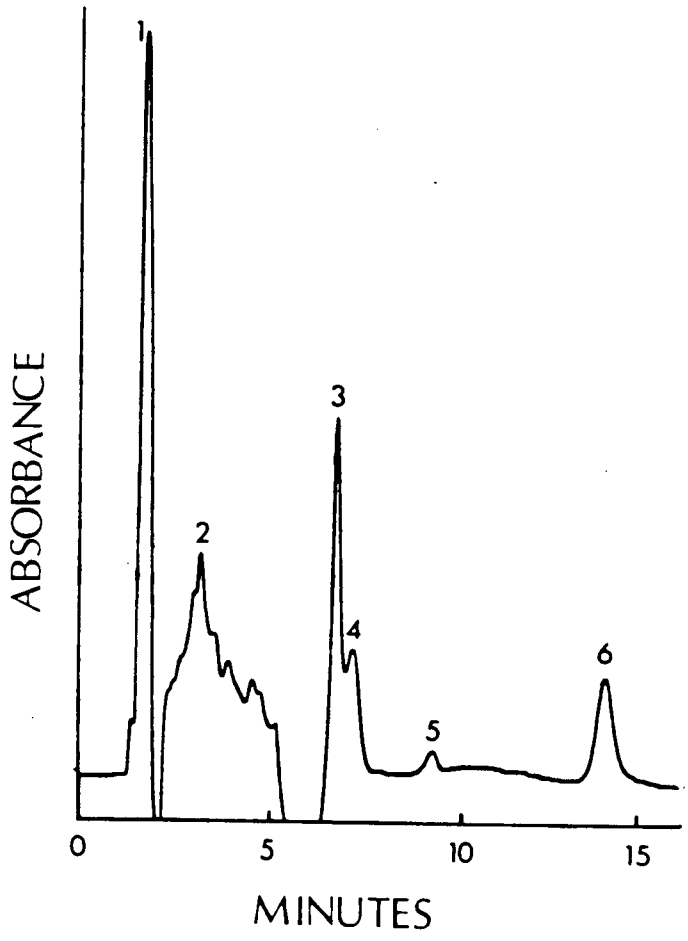


Fig. 63. A Chromatogram for the Isocratic HPLC Separation of Digitoxin from Other Components of Digitalis purpurea Leaf. Peak identities: (1, 2, 4, 5) unknowns; (3) gitoxin; (6) digitoxin. HPLC conditions: solvent system, water/methanol/isopropanol/dichloromethane: 45/38/11/6; flow rate, 1.1 ml/min; UV detection at 220 nm; Chart speed, 0.5 cm/min.

has the same retention time as that of an authentic digitoxin sample) is very well separated from any of the other components. Peak 3 was found to represent gitoxin. Peaks 1, 2, 4 and 5, however, were not identified. Digitalis powder, which is currently official in the USP contains a high proportion of digitoxin and a bioassay method is used to monitor its potency. It appears, therefore, that this HPLC method can be used: (1) at least as a supplementary technique for fast monitoring of the digitoxin content in Digitalis powder and (2) for preparative HPLC work involving isolation and purification of digitoxin drug substance.

6. Development of HPLC Methods for the Analysis of Digoxin in its Dosage Forms

The analytic methods reported in the literature, dealing with digoxin tablet formulations are essentially colorimetric (Myrick, 1969; USP XX, 1980), fluorometric (Cullent et al., 1970; Nyberg et al., 1974) and gas-liquid chromatographic (Kibbe and Araujo, 1973) techniques. The colorimetric methods have the disadvantage of insufficient sensitivity and/or lack selectivity. The fluorometric method is based on the determination of the dehydration products of the steroid moiety of the digoxin molecule, after reaction with concentrated hydrochloric acid in the presence of hydrogen peroxide and ascorbic acid. Even though the fluorometric methods have the advantage of high sensitivity, they are non specific. The gas liquid chromatographic methods, in general, involve the production of derivatives and require a great deal of time as well as laborious manipulative steps.

The sensitive GLC method reported by Watson et al. (1972) employs electron capture detection after derivatization to genin-diheptafluorobutyrate. Any degradation product of digoxin that may be present, however, will not be differentiated from digoxin. The GLC method (Kibbe and Aranjo, 1973) for the analysis of digoxin tablets is again non-specific (since digoxin is hydrolyzed to digoxigenin prior to detection) and not sensitive enough for single tablet assay. The lack of a mobile phase that has sufficient UV transparency at the wavelength of maximum absorption of digoxin (220 nm) and a solvent that is appropriate for sample preparation, appears to have prevented the use of HPLC in the analysis (after UV detection) of digoxin dosage forms. It is clear that there is a need for methods of analysis of digoxin in its dosage forms, that are selective enough to preclude assay interference from degradation products and sufficiently sensitive to allow single tablet assay. The possibility for such methods, using HPLC, was therefore investigated.

The representative chromatogram (Fig. 64) for the analysis of digoxin in tablet dosage forms shows the peaks of digoxin and 17α-ethynylestradiol (internal standard) with retention times of 4.7 and 8.9 minutes, respectively. This chromatogram was obtained with a solvent system of water/methanol/isopropanol/dichloromethane: 47/40/9/4. It can be seen that the digoxin and internal standard peaks are free from interference by tablet excipients. Peak 1 was found to be an impurity from the filter paper. The two negative peaks immediately after peak 1 and the small negative peak between the digoxin and internal standard peaks were due to solvent effect. The baseline perturbation that occurs between the peaks of digoxin and the internal standard was found to appear in the

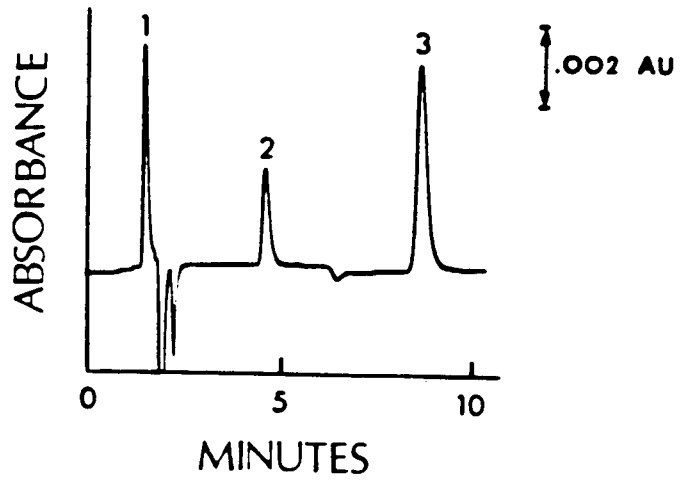


Fig. 64. A Representative Chromatogram for the Isocratic HPLC Analysis of Digoxin Tablets. Peak identity: (1) unknown impurity; (2) digoxin; and (3) 17α-ethynylestradiol (internal standard). HPLC conditions: solvent system, water/methanol/isopropanol/dichloromethane: 47/40/9/4; flow rate, 1.2 ml/min; UV detection at 220 nm; Chart speed, 0.5 cm/min.

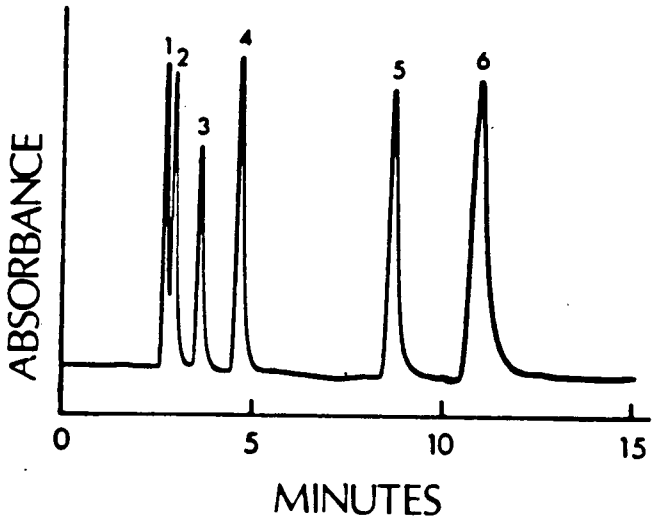


Fig. 65. A Chromatogram for the Isocratic HPLC Separation of Digoxin, its probable Degradation Products, 17α-ethynylestradiol (internal standard) and gitoxin: sequence of elution: (1) digoxigenin; (2) digoxigenin monodigitoxoside; (3) digoxigenin bisdigitoxoside; (4)digoxin; (5) 17α-ethynylestradiol; and (6) gitoxin. HPLC conditions: same as in Fig. 64.

form of a peak or a baseline depression depending upon the proportion of water and methanol in the solvent used for sample preparation. It was observed that a methanol content of about 35% v/v in the hydroalcoholic solvent in which the sample was dissolved prior to injection in the liquid chromatograph, eliminated the perturbation effect and maintained baseline stability. A proportion of methanol that was higher or lower than about 35% v/v introduced a negative or positive peak, respectively. The peak that is associated with a higher water content appears to be due to the formation of an emulsion between the mobile phase and injected sample at the point of first contact. Whereas the baseline depression that occurs with a higher methanol content seems to be due to a dilution effect of the injected sample solution on the mobile phase at the point of contact, thus momentarily reducing the baseline absorbance level.

The HPLC method for digoxin tablet assay was found to be capable of accounting for each of the probable degradation products of digoxin as well as gitoxin which is a common contaminant of digoxin, as shown in Fig. 65. Digoxigenin, digoxigenin monodigitoxoside, digoxigenin bis-digitoxoside (peaks 1, 2 and 3, respectively) elute prior to digoxin (peak 4) and gitoxin (peak 6) appears after 17α-ethynylestradiol (peak 5). For purposes of assay validation, common tablet excipients (e.g. starch, lactose, cellulose, stearate lubricants) were taken through the assay and found to be non-interfering.

The calibration curve of digoxin (Fig. 25) obtained for the analysis of digoxin (Fig. 25) dosage forms was found to be linear with the line passing through the origin, and a response factor of 1.4261 was derived for the quantitation step. The results of the composite sample analysis of digoxin tablets are shown in Table XI. Average label claim values (n = 4)

Table XI. Results of the HPLC Analysis of Composite Samples of Digoxin Tablets

N	Percent of Label Claim			
	Brand A		Brand B	Brand C
	0.125 mg	0.25 mg	0.25 mg	0.25 mg
1	98.0	94.0	98.8	101.6
2	101.2	96.8	97.2	100.0
3	99.2	95.2	98.0	104.8
4	101.2	95.2	97.6	98.4
Mean	99.9	95.3	97.9	101.2

of 95.3%, 97.9% and 101.1% were obtained for Brands A, B and C, all of which were 0.25 mg tablets. The average label claim value for the 0.125 mg tablet (Brand A) was found to be 99.9%. Since the range of label claim values that the USP allows for digoxin tablets is 92-108%, it can be observed that all brands meet the assay requirements. The precision data in Table XII indicate that the analysis of the tablets was accomplished with a relative standard deviation of 1.5% (n = 6).

The results of the HPLC single tablet assay of digoxin tablets (0.125 mg per tablet and 0.25 mg per tablet) of Brand A, are presented in Table XIII. Average label claim values of 97.1% and 96.6% were obtained for 0.125 mg and 0.25 mg tablets, respectively, with a corresponding precision of 5.1% and 4.6%. The recovery data for digoxin tablet assay are presented in Table XIV. The average recovery value of digoxin from tablet material was found to be 99.8% with a relative standard deviation of 3.2% (n = 6).

The representative chromatogram for the analysis of digoxin injection (Fig. 66) indicates that digoxin (peak 2) and the internal standard (peak 3) are free from interference by pharmaceutical excipients. Peak 1 and the negative peaks at the initial portion of the chromatogram and the small negative peak between the digoxin and internal standard peaks are due to solvent effects. This chromatogram is essentially similar to that obtained for the tablet assay and was generated using the solvent system, water/methanol/isoproanol/dichloromethane: 47/40/9/4. The results of the analysis of digoxin injection (Table XV) indicate average label claim values of 99.4% and 99.1%, for the 0.05 mg/ml and 0.25 mg/ml injections, respectively. These values were obtained with relative

Table XII. Precision Data for the HPLC Analysis of Digoxin Tablets[a]

N	Percent of Label Claim
1	98.0
2	99.4
3	98.2
4	98.0
5	101.4
6	100.5
Mean ($\bar{x}$)	99.2
R.S.D., %[b]	1.45

[a] 0.25 mg digoxin tablets, Brand A

[b] Relative standard deviation

Table XIII. Results of HPLC Single Tablet Assay of Digoxin Tablets

N	Percent of Label Claim[a]	
	0.125 mg	0.25 mg
1	90.0	92.8
2	91.4	96.4
3	105.8	92.6
4	102.4	106.0
5	93.9	102.0
6	96.8	94.8
7	94.6	98.6
8	96.8	96.9
9	101.2	93.2
10	98.4	92.8
Mean ($\bar{x}$)	97.1	96.6
R.S.D.[b], %	5.1	4.6

[a] Brand A

[b] Relative standard deviation

Table XIV. Recovery Data for Digoxin Tablet Assay[a]

N	Theoretical Amount of Digoxin (mg)	Amount of Digoxin Added (mg)	% Recovery
1	1.25	0.625	99.7
2	1.25	0.625	101.3
3	1.25	0.625	96.8
4	1.25	0.625	102.6
5	1.25	0.625	95.2
6	1.25	0.625	103.4
Mean ($\bar{x}$)			99.8
R.S.D. %[b]			3.2
95% Confidence limits(±)			3.4

[a] 0.25 mg digoxin tablet; Brand A

[b] Relative standard deviation

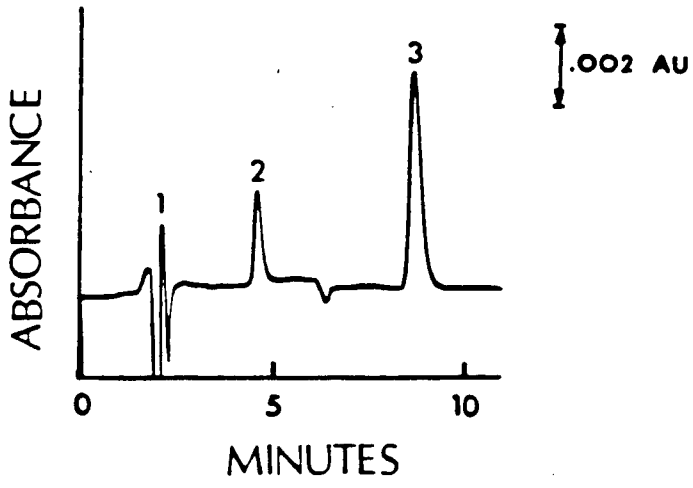


Fig. 66. A Representative Chromatogram for the Isocratic HPLC Analysis of Digoxin Injection. Peak Identity and HPLC conditions: same as in Fig. 64.

Table XV. Results of the HPLC Analysis of Digoxin Injection and Elixir

N	Percent of Label Claim		
	Injection		Elixir
	0.05 mg/ml	0.25 mg/ml	0.05 mg/ml
1	102.5	98.4	100.5
2	98.5	102.4	98.6
3	97.5	99.2	97.4
4	99.0	98.9	101.2
5	100.0	97.6	-
6	99.0	98.4	-
Mean ($\bar{x}$)	99.4	99.1	99.4
R.S.D.[a], %	1.7	1.6	1.8

[a] Relative standard deviation

standard deviations of 1.7% and 1.6%, respectively.

Analysis of digoxin elixir could not be carried out using the solvent system utilized for the assay of digoxin tablets and injection because of excipient interference with the digoxin peak. Various alterations in the proportion of the components of the solvent system were made in order to obtain a relatively more polar mobile phase that would have greater differentiating properties. A solvent system of water/methanol/isopropanol/dichloromethane: 51/42/5/2 resulted in a chromatogram (Fig. 67) that resolves digoxin (peak 5) and the internal standard (17α-ethynylestradiol, peak 7) from any of the peaks due to pharmaceutical excipients. Peaks 1-4 and 6 were due to pharmaceutical excipients. Peak 4 was found to be methylparaben; the other peaks, however, were not identified. It can be observed that the internal standard, 17α-ethynylestradiol, which has a retention time of about 18 minutes, causes a relatively long chromatographic time. In an attempt to reduce the chromatographic time, a number of steroids were tested for use as internal standards. 17β-dihydroequilin was found to satisfy the chromatographic requirements as shown in Fig. 68. Peaks 6 and 9 represent digoxin and 17β-dihydroequilin, respectively while the other peaks are due to elixir excipients. The results of the analysis of digoxin elixir, however, were obtained with 17α-ethynylestradiol as the internal standard, since it has the advantage of ready availability.

The calibration curve of digoxin obtained for the analysis of digoxin elixir (Fig. 26) was found to be linear with the line passing through the origin; and a response factor of 1.3652 was used for quantitation. The results of the analysis of digoxin elixir (Table XV) show an average label claim value of 99.4% with a relative standard deviation of 1.8%. It is to be noted that the analysis of digoxin elixir was achieved by direct dilution of the elixir sample without having to resort to any pre-extraction

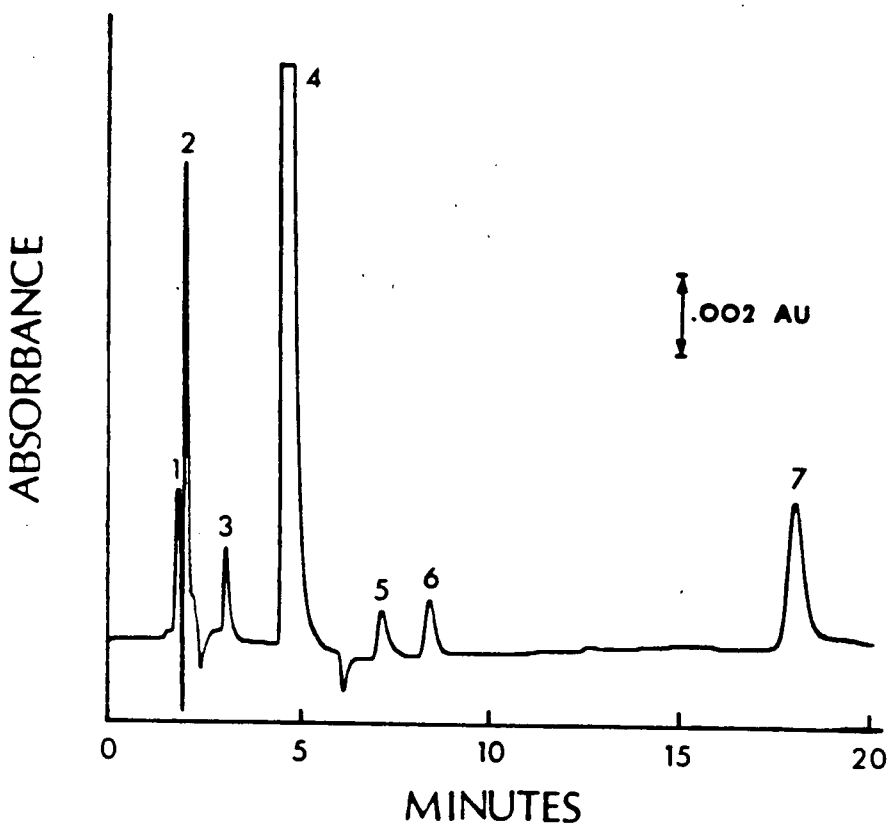


Fig. 67. A Representative Chromatogram for the Isocratic HPLC Analysis of Digoxin Elixir as obtained with a solvent system of water/methanol/isopropanol/dichloromethane: 51/42/5/2. Peak identity: (1-3, 6) unknown; (4) methylparaben; (5) digoxin; and (7) 17α-ethynylestradiol (internal standard). Other HPLC conditions: same as in Fig. 64.

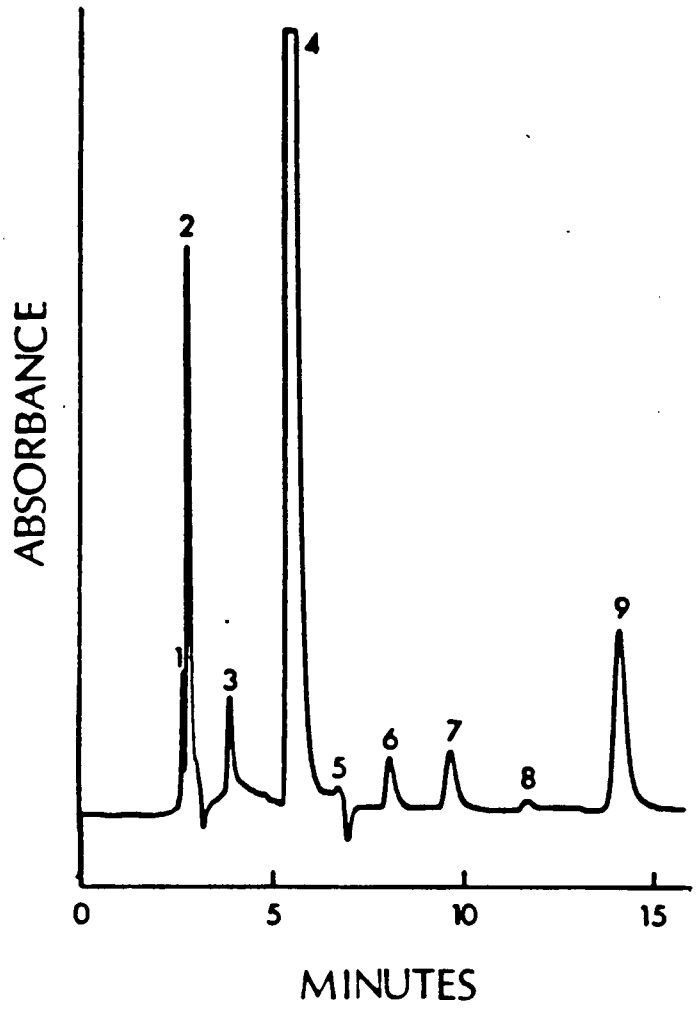


Fig. 68. A Representative Chromatogram for the Isocratic HPLC Analysis of Digoxin Elixir using 17β-dihydroequilin as the Internal Standard. Peak identity: (1-3, 5, 7, 8) unknown; (6) digoxin; and (9) 17β-dihydroequilin. HPLC conditions: same as in Fig. 67.

step.

The results of the HPLC analysis, as described above, indicate that the methods are fast, selective, accurate, sensitive, relatively simple and therefore, convenient for the assay of digoxin dosage forms.

7. Development of an HPLC Method for the Analysis of Digitoxin in its Dosage Forms

Due to the relatively long half-life of digitoxin in the body, the unit tablet dose is very low (usually 0.1 mg per tablet). Hence assurance of content uniformity of tablets would necessitate an analytical method of high sensitivity that would allow single tablet assay. Furthermore, the presence of pharmaceutical excipients and probable formation of degradation products and/or contaminants would require a high degree of selectivity. Most of the earlier methods reported for the quantitative analysis of digitoxin have been colorimetric (James et al., 1947; Bell and Krantz, 1948; Soos, 1948; Kennedy, 1950; Rowson, 1952; Tattje, 1954; Tattje, 1957; USP, 1980) or fluorometric (Sciarini and Salter, 1951; Jakovljevic, 1963; Wells et al., 1961; Khowrz, 1967; Cullen et al. , 1970). Many investigators have later used thin-layer (Jellife, 1967; Bican-Fister and Merkas, 1969; Frigns, 1970; Evans et al., 1974), gas-liquid (Watson et al., 1972) and high-performance liquid (Castle, 1975; Lindner and Frei, 1976; Nachtmann et al., 1976; Cobb, 1976; Ernie and Frei, 1977) chromatographic methods for the separation and quantitative determination of digitoxin.

The colorimetric methods generally lack sensitivity. Moreover, they have no selectivity because the color-forming derivatizing reagents react with the digitoxose sugar moiety or the lactone ring of the digitoxin molecule, both of which are shared by all cardenolides. Since the

fluorometric methods are based on the reaction of the derivatizing agent with the steroid moiety of the digitoxin molecule, they are non-specific with respect to other digitalis glycosides. Even though the gas chromatographic-ECD method reported by Watson et al. is highly sensitive, it is again non-specific. The electron capture detection requires derivatization to genin-diheptafluorobutyrate, and hence any degradation product that may be present will not be differentiated from digitoxin. Even though HPLC separations and in some cases minimum quantifiable amounts of digitoxin have been reported, application of any of these methods to the analysis of digitoxin dosage forms has not been shown. Quantitative analysis of digitoxin dosage forms would need an appropriate sample preparation that would ensure a high percentage of sample recovery. The possibility of an HPLC method that fulfills the requirements of sufficient UV transparency and sample recovery in the analysis of digitoxin in its dosage forms was, therefore, investigated.

A solvent system of water/methanol/isopropanol/dichloromethane: 45/38/11/6 resulted in the representative chromatogram (Fig. 69) that was used for the analysis of digitoxin in tablet and injection dosage forms. This chromatogram indicates the separation of 17α-methyltestosterone (internal standard, peak 2) and digitoxin (peak 3) without any interference from pharmaceutical excipients. The internal standard and digitoxin elute at retention times of 12.7 and 14.9 minutes, respectively. Peak 1 is an impurity from the filter paper and the two negative peaks are due to solvent effect.

In order to test the selectivity of the HPLC system, a sample of a solution of a standard mixture of the internal standard, digitoxin,

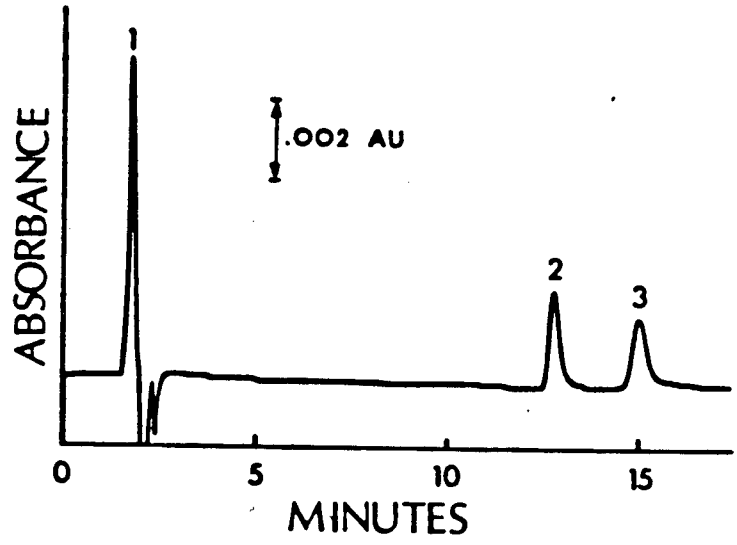


Fig. 69. A Representative Chromatogram for the Isocratic HPLC Analysis of Digitoxin Tablets and Injection. Peak identity: (1) unknown; (2) 17α-methyltestosterone (internal standard); and (3) digitoxin. HPLC conditions: solvent system, water/methanol/isopropanol/dichloromethane: 45/38/11/6; flow rate, 1.2 ml/min; UV detection at 220 nm; Chart speed, 0.5 cm/min.

digoxin, and the degradation products of the latter two was injected into the liquid chromatograph. The chromatogram that was obtained (Fig. 70) shows the resolution of all the components, thus indicating the possibility of simultaneously quantitating all of the compounds in a chromatographic time of about 15 minutes. Digoxin and its degradation products/metabolites were included in this separation in order to indicate the possibility of applying this method for monitoring the metabolites of digitoxin in biological samples.

The calibration curve (Fig. 30) of digitoxin, obtained for the analysis of tablet and injection dosage forms was found to be linear with the line passing through the origin and a response factor of 0.9348 was used for quantitation. The results of the HPLC analysis of digitoxin tablet and injection dosage forms are presented in Table XVI. It can be observed that the average label claim value for the composite sample analysis of digitoxin tablets is 97.2% with a relative standard deviation of 1.4% (n = 6). The data for the single tablet assay of digitoxin indicate an average label claim value of 98.8% with a relative standard deviation of 4.8% (n = 10). The recovery data for the analysis of digitoxin tablets (Table XVII) indicates an accuracy of 99.7% with a relative standard deviation of 3.1% (n = 4). The results of the analysis of digitoxin injection (Table XVI) show an average percentage label claim of 96.9 with a relative standard deviation of 3.3% (n = 3). Since the percentage label claim range allowed by the USP is 90-110; it can be seen that the digitoxin dosage forms comply with the official assay requirements.

The results of the HPLC analysis as described above, indicate that

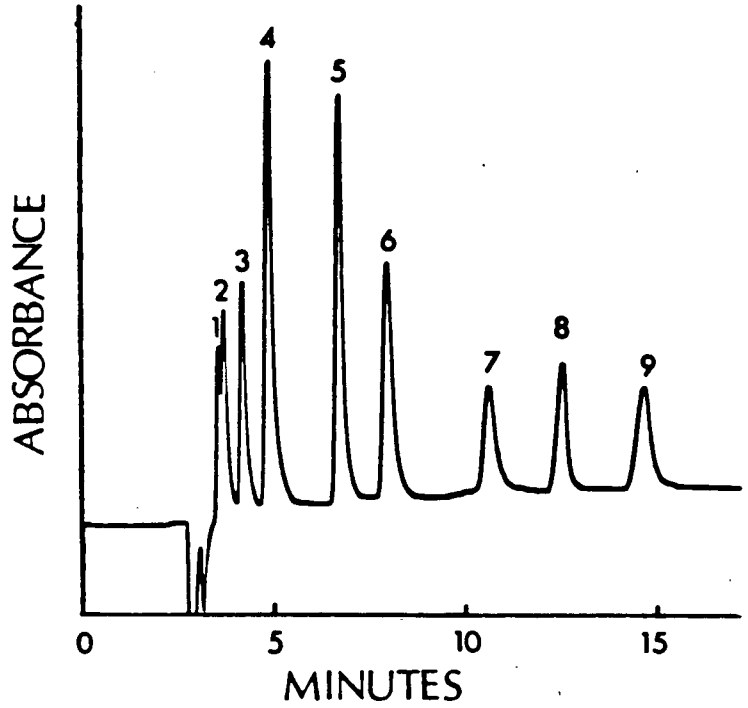


Fig. 70. A Chromatogram for the Isocratic HPLC Separation of Digitoxin, Digoxin and their potential Degradation Products and 17α-methyltestosterone (internal standard). Peak identity: (1) digoxigenin; (2) digoxigenin monodigitoxoside; (3) digoxigenin bisdigitoxoside; (4) digoxin; (5) digitoxigenin; (6) digitoxigenin monodigitoxoside; (7) digitoxigenin bisdigitoxoside; (8) 17α-methyltestosterone; and (9) digitoxin. HPLC conditions: same as in Fig. 69.

Table XVI. Results of the HPLC Analysis of Digitoxin Tablets and Injection

N	Percent of Label Claim		
	Tablet Composite Assay[a]	Single Tablet Assay[a]	Injectable[b]
1	95.1	94.8	95.9
2	97.1	104.6	94.3
3	96.8	92.8	100.5
4	99.0	98.4	-
5	98.4	96.5	-
6	96.8	94.8	-
7	-	97.9	-
8	-	107.0	-
9	-	98.2	-
10	-	103.8	-
Mean	97.2	98.8	96.9
R.S.D.(%)[c]	1.4	4.8	3.3

[a] 0.1 mg Tablet

[b] 0.2 mg/ml

[c] Relative standard deviation

Table XVII. Recovery Data for the HPLC Analysis of Digitoxin Tablets[a]

N	Theoretical Amount of Digitoxin (mg)	Amount Added (mg)	% Recovery
1	1.0	0.5	98.6
2	1.0	0.5	102.6
3	1.0	0.5	95.8
4	1.0	0.5	101.9
Mean			99.7
R.S.D.[b]			3.1
95% Confidence limits (±)			4.9

[a] 0.1 mg tablet

[b] Relative standard deviation

the method fulfills the requirements of sensitivity, selectivity, reproducibility, accuracy, simplicity and short of time of analysis, for the assay of digitoxin dosage forms.

8. Comparison of the Analytic Data of Digoxin and Digitoxin Dosage Forms by HPLC and USP XX Methods

In order to determine the relative merits of the HPLC methods that were developed for the analysis of digoxin and digitoxin in their respective dosage forms, comparative studies were carried out with reference to the USP methods. The same batches of digoxin and digitoxin dosage forms were analysed by HPLC and the USP methods. The HPLC and USP procedures were evaluated in terms of time of analysis, sensitivity, selectivity and convenience. The data for the analytical results were compared with regard to their precision and accuracy.

As shown in the flow charts of Figures 34, 35, 36 and 37, the current USP assays for digoxin tablets, injection and elixir involve extraction of the drug, evaporation to dryness, cooling in a vacuum desiccator over phosphorus pentoxide for 60 minutes, derivatization with acid-ferric chloride and determination of maximum absorbance after repeated measurements at two-minute intervals. It has been observed that the USP procedures involve laborious and manipulative steps that require periods of about four hours for completion of one assay. The tediousness of the procedure is particularly significant in the USP single tablet assay (content uniformity test) in which the analysis of 20 individual tablets is required. The HPLC methods, on the other hand, have chromatographic times of less than twenty minutes and sample preparations can be done in about 15 minutes. Thus the analyses can be completed in a period of

less than forty minutes.

The USP methods for the analysis of composite tablets, injection and elixir require samples equivalent to 2.5 mg of digoxin. Whereas, HPLC analysis can be carried out with composite tablet, injection and elixir samples of 1.25 mg, 0.1 mg and 1.0 mg of digoxin, respectively. The minimum amount of digoxin that is quantified using the USP procedure is about 25 mcg, while the HPLC method has been used for the quantitative determination of about 50 ng of the drug.

Digoxin quantitation using the USP method is based on the reaction of the derivatizing agent with the sugar moiety to form a colored derivative. Therefore, any digitoxosides that may be present as degradation products will not be differentiated from digoxin; whereas the HPLC methods have been shown to account for the probable degradation products (Fig. 65).

Results of the analysis of composite samples of three different brands of digoxin tablets by HPLC and the USP method are shown in Table XVIII. It can be seen that while the mean percentage label claim values obtained by both methods are quite comparable the HPLC data show better precision. HPLC and USP results for the analysis of digoxin injection and elixir (Table XIX) indicate that the mean percentage label claim and relative standard deviation values obtained with the HPLC method compare favourably with those derived from the USP data.

The accuracy data for the analysis of digoxin tablets as obtained by HPLC and the USP method(Table XX) indicate mean recovery values of 99.8% and 101.2%, respectively with a corresponding relative standard deviation of 3.3% and 3.2%. The above data, therefore, indicate that the accuracy of the HPLC method compares favourably with that of the USP method.

Table XVIII. Results of the Analysis of Composite Samples of Digoxin Tablets by HPLC and the USP XX Method

N	Percent of Label Claim							
	Brand A				Brand B		Brand C	
	0.125 mg		0.25 mg		0.25 mg		0.25 mg	
	HPLC Method	USP XX Method	HPLC Method	USP XX Method	HPLC Method	USP XX Method	HPLC Method	USP XX Method
1	98.0	103.0	94.0	105.6	98.8	101.4	101.6	97.7
2	101.2	96.1	96.8	96.2	97.2	98.8	100.0	102.7
3	99.2	94.9	95.2	98.4	98.0	103.0	104.8	103.2
4	101.2	-	95.2	-	97.6	-	98.4	-
Mean	99.9	98.0	95.3	100.1	97.9	101.0	101.2	101.2
R.S.D.[a] %	1.6	4.4	1.1	4.9	0.7	2.7	2.7	3.0

[a] Relative standard deviation

Table XIX. Results of the Analysis of Digoxin Injection and Elixir by HPLC and the U.S.P. XX Method

N	Percent of Label Claim					
	Injection				Elixir	
	0.05 mg/ml		0.25 mg/ml		0.05 mg/ml	
	HPLC Method	USP XX Method	HPLC Method	USP XX Method	HPLC Method	USP XX Method
1	102.5	103.2	98.4	101.2	100.5	101.6
2	98.5	101.2	102.4	101.9	98.6	97.4
3	97.5	99.6	99.2	102.7	97.4	98.2
4	99.0	-	98.9	-	101.2	-
5	100.0	-	97.6	-	-	-
6	99.0	-	98.4	-	-	-
Mean ($\bar{x}$)	99.4	101.3	99.1	101.9	99.4	99.1
R.S.D.[a], %	1.7	1.8	1.6	0.8	1.8	2.2

[a] Relative standard deviation

Table XX. Recovery Data for Digoxin Tablet[a] Assay by HPLC and the USP XX Method

N	Theoretical Amount of Digoxin (mg)		Weight of Digoxin Added (mg)		Recovery %	
	HPLC[b]	USP[c]	HPLC	USP	HPLC	USP
1	1.25	2.5	0.625	1.25	99.7	101.3
2	1.25	2.5	0.625	1.25	101.3	102.7
3	1.25	2.5	0.625	1.25	96.8	96.8
4	1.25	2.5	0.625	1.25	102.6	101.9
5	1.25	2.5	0.625	1.25	95.2	98.6
6	1.25	2.5	0.625	1.25	103.4	105.8
Mean ($\bar{x}$)					99.8	101.2
R.S.D.[d], %					3.3	3.2
95% Confidence limits (±)					3.5	3.4

[a] 0.25 mg Digoxin Tablets, Brand A
[b] According to preliminary assay, percent label claim is 100.1
[c] According to preliminary assay, percent label claim is 99.7
[d] Relative standard deviation

The USP procedure for the assay of digitoxin tablets and injection generally involves extraction, column chromatographic separation, evaporation of the eluent, reconstitution in alcohol, derivatization with alkaline picrate reagent and determination of maximum absorbance after repeated measurements at intervals of two minutes. The USP single tablet assay (content uniformity test) comprises extraction, evaporation of the solvent, reconstitution in 80% alcohol, fluorogenic derivatization and fluorometric determination. It can be observed that the methods are labour intensive and completion of one assay generally requires a period of over four hours. The HPLC procedure for the assay of digitoxin tablets and injection, however, requires less than forty-five minutes for completion of one assay.

The USP assays for digitoxin composite tablets and injection require samples equivalent to 2 mg of digitoxin. The USP colorimetric method used for composite tablet assay is not sensitive enough to be employed in single tablet assay. Therefore, the USP method for digitoxin single tablet assay employs fluorometric measurement. The HPLC method for the analysis of digitoxin composite tablets, single tablets and injection requires samples equivalent to 1 mg, 0.1 mg and 0.2 mg, respectively. Moreover, the assay procedure for single tablet assay is the same as in the composite tablet assay.

Both the colorimetric and fluorometric determinations of digitoxin will not be able to differentiate between the drug and its probable degradation products. Whereas, the HPLC method has been shown (Fig. 70) to be specific to digitoxin and also capable of accounting for each of the probable degradation products.

Results of the analysis of digitoxin composite tablets by HPLC and

Table XXI. Results of the Analysis of Composite Samples of Digitoxin Tablets[a] by HPLC and the USP XX Method

N	Percent of Label Claim	
	HPLC	USP
1	95.1	103.1
2	97.1	101.1
3	96.8	97.4
4	99.0	-
5	98.4	-
6	96.8	-
Mean ($\bar{x}$)	97.2	100.5
R.S.D.[b], %	1.4	2.9

[a] 0.1 mg per tablet

[b] Relative standard deviation

Table XXII. Recovery Data for Digitoxin Tablet[a] Assay by HPLC and the USP XX Method

N	Theoretical Amount of Digoxin (mg)		Weight of Digoxin Added (mg)		Recovery %	
	HPLC	USP	HPLC	USP	HPLC	USP
1	1.0	2.0	0.5	1.0	98.6	105.2
2	1.0	2.0	0.5	1.0	102.6	101.7
3	1.0	2.0	0.5	1.0	95.8	96.0
4	1.0	2.0	0.5	1.0	101.9	102.4
Mean ($\bar{x}$)					99.7	101.3
R.S.D.[b], %					3.1	3.8
95% Confidence limits (±)					4.9	6.2

[a] 0.1 mg per tablet

[b] Relative standard deviation

USP methods (Table XXI) indicate mean label claim values of 97.2% and 100.5% with relative standard deviations of 1.4% and 2.9% respectively. Comparative data for digitoxin injection are not presented because of insufficient sample. The mean recovery values of digitoxin as obtained by HPLC and the USP method (Table XXII) were found to be 99.7% and 101.3%, respectively.

In comparison with the USP procedures for the analysis of digoxin and digitoxin in their respective dosage forms, the HPLC methods appear to be more advantageous from the standpoints of selectivity, sensitivity, simplicity, convenience and time of analysis.

9. Stability Study of Digoxin and Digitoxin in their respective Dosage Forms using HPLC Methods

It has been reported (Foss and Benezra, 1980) that digoxin is stable indefinitely if it is kept in the dark in tightly closed containers. The above authors have also reported that digoxin tablets and neutral solutions of digoxin in ethyl alcohol and propylene glycol are stable for periods of up to five years. According to Jakovljevic (1974), no degradation of digitoxin in tablets, injectables or solutions was observed when stored for five years in the dark at temperatures of up to 30°C. It is generally known, however, that both digoxin and digitoxin undergo hydrolysis under acidic conditions. Sternson and Shaffer (1978) have reported the kinetics of *in vitro* digoxin degradation in acidic solutions, as monitored by HPLC. Even though the stability of digoxin and digitoxin has been studied using colorimetric and chromatographic techniques, it appears that no HPLC methods have been reported for the study of the degradation of these products in dosage forms. The HPLC methods developed

for the assay of digoxin and digitoxin dosage forms have been shown to be capable of accounting for each of the potential degradation products. The possibility of applying these methods for monitoring the stability of digoxin and digitoxin in their respective dosage forms and determination of each of the degradation products was, therefore, investigated.

Samples of dosage forms of digoxin and digitoxin stored at ambient conditions; 60°C and 70.4% relative humidity; and 80°C and 37.1% relative humidity were periodically analysed. Representative chromatograms for the stability study of Lanoxin tablets (Fig. 71) and Lanoxin injection (Fig. 72) show that digoxin (peak 2) and 17α-ethynylestradiol (internal standard, peak 3) are well resolved without any interfering peaks from excipients. Both of the above chromatograms were obtained with a solvent system of water/methanol/isopropanol/dichloromethane: 47/40/9/4 which is the same as that used for the analysis of digoxin tablets and injection. The representative chromatogram obtained for the stability study of Natigoxin tablets is shown in Fig. 73. The representative chromatogram for the stability monitoring of digitoxin tablets (Fig. 74) shows the resolution of digitoxin (peak 4) and 17α-methyltestosterone (internal standard, peak 3) without any interference from tablet excipients. This chromatogram was obtained with a solvent system of water/methanol/isopropanol/dichloromethane: 45/38/11/6 and is similar to that previously used for the analysis of digitoxin dosage forms.

The solvent system of water/methanol/isopropanol/dichloromethane: 51/42/5/2 which was previously used for the assay of Lanoxin elixir was tried for the stability study of digoxin in the elixir. The chromatogram that was obtained (Fig. 75) for a sample of elixir spiked with digoxin and its degradation products shows a massive excipient peak (peak 1) and

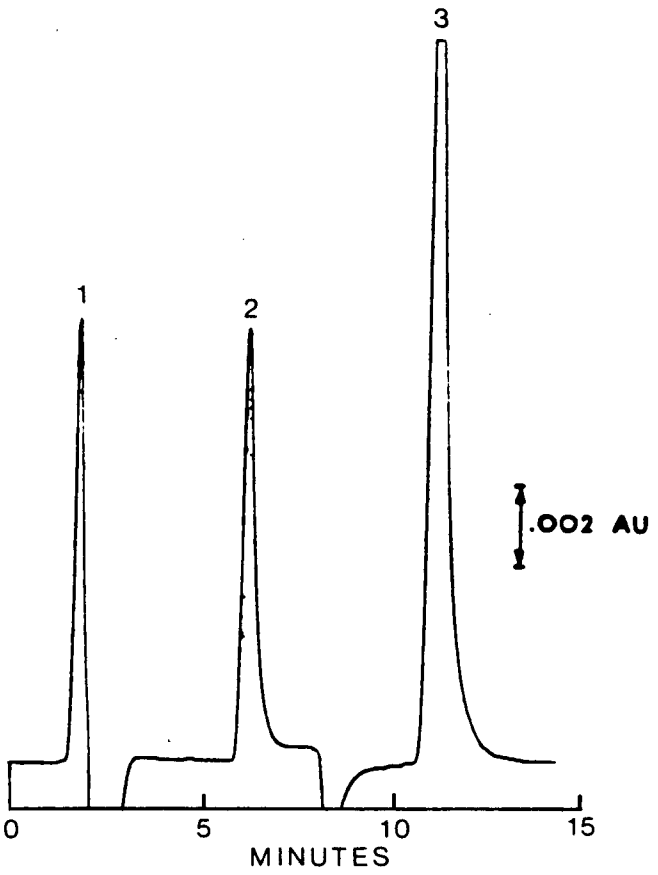


Fig. 71. A Representative Chromatogram for the Stability Monitoring of Digoxin Tablets by HPLC. Peak identity: (1) unknown; (2) digoxin; and (3) 17α-ethynylestradiol. HPLC conditions: same as in Fig. 64.

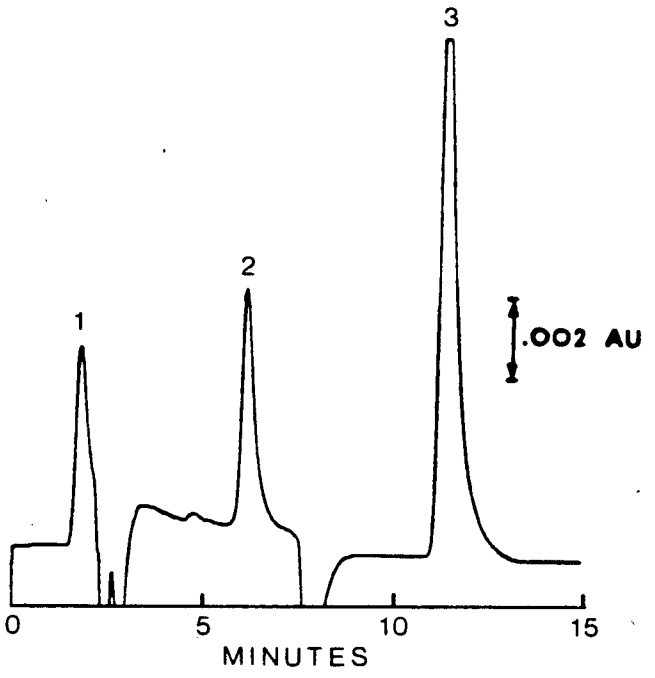


Fig. 72. A Representative Chromatogram for the Stability Monitoring of Digoxin Injection by HPLC. Peak identity: (1) unknown; (2) digoxin; and (3) 17α-ethynylestradiol. HPLC conditions: same as in Fig. 64.

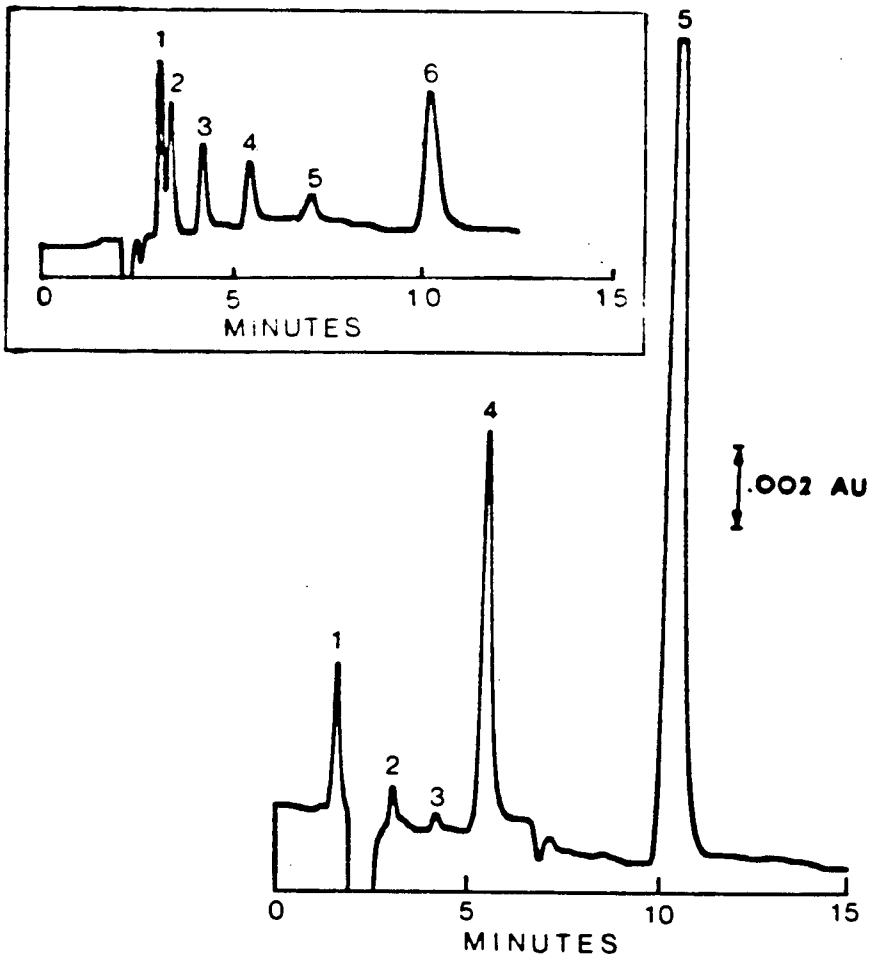


Fig. 73. A Representative Chromatogram for the Stability Monitoring of Natigoxin^{R} Tablets by HPLC. Peak identity: (1) unknown; (2) digoxigenin; (3) digoxigenin bisdigitoxoside; (4) digoxin; and (5) 17α-ethynylestradiol. HPLC conditions: same as in Fig. 64. Inset: A chromatogram of a standard sample of digoxigenin (1), digoxigenin monodigitoxoside (2), digoxigenin bisdigitoxoside (3), digoxin (4) and 17α-ethynylestradiol (6) obtained under the same HPLC conditions. Peak 5 is due to solvent perturbation.

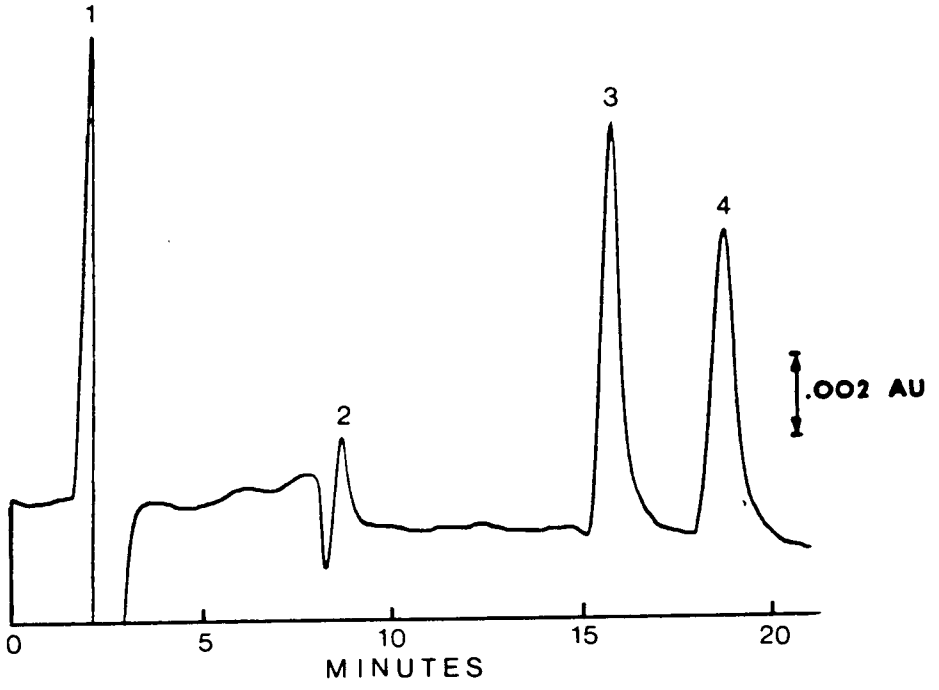


Fig. 74. A Representative Chromatogram for the Stability Monitoring of Digitoxin Tablets by HPLC. Peak identity: (1) unknown; (2) solvent effect; (3) 17α-methyltestosterone; and (4) Digitoxin. HPLC conditions: same as in Fig. 69.

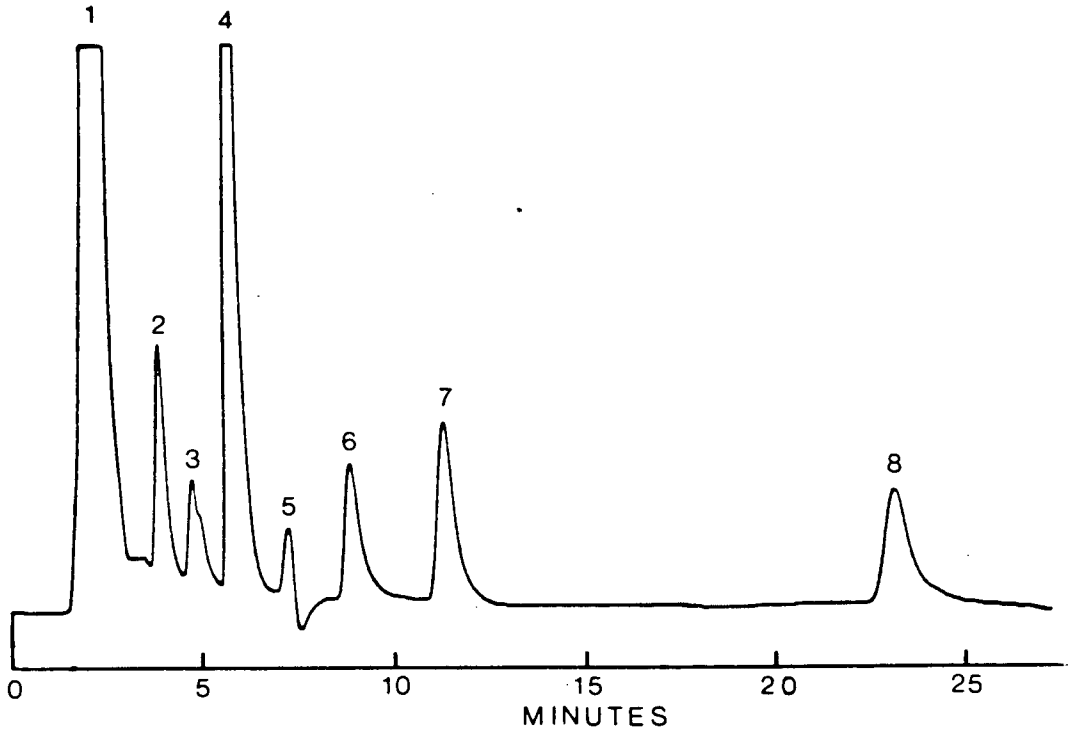


Fig. 75. A Chromatogram of a Sample of Lanoxin^{R} Elixir spiked with Digoxin and its potential Degradation Products. Peak identity: (1) unknown; (2) digoxigenin; (3) digoxigenin monodigitoxoside; (4) methylparaben; (5) digoxigenin bisdigitoxoside; (6) unknown; (7) digoxin; and (8) 17α-ethynylestradiol. HPLC conditions: same as in Fig. 67.

peaks 2, 3, 4, 5, 6, 7 and 8 representing digoxigenin, digoxigenin monodigitoxoside, methylparaben, digoxigenin bisdigitoxoside, unknown, digoxin and 17α-ethynylestradiol (internal standard), respectively. It can be observed that the peaks for the mono- and bisdigitoxosides are interfered with. Therefore, the direct dilution method of sample preparation used for the assay of Lanoxin elixir could not be applied to the simultaneous monitoring of digoxin and its degradation products.

Preliminary extraction of the elixir sample (which was spiked with digoxigenin and the mono- and bisdigitoxosides) with dichloromethane and reconstitution with the eluting solvent system resulted in a chromatogram (Fig. 76) which shows the elimination of all excipient and unknown peaks except peak 3 (methylparaben) and peak 5 (unknown). Preliminary addition of sodium carbonate solution, dichloromethane extraction of the elixir sample (spiked with digoxin and its potential degradation products) and subsequent sample preparation as previously described for the analysis of digoxin elixir resulted in the chromatogram shown in Fig. 77. In the chromatogram obtained for a sample of elixir stored at room temperature (Fig. 78), it can be seen that all excipient peaks except that of methylparaben are eliminated. The peak representing digoxigenin bisdigitoxoside (Fig. 77, peak 4), however, is interfered with by the solvent perturbation effect at the retention time of about 6.7 minutes. Therefore, the solvent system previously used for the analysis of digoxin elixir could not be applied for the simultaneous monitoring of digoxin and its degradation products in the elixir.

It can be observed that the problem of interference with the digoxigenin bisdigitoxoside peak can be solved if the methylparaben and bisdigitoxoside peaks could be pushed forward so that the former would coincide with the baseline perturbation and the latter would appear

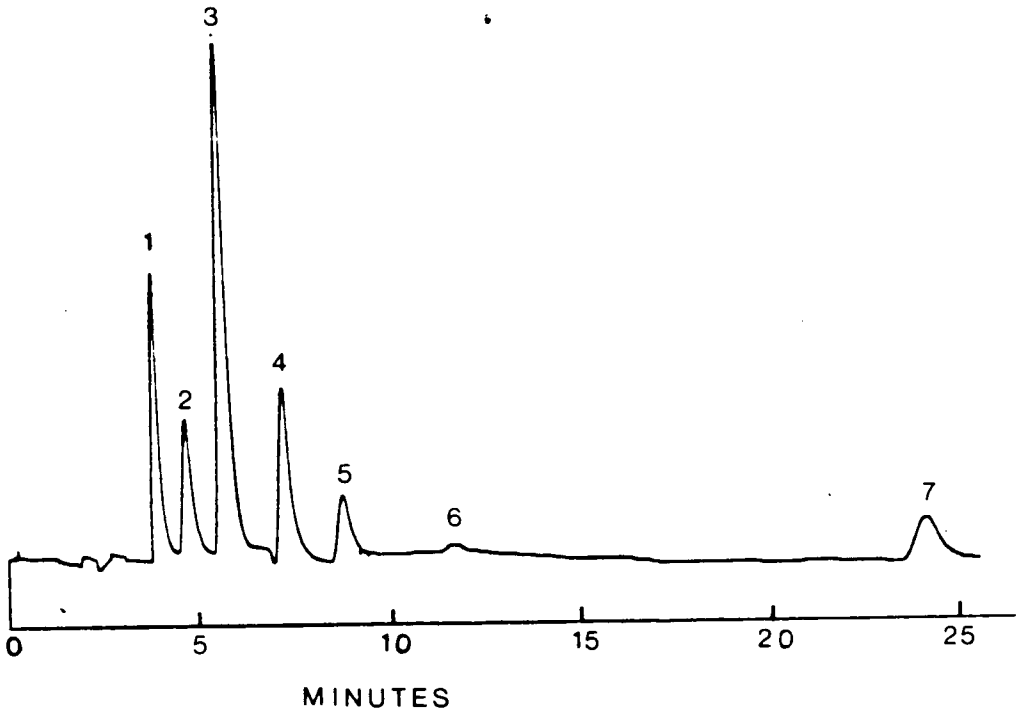


Fig. 76. A Chromatogram of an Extracted Sample of Lanoxin^{R} Elixir spiked with digoxigenin and the mono- and bisdigitoxosides. Peak identity: (1) digoxigenin; (2) digoxigenin monodigitoxoside; (3) methylparaben; (4) digoxigenin bisdigitoxoside; (5) unknown; (6) digoxin; and (7) 17α-ethynylestradiol. HPLC conditions: same as in Fig. 67.

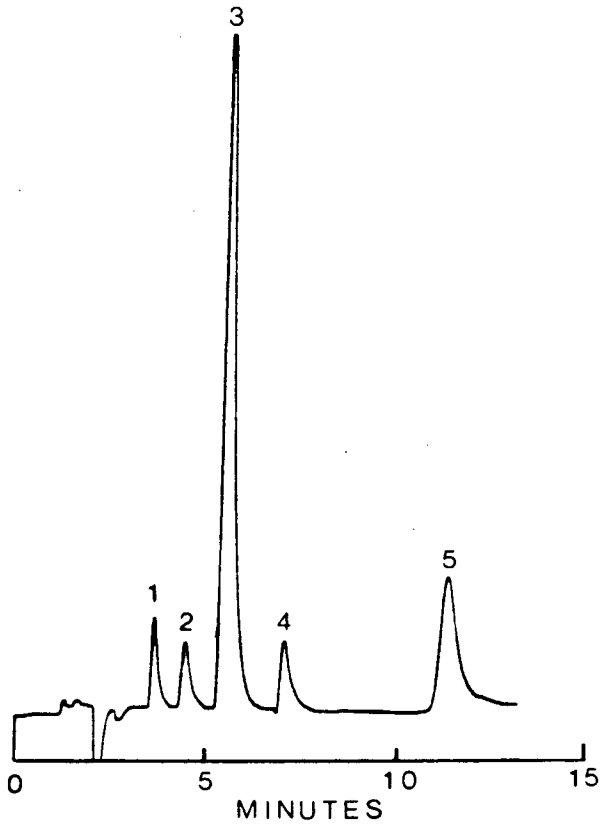


Fig. 77. A Chromatogram of an Extracted Sample of Lanoxin^{R} Elixir after preliminary addition of Sodium Carbonate and spiking with Digoxin and its potential Degradation Products. Peak identity: (1) digoxigenin; (2) digoxigenin monodigitoxoside; (3) methylparaben; (4) digoxigenin bisdigitoxoside; and (5) digoxin. HPLC conditions: same as in Fig. 67.

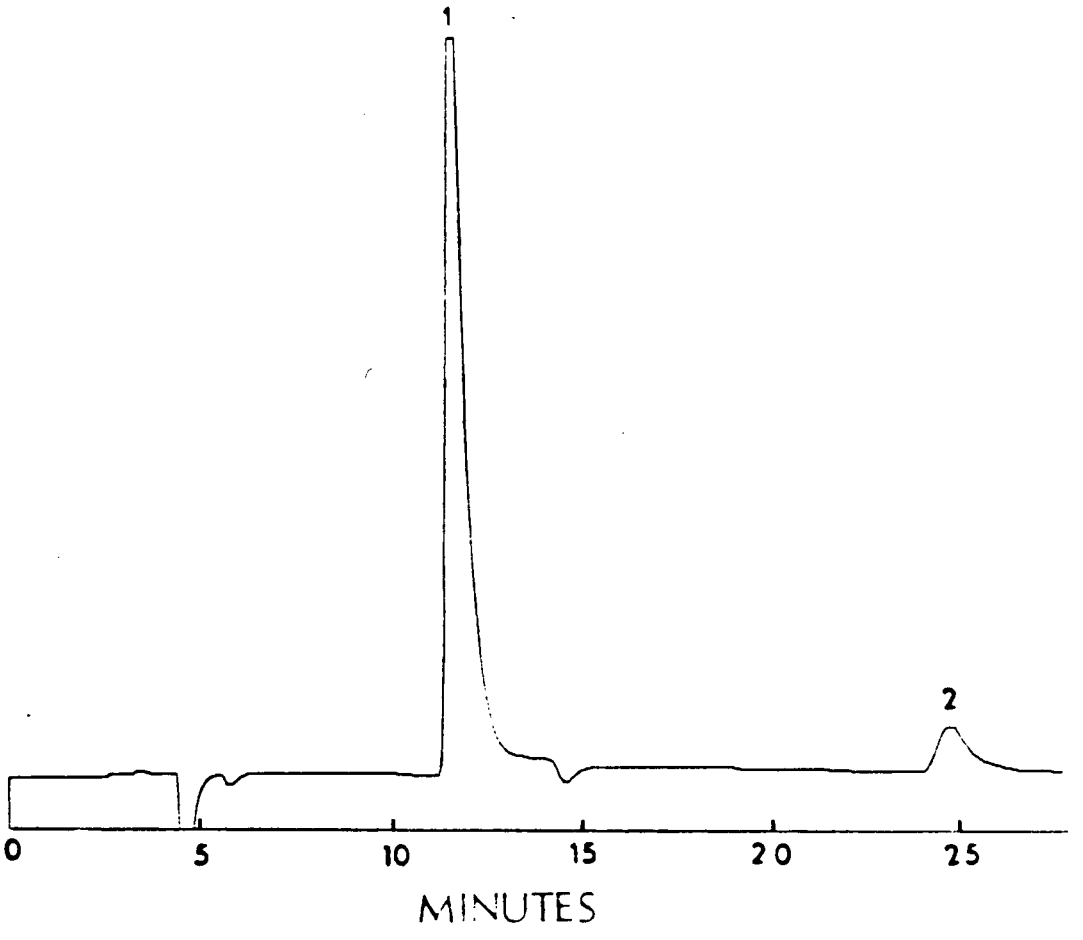


Fig. 78. A Chromatogram of a Sample of Lanoxin^{R} Elixir stored at room temperature, after preliminary addition of sodium carbonate and extraction with dichloromethane. Peak identity: (i) methylparaben; (2) digoxin. HPLC conditions: same as in Fig. 67 except that the flow rate was 0.6 ml/min.

at a later retention time thus disengaging itself from the interference. This was accomplished with a solvent system of water/methanol/isopropanol/dichloromethane: 51/43/4/2 as shown in Fig. 79. This chromatogram shows the complete separation of digoxigenin (peak 2), digoxigenin monodigitoxoside (peak 3), methylparaben (peak 4), digoxigenin bisdigitoxoside (peak 5), hydrocortisone (internal standard, peak 6) and digoxin (peak 7) in a chromatographic time of about 14 minutes. The retention times in Fig. 79 are larger than would be expected from the slight modification of solvent composition probably due to a change in the status of the column. A representative chromatogram for the stability study of Lanoxin elixir is shown in Fig. 80. The chromatogram obtained for 1% levels of the potential degradation products of digoxin under the HPLC conditions of the assay of tablets (and injection) is presented in Fig. 81. The chromatogram obtained for 1% levels of the potential degradation products of digitoxin under the HPLC conditions of digitoxin tablet assay is shown in Fig. 82.

The results of the stability study of digoxin tablets stored at 60°C and 70.4% relative humidity are presented in Table XXIII. It can be seen that digoxin in Lanoxin tablets does not show any degradation for storage periods of up to 16 weeks. Digoxin in Natigoxin tablets, however, undergoes gradual degradation to a level of 73.4% with 22.3% and 10.8% appearing as digoxigenin and digoxigenin bisdigitoxoside, respectively, after 16 weeks of storage. It is interesting to note that no digoxigenin monodigitoxoside was detected as a degradation product.

The stability data for digoxin tablets stored at 80°C and 37.1%

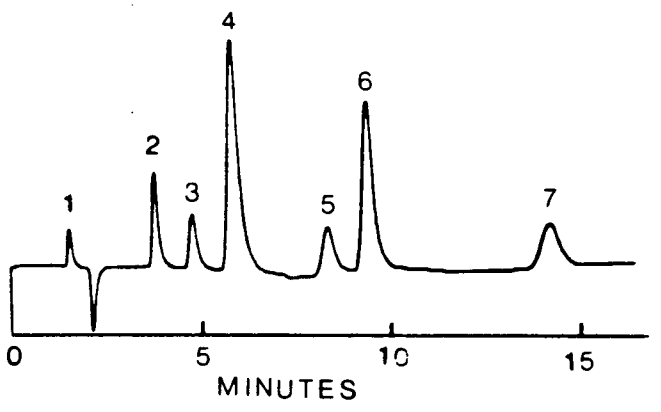


Fig. 79. A Chromatogram of an Extracted sample of Lanoxin^{R} Elixir after preliminary addition of Sodium Carbonate and spiking with Hydrocortisone (internal standard), Digoxin and its potential Degradation Products. Peak identity: (1) unknown; (2) digoxigenin; (3) digoxigenin monodigitoxoside; (4) methylparaben; (5) digoxigenin bisdigitoxoside; (6) hydrocortisone and (7) digoxin. Solvent system: water/methanol/isopropanol/dichloromethane: 51/43/4/2. Other HPLC conditions: same as in Fig. 67.

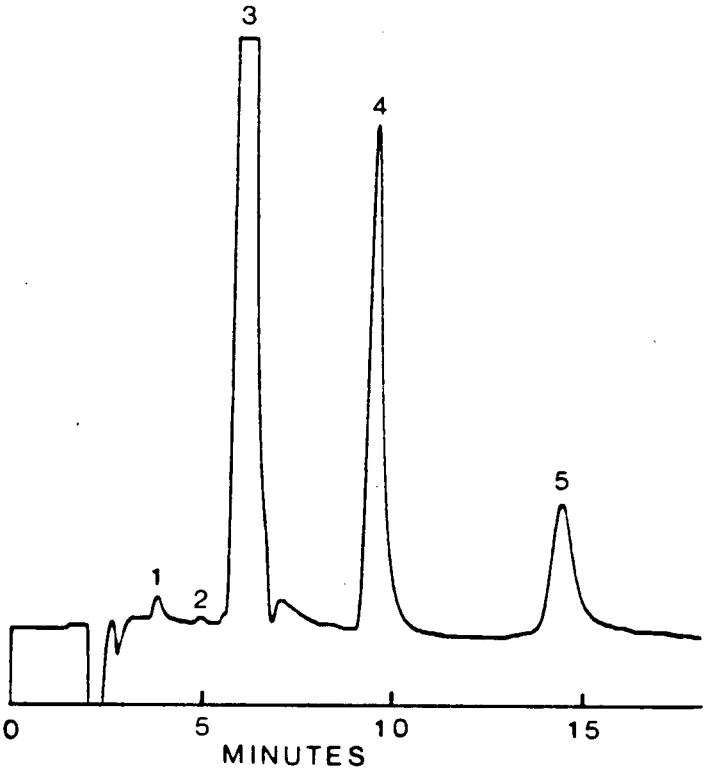


Fig. 80. A Representative Chromatogram for the Stability Monitoring of Lanoxin^{R} Elixir by HPLC. Peak identity: (1) digoxigenin; (2) digoxigenin monodigitoxoside; (3) methylparaben; (4) hydrocortisone; and (5) digoxin. Solvent system: water/methanol/isopropanol/dichloromethane: 51/43/4/2. Other HPLC conditions: same as in Fig. 67.

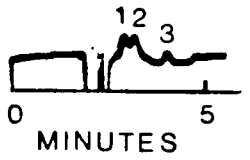


Fig. 81. A Chromatogram for the 1% levels (10 ng) of the potential Degradation Products of Digoxin. Peak identity: (1) digoxigenin; (2) digoxigenin monodigitoxoside; and (3) digoxigenin bisdigitoxoside. HPLC conditions: same as in Fig. 64.

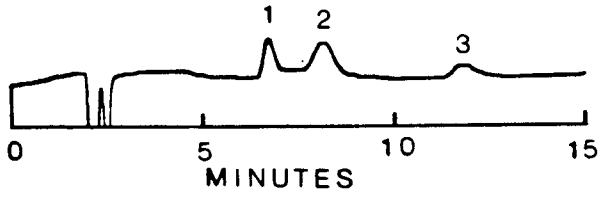


Fig. 82. A Chromatogram for the 1% levels (20 ng) of the potential Degradation Products of Digitoxin. Peak identity: (1) digitoxigenin; (2) digitoxigenin monodigitoxoside; and (3) digitoxigenin bisdigitoxoside. HPLC conditions: same as in Fig. 69.

Table XXIII. Results of the Stability Study of Digoxin Tablets stored at 60°C and 70.4% Relative Humidity

Product	Storage Time (weeks)	Assay (ng)[a]						
		Digoxigenin		Digoxigenin mono-digitoxoside		Digoxigenin bis-digitoxoside		Digoxin[b]
		Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount
Lanoxin 0.25 mg	4	-	-	-	-	-	-	101.2
Lanoxin 0.125 mg	4	-	-	-	-	-	-	98.9
Natigoxin 0.25 mg	4	-	-	-	-	7.8	8.9	90.1
Lanoxin 0.25 mg	8	-	-	-	-	-	-	103.2
Lanoxin 0.125 mg	8	-	-	-	-	-	-	96.9
Natigoxin 0.25 mg	8	8.0	16.0	-	-	8.2	9.4	84.0
Lanoxin 0.25 mg	12	-	-	-	-	-	-	105.6
Lanoxin 0.125 mg	12	-	-	-	-	-	-	101.3
Natigoxin 0.25 mg	12	10.6	21.2	-	-	8.8	10.0	79.3
Lanoxin 0.25 mg	16	-	-	-	-	-	-	99.7
Lanoxin 0.125 mg	16	-	-	-	-	-	-	95.4
Natigoxin 0.25 mg	16	11.2	22.3	-	-	9.5	10.8	73.4

[a] Preliminary assay values for Lanoxin tablets 0.25 mg, Lanoxin tablets 0.125 mg and natigoxin tablets 0.25 mg are 101.6%, 99.6% and 104.5%, respectively.

[b] According to label claim, theoretical amount is 100 ng.

relative humidity (Table XXIV) indicates the same pattern of digoxin degradation (in Natigoxin tablets) as in the results shown in Table XXIII. The initial degradation is marked by the appearance of the bisdigitoxoside with the subsequent formation of a relatively higher percentage of the genin. Digoxin is degraded to a level of 64.5% with the formation of 11.5% of digoxigenin bisdigitoxoside and 32.4% of digoxigenin.

The appearance of degradation products only in Natigoxin tablets may be due to the use of sulfuric acid solutions to control humidity. This assumption appears to be supported by differences in the buffer capacity of Lanoxin and Natigoxin tablets (Fig. 83). It was observed that Lanoxin tablets 0.25 mg and Natigoxin tablets 0.25 mg had buffer capacities of 0.0013 and 0.0004 gram - equivalent, respectively. The higher buffer capacity of Lanoxin tablets (0.125 mg and 0.25 mg) may account for the ability of these formulations to withstand any probable effects of sulfuric acid.

The data for the stability study of Lanoxin injection stored at 60°C and 70.4% relative humidity is shown in Table XXV. Digoxin degradation begins at about 8 weeks of storage showing a digoxin level of 75.1% with 22.0% and 14.0% appearing as bisdigitoxoside and digoxigenin, respectively, in a period of 12 weeks. The pattern of degradation is similar to that of Natigoxin tablets in the sense that digoxigenin monodigitoxoside is not present as a degradation product.

The results for the stability study of Lanoxin injection stored at 80°C and 37.1% relative humidity (Table XXVI) indicate that digoxin degradation begins at about four weeks and results in a digoxin level of 37.0% with 28.1%, 11.2% and 34.5% respectively appearing as the bisdigitoxoside, monodigitoxoside and genin in a period of 12 weeks of storage. The pattern of degradation in this case is quite different from that shown in Table XXV in that digoxigenin monodigitoxoside is

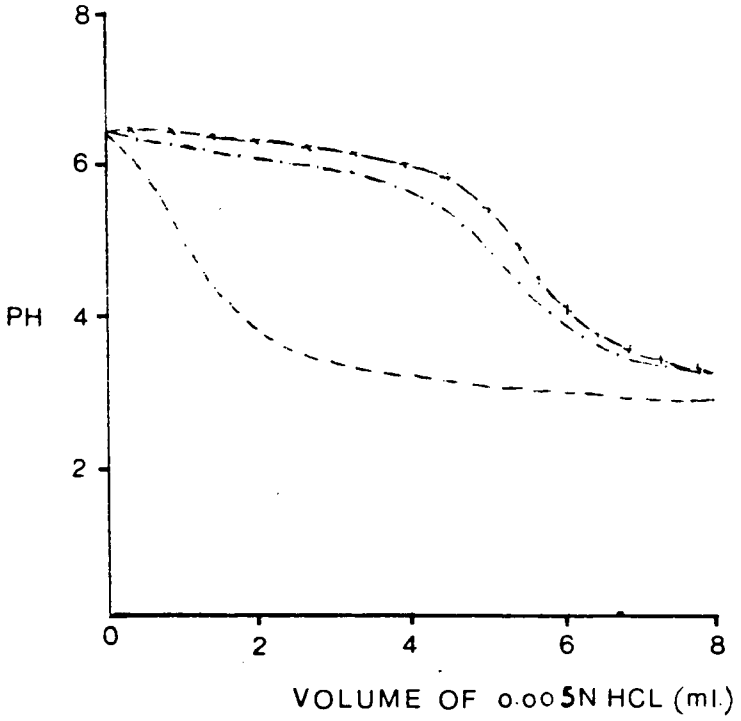


Fig. 83. The pH Profile of powdered Tablet Material (equivalent to 20 tablets) suspended in 20 ml of distilled water, after gradual addition of 0.005 N HCL. Lanoxin tablets 0.125 mg (-x-); Lanoxin tablets 0.25 mg (-.-); Natigoxin tablets (--).

Table XXIV. Results of the Stability Study of Digoxin Tablets stored at 80°C and 37.1% Relative Humidity

Product	Storage time (weeks)	Assay (ng)						
		Digoxigenin		Digoxigenin mono-digitoxoside		Digoxigenin bis-digitoxoside		Digoxin[a]
		Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount
Lanoxin 0.25 mg	4	-	-	-	-	-	-	94.0
Lanoxin 0.125 mg	4	-	-	-	-	-	-	101.3
Natigoxin 0.25 mg	4	-	-	-	-	8.1	9.2	88.0
Lanoxin 0.25 mg	8	-	-	-	-	-	-	96.0
Lanoxin 0.125 mg	8	-	-	-	-	-	-	97.7
Natigoxin 0.25 mg	8	8.3	16.5	-	-	8.2	9.3	77.2
Lanoxin 0.25 mg	12	-	-	-	-	-	-	98.4
Lanoxin 0.125 mg	12	-	-	-	-	-	-	95.2
Natigoxin 0.25 mg	12	15.4	30.7	-	-	9.1	10.4	69.6
Lanoxin 0.25 mg	16	-	-	-	-	-	-	96.2
Lanoxin 0.125 mg	16	-	-	-	-	-	-	102.4
Natigoxin 0.25 mg	16	16.3	32.4	-	-	10.1	11.5	64.5

[a] According to label claim, theoretical amount is 100 ng.

Table XXV. Results of the Stability Study of Lanoxin Injection 0.05 mg/ml Stored at 60°C and 70.4% Relative Humidity

Time (weeks)	Assay (ng)						
	Digoxigenin		Digoxigenin mono-digitoxoside		Digoxigenin Bis-digitoxoside		Digoxin[a,b]
	Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount
2	-	-	-	-	-	-	103.2
4	-	-	-	-	-	-	101.8
6	-	-	-	-	-	-	96.0
8	3.7	7.4	-	-	15.9	18.1	87.4
10	5.1	10.2	-	-	15.1	17.2	80.6
12	7.0	14.0	-	-	19.3	22.0	75.1

[a] According to label claim theoretical amount is 100 ng.

[b] Initial label claim is 95.2%.

Table XXVI. Results of the Stability Study of Lanoxin Injection 0.05 mg/ml stored at 80°C and 37.1% Relative Humidity

Time (weeks)	Assay (ng)						
	Digoxigenin		Digoxigenin mono-digitoxoside		Digoxigenin Bis-digitoxoside		Digoxin[a]
	Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount
2	-	-	-	-	-	-	94.8
4	4.6	9.2	-	-	10.5	12.0	80.0
6	7.0	14.0	-	-	16.8	19.2	70.8
8	10.1	20.3	7.0	10.1	23.2	26.4	56.3
10	11.5	23.0	5.9	8.6	27.7	31.6	44.2
12	17.3	34.5	7.7	11.2	24.6	28.1	37.0

[a] According to label claim, theoretical amount is 100 ng.

present as a degradation product. Moreover, the relative amounts of the degradation products show a marked shift towards digoxigenin.

The stability data for Lanoxin elixir stored at 60°C and 70.4% relative humidity (Table XXVII) show that digoxin degradation begins at about three weeks with the appearance of digoxigenin. At about five weeks the degradation of digoxin is characterized by the formation of both the genin and the monodigitoxoside. By the end of eight weeks of storage digoxin is reduced to a level of 88.2% with 14.6% and 5.9% appearing as digoxigenin and digoxigenin monodigitoxoside. It can be seen that the pH of the elixir shows a gradual decline from 6.9 to 6.7 in a period of five weeks.

The results of the stability study of Lanoxin elixir stored at 80°C and 37.1% relative humidity (Table XXVIII) indicate that digoxin degradation follows the same pattern as shown in Table XXVII except that the rate of degradation is much higher in this case. Digoxin is reduced to 16.4% within three weeks with 28.8% and 48.9% appearing as digoxigenin and digoxigenin monodigitoxoside, respectively. Even though no digoxin is detectable by the fourth week, the study was continued up to a period of six weeks in order to find out if there would be a shift inthe relative amounts of the degradation products. The data for the sixth week indicate an eventual decline in the relative amount of digoxigenin monodigitoxoside possibly due to its breakdown into the genin species. It is to be noted that digoxigenin bisdigitoxoside was not detected in any of the elixir samples and therefore, the pattern of degradation is quite different from that observed in the stability study of digoxin tablets and injection.

The data in Table XXVIII show that the pH of the elixir drops from an initial value of 6.5 to 3.9 over a period of six weeks indicating

Table XXVII. Results of the Stability Study of Lanoxin Elixir stored at 60°C and 70.4% Relative Humidity

Time (weeks)	Assay (ng)							pH
	Digoxigenin		Digoxigenin mono-digitoxoside		Digoxigenin bis-digitoxoside		Digoxin[a]	
	Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount	
3	3.8	7.5	-	-	-	-	100.3	6.9
4	3.9	7.8	-	-	-	-	103.0	6.9
5	4.0	8.0	3.0	4.5	-	-	93.0	6.8
6	6.8	13.6	4.2	6.3	-	-	90.9	6.7
8	7.3	14.6	3.9	5.9	-	-	88.2	6.7

[a] According to label claim theoretical amount is 100 ng

Table XXVIII. Results of the Stability Study of Lanoxin Elixir stored at 80°C and 37.1 % Relative Humidity

Time (weeks)	Assay (ng)							pH
	Digoxigenin		Digoxigenin mono-digitoxoside		Digoxigenin bis-digitoxoside		Digoxin[a,b]	
	Amount	Digoxin Equivalent	Amount	Digoxin Equivalent	Amount	Equivalent	Amount	
1	7.4	14.8	18.5	27.7	-	-	60.0	6.5
2	12.2	24.4	34.1	51.1	-	-	30.2	6.0
3	14.4	28.8	34.9	48.9	-	-	16.4	5.6
4	21.7	43.4	43.1	64.8	-	-	-	5.1
5	23.9	47.8	48.6	72.9	-	-	-	4.5
6	27.1	54.2	31.1	46.6	-	-	-	3.9

[a] According to label claim theoretical amount is 100 ng.

[b] Initial label claim is 101.2%.

the gradual formation of an acidic species. An attempt was therefore made to identify the compound that was mainly responsible for the increase in acidity. A sample of the elixir which had been stored for six weeks at 80°C and 37.1% relative humidity was prepared using the procedure described for sample preparation of digoxin elixir. The chromatogram obtained for this sample using a solvent system of water/methanol/isopropanol/dichloromethane: 47/40/9/4 as monitored with a UV detector set at the wave length of 280 nm is shown in Fig. 84. Peak 1 was found to be p-hydroxybenzoic acid, peaks 2, 3 and 5 were unknown and peak 4 was identified as methylparaben. The chromatogram obtained under the same conditions for a sample of Lanoxin elixir which was stored at room temperature (Fig. 85) shows a very small peak (peak 1) and a large peak (peak 3) corresponding to p-hydroxybenzoic acid and methylparaben, respectively. The formation of a relatively higher proportion of p-hydroxybenzoic acid as shown in Fig. 84 corresponds with the drop in pH shown in Table XXVIII. The presence of sulfuric acid in the storage chambers may also contribute to the increase in acidity observed in Tables XXVII and XXVIII. The formation of p-hydroxybenzoic acid was confirmed by mass spectrometric data (Fig. 86) that matches the reference spectra in the EPA/NIH Mass Spectral Data Base (1978).

The results of the stability study of digitoxin tablets stored at 80°C and 37.1% relative humidity; and 60°C and 70.4% relative humidity are shown in Table XXIX. The data indicate that digitoxin is stable over the period of sixteen weeks under both conditions of storage and none of thepotential degradation products were detected.

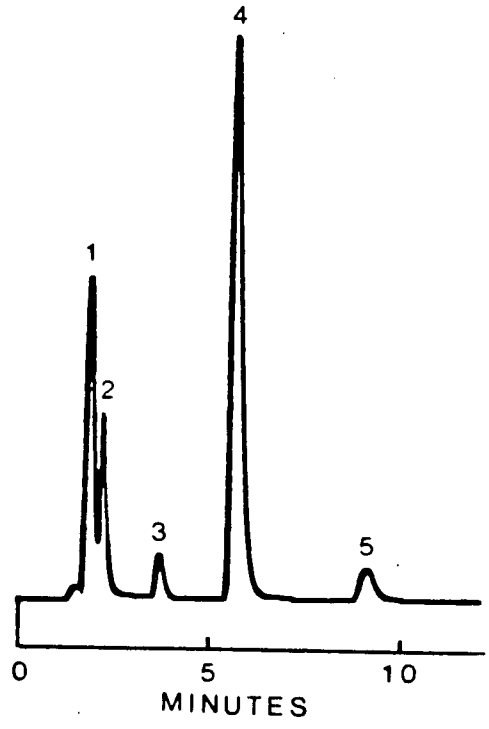


Fig. 84. A Chromatogram for the Isocratic HPLC Separation of the Degradation Products of methylparaben in a sample of Lanoxin^{R} Elixir stored for six weeks at 80°C and 37.1% Relative Humidity. Peak identity: (1) p-hydroxybenzoic acid; (2,3 and 5) unknown; and (4) methylparaben HPLC conditions: solvent system, water/methanol/isopropanol/dichloromethane: 47/40/9/4; UV detection at 280 nm; flow rate, 1.2 ml/min; Chart speed, 0.5 cm/min.

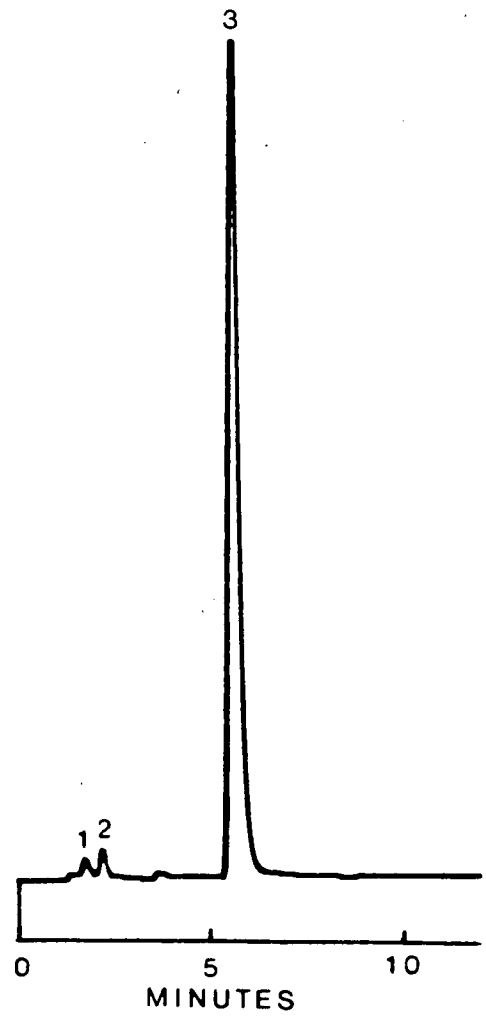


Fig. 85. A Chromatogram for a sample of Lanoxin^{R} Elixir stored under ambient conditions. Peak identity: (1) p-hydroxybenzoic acid; (2) unknown; and (3) methylparaben. HPLC conditions: same as in Fig. 83.

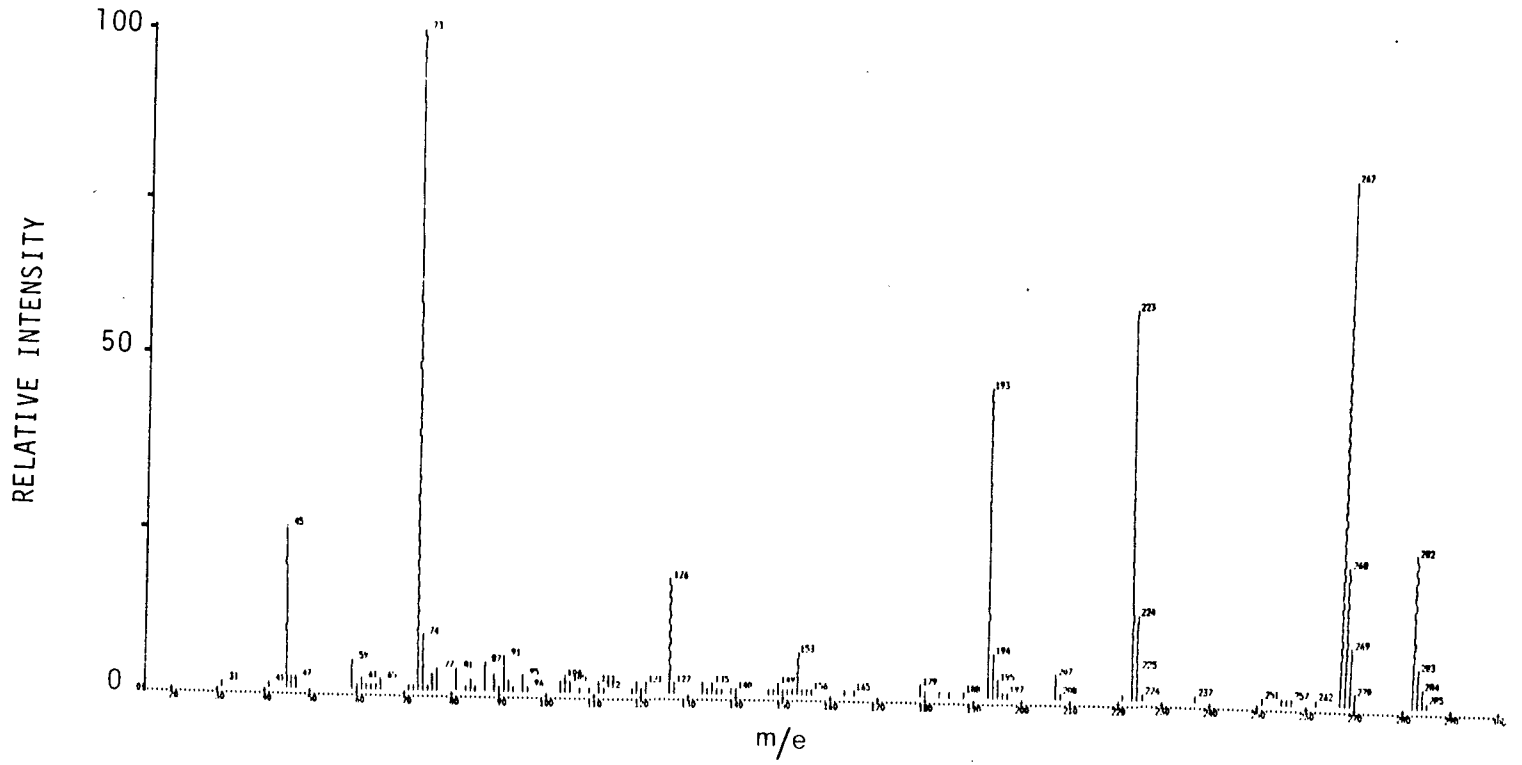


Fig. 86. Mass Spectrum of a TMS derivative of p-hydroxybenzoic acid after GLC separation of a sample of Lanoxin^{R} Elixir stored for six weeks at 80°C and 37.1% Relative Humidity. GLC and MS conditions: OV-17 (Packed column); initial temperature, 100 °C and temperature program of 8°C/min up to 220°C; electron energy of 80 eV.

Table XXIX. Results of the Stability Study of Digitoxin Tablets[a,b] stored at 80°C and 37.1% Relative Humidity and 60°C and 70.4% Relative Humidity

Storage time (weeks)	Percent of Label Claim	
	80°C & 37.1% R.H.	60°C & 70.4% R.H.
4	104.2	99.6
8	105.9	101.2
12	98.4	103.5
16	99.2	98.3

[a] initial percent of label claim is 101.8

[b] no degradation products were observed

It is known that digoxin has a number of possible pathways of degradation (Sternson and Shaffer, 1978). A schematic diagram of the pathways of digoxin degradation is shown in Fig. 87. According to the results of the stability study, degradation of digoxin in Natigoxin tablets may follow pathways 1, 5 and 3. Digoxin degradation in the injection stored at 60°C appears to follow pathways 1 and 3. Since the relative amounts of the genin and bisdigitoxoside determined at different storage times remains the same, it appears that pathway 5 is inoperative. In the case of the injection sample stored at 80°C, however, the presence of all three degradation products appears to indicate that digoxin breakdown follows pathways 1, 2 and 3. The relative decrease in the amounts of the bis- and monodigitoxosides at the later part of the study seems to indicate that pathways 4, 5 and 6 may also be operative as parallel routes.

In the case of the elixir sample stored at 60°C, the results indicate that digoxin degradation follows pathways 3 and 2 and that the former is the major route. The data for the elixir sample stored at 80°C show that pathway 2 is initially the major route of degradation with pathway 3 also being operative as a parallel route. The relatively greater amounts of digoxigenin observed at the end of the storage period seem to suggest that pathway 6 may also be operative.

From the above discussion it can be observed that: (1) digoxin degradation may follow all of the three or any two pathways; (2) different storage conditions for the same sample may result in different degradation pathways especially in the injection; and (3) different periods of storage of the same sample may be associated with different pathways of degradation. It appears, therefore, that the assortment of pathways that may be operative at different conditions and times of storage and the probable effects

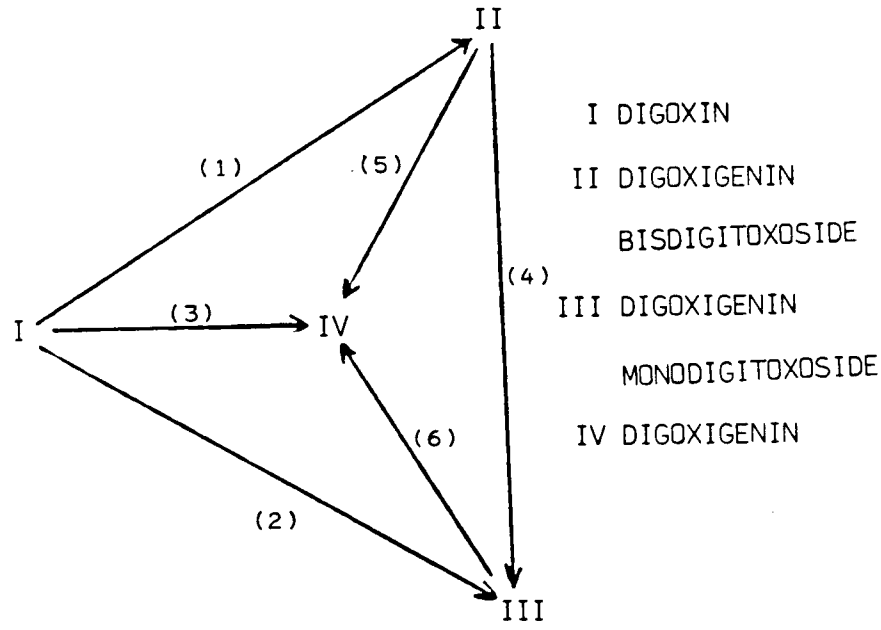


Fig. 87. Schematic Diagram of Pathways of Digoxin Degradation.

of sulfuric acid that was used to control humidity would make it impractical to estimate digoxin shelf-life from data obtained by this study. Nevertheless, the data in this stability study demonstrate the possibility of simultaneously monitoring all of the probable degradation products of digoxin and digitoxin by HPLC. The HPLC methods described in this investigation can be useful for the stability monitoring of digoxin and digitoxin in dosage forms that may be studied under ambient conditions of storage. The absence of digoxin degradation products in Lanoxin and digitoxin tablets, under the conditions of relatively high temperatures used in this study, confirms literature reports of high stability of these products.

IV. SUMMARY AND CONCLUSIONS

1. An HPLC system that employs a reverse-phase column; UV detection at 220 nm and solvent systems consisting of various proportions of water, methanol, isopropanol and dichloromethane was developed for the separation of a mixture of digoxin, digitoxin and their potential degradation products and metabolites. Separation of the above compounds by isocratic, solvent switchover and gradient elution modes was carried out in a chromatographic time of 27, 16 and 13 minutes, respectively. Isocratic separation of: (1) digoxin and its metabolites; (2) digitoxin and its metabolites; (3) gitoxin, digoxin and its metabolites and (4) α and β-acetyldigoxin, digoxin and its metabolites was achieved in less than 15 minutes in most cases.

2. HPLC separation of digoxin and its metabolites including dihydrodigoxigenin was monitored after fluorogenic post-column derivatization using the air segmentation principle with 100% fluid recovery. The HPLC solvent system consisting of water, methanol, isopropanol and dichloromethane was found to be compatible with the aqueous media of the reagents used for fluorogenic derivatization.

3. Nine estrogen steroids were resolved with a solvent system consisting of water, methanol, isopropanol and dichloromethane in a total elution time of about 35 minutes.

4. The HPLC solvent system mentioned in (1) was used to examine an extract of digitalis leaf. A peak appeared at a retention time corresponding to that of authentic digitoxin.

5. Isocratic HPLC systems were developed for the quantitative analysis of digoxin in tablets, injectables and elixir.

   (a) A solvent system of water/methanol/isopropanol/dichloromethane: 47/40/9/4 and an internal standard of 17α-ethynylestradiol were used for the assay of digoxin tablets and injection in a chromatographic time of less than 10 minutes.

   (b) The HPLC method for the analysis of digoxin tablets and injection was found to be capable of accounting for gitoxin and each of the probable degradation products of digoxin.

   (c) The calibration curve of digoxin obtained for the assay of tablets and injection dosage forms was found to be linear with the line passing close to the origin.

   (d) The mean label claim values for the composite tablet analysis of 0.25 mg digoxin tablets of Brands A, B and C were found to be 95.3%, 97.9% and 101.1%, respectively. The composite assay of 0.125 mg digoxin tablets (Brand A) resulted in a mean label

claim value of 99.9%. Digoxin composite tablet analysis was accomplished with a relative standard deviation of 1.5%. The results of single tablet assay of 0.125 mg and 0.25 digoxin tablets indicated mean label claim values of 97.1% and 96.6% with relative standard deviations of 5.1% and 4.6%, respectively.

(e) The average recovery value of digoxin from tablet material was found to be 99.8% with a relative standard deviation of 3.2%.

(f) The HPLC analysis of 0.05 mg/ml and 0.25 mg/ml digoxin injectables resulted in mean label claim values of 99.4% and 99.1% with relative standard deviations of 1.7% and 1.6%, respectively.

(g) A solvent system of water/methanol/isopropanol/dichloromethane: 51/42/5/2 was used for the assay of digoxin elixir. The chromatographic time was found to be 18 minutes, using 17α-ethynylestradiol as the internal standard. The HPLC analysis was performed by direct injection of the diluted elixir sample

(h) The calibration curve of digoxin obtained for the analysis of the elixir was found to be linear with the line passing close to the origin.

(i) The HPLC assay of digoxin elixir resulted in a mean label claim value of 99.4% with a relative standard deviation of 1.8%.

The results of the HPLC analysis indicate that the methods are fast, selective, accurate, sensitive and, therefore, convenient for the assay of digoxin dosage forms.

6. An isocratic HPLC system was developed for the quantitative analysis of digitoxin in tablet and injection dosage forms.
   (a) a solvent system of water/methanol/isopropanol/dichloromethane: 45/38/11/6 and an internal standard of 17α-methyltestosterone were used for the assay of digitoxin tablets and injection in a chromatographic time of about 15 minutes.
   (b) The HPLC method was found to be capable of accounting for each of the probable degradation products of digitoxin.
   (c) The calibration curve of digitoxin was found to be linear with the line passing close to the origin.
   (d) The results of the assay of digitoxin tablets (composite tablet analysis) and injection indicated mean label claim values of 97.2% and 96.9% with relative standard deviations of 1.4% and 3.3%, respectively.
   (e) The mean label claim value for the digitoxin single tablet assay was found to be 98.8% with a relative standard deviation of 4.8%.
   (f) The mean recovery value of digitoxin from tablet material was found to be 99.7% with a relative standard deviation of 3.1%.

   The analytic data, therefore, indicate that the HPLC method has the desirable characteristics of sensitivity, selectivity, reproducibility, accuracy, simplicity and short time of analysis for the assay of digitoxin dosage forms.

7. Comparison of the analysis of digoxin and digitoxin dosage forms by HPLC and the USP methods was made with reference to the following

factors:

(a) Time. The HPLC and USP assay procedures for digoxin dosage forms were found to require periods of less than 40 minutes and about 4 hours, respectively. The times of analysis required for the assay of digitoxin dosage forms by HPLC and the USP method were found to be less than 45 minutes and over 4 hours, respectively.

(b) Sensitivity. The USP methods for the analysis of composite tablets, injection and elixir required samples equivalent to 2.5 mg of digoxin. HPLC analysis could be carried out with composite tablet, injection and elixir samples equivalent to 1.25 mg, 0.1 mg and 1.0 mg of digoxin, respectively. The USP colorimetric assays for digitoxin composite tablets and injection were carried out with samples equivalent to 2.0 mg of digitoxin and, therefore, were not applicable for single tablet assay. The HPLC methods for the assay of digitoxin composite tablets, single tablets and injection required samples equivalent to 1.0 mg, 0.1 mg and 0.2 mg of digitoxin, respectively.

(c) Selectivity. Unlike the colorimetric and fluorometric methods of the USP, the HPLC methods for the analysis of digoxin and digitoxin dosage forms were found to be capable of accounting for each of the probable degradation products of both drugs.

(d) Precision. The relative standard deviations obtained for the analysis of digoxin and digitoxin dosage forms using HPLC and USP methods were found to be comparable.

(e) Accuracy. The mean recovery values of digoxin and digitoxin from tablet material as obtained by HPLC were found to be comparable with those derived from the USP data.

In comparison with the USP procedures, the HPLC methods for the analysis of digoxin and digitoxin in their dosage forms appear to be more advantageous from the standpoints of selectivity, sensitivity, simplicity, and time of analysis.

8. The HPLC systems developed for the assay of tablets and injectables of both digoxin and digitoxin may be applicable for the stability study of these dosage forms.

   (a) A solvent system of water/methanol/isopropanol/dichloromethane: 51/43/4/2 was used for the stability study of digoxin elixir. The chromatographic time was found to be less than 15 minutes using hydrocortisone as the internal standard.

   (b) The HPLC methods were found to be suitable for simultaneous quantitation of digoxin, digitoxin and their respective degradation products.

   (c) Lanoxin^{R} and digitoxin tablets were found to be stable under all the conditions of storage used in this study. Natigoxin^{R} tablets, Lanoxin^{R} injection and elixir were found to be subject to varying degrees and patterns of degradation.

   (d) The stability data were found to indicate that digoxin degradation may follow all of the three or any two pathways. The degradation of some samples is probably a result of the sulfuric acid atmosphere. It appears thant the Lanoxin tablet formulation is able to resist this effect.

This study demonstrated that the HPLC method developed could be useful for conventional accelerated stability testing for the digoxin and digitoxin formulations studied.

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