THE DETERMINATION OF DRUGS USING CIRCULAR DICHROISM

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PREFACE

The objective of this study was to develop methods for the determination of drugs using circular dichroism spectropolarimetry.

I wish to express my appreciation to my adviser, Dr. Neil Purdie, for his invaluable guidance, patience, understanding, and unending support throughout this study. I also thank to the members of my committee, Drs. Mottola, McGown, Varga, Mitchell, for their assistance and teaching. Thanks also go to all the faculty and staff of the chemistry department and to my fellow graduate students.

I would also like to give thanks for the financial support from the chemistry department and Dow chemical Company in the way of assistantships and fellowships.

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INTRODUCTION

In the past two decades, remarkable progress has been made in the methods used for determinations in the analysis of drugs. In great measure, these advances can be attributed to notable technological advances in developing analytical instrumentation, for example in absorption spectrometry, flame photometry, refractometry, polarimetry, polarography, chromatography, et cetera. None is without limitations and very often, several complementary methods are required in order to identify, confirm, and quantitate drugs. Numerous papers contain results wherein different methods are simultaneously reported. The methods most commonly used for drug analysis include color tests (1), gas and liquid chromatography (2,3), mass spectrometry (4,5), and spectrophotometric methods such as IR (6), fluorescence (7), NMR (8), and UV absorption (9,10). The common opinion is that chromatographic methods are the most successful in the analysis of drugs, particularly for the more difficult problems involving complex mixtures where separation is an essential step.

One of the oldest methods still in use in drug analysis is UV-visible spectrophotometry, in spite of the fact that

it does not give much information on the detailed structural features of molecules. The applications of UV-visible spectrophotometry are : (1) qualitative analysis, in which the goal is the identification of a pure compound, the confirmation of the presence or absence of a particular species in a mixture, and the identification of certain functional groups in a compound under structural investigation; (2) quantitative analysis, that is, the determination of one or more species in a mixture. Recently experimental or technological innovations have restored interest in UV-visible spectrophotometry.

Circular dichroism(CD) is currently recognized as one of the most fundamental and useful modern physical methods in the study of molecular stereochemistry. Numerous new applications of CD have appeared in the past few years, notably in the fields of organic chemistry and biological sciences. CD studies have also been employed in elucidating the structure of metal ligand complexes (11,12). CD measurements continue to be used in conjunction with optical rotatory dispersion for verifying configurations.

There are two recognized requirements for CD activity. The analyte (drug) must be optically active and must contain a chromophore for the absorption of electromagnetic radiation. Since most drugs meet both of these requirements, the technique is particularly useful for their identification and determination.

CHAPTER II

BACKGROUND AND THEORY

Circular Dichroism

History

The French physicist Biot discovered in the nineteenth century that some organic compounds had the unusual property of rotating the plane of polarization of a linearly polarized incident light beam (13,14). These compounds, which are said to be optically active or to possess optical rotatory power, are characterized by a lack of symmetry in their molecular or crystalline structure. The study of this property is referred to as polarimetry. Data are usually obtained at a single wavelength.

Fresnel established that the extent of the optical rotation due to a compound increases as one uses light of increasingly shorter wavelength for the measurement. This was subsequently to become known as optical rotatory dispersion (ORD). He is credited with the discovery of the quarter wave plate, the Fresnel rhomb, and the triprism which are composed of optically (+) and (-) quartz elements, and the relationship of optical rotation to circular birefringence of the active medium. He supposed that the

molecules of an active medium have a left- and right-handed helical morphology. The helix model was developed by Drude (13). His theory of optical activity was based on the assumption that in a disymmetric structure, charged particles are constrained to move along helical paths.

Besides rotation, Haidenger (13) reported his observations on the differences in the absorption of the left- and right-handed components of circularly polarized light by crystals of amethyst quartz. The measurement of this differential absorption as a function of wavelength became known as circular dichroism (CD) spectropolarimetry. Circular dichroism in aqueous solutions of chiral transition metal complexes had previously been discovered by Cotton (13). Work on the stereochemical configuration in chiral molecules by Fischer (13) led him to believe that these could be derived from Drude's model using the sign of the optical rotatory power as a basis.

The Drude model was criticized by Kuhn (14). In Kuhn's model, which is essentially the classical forerunner of the quantum-mechanical exciton models, optical activity is generated by the coulombic coupling of two or more anisotropically charged oscillators located at a finite separation in the molecule. The application of Kuhn's model was circumscribed by the limited development of molecular electronic spectroscopy in the 1930's. Although optical rotation was used in some of the earliest structural studies, the fundamental theory is still not thoroughly

understood. It is not possible to calculate precisely the optical activity to be expected for a single asymmetric molecule. There have been many complex molecular theories and numerous empirical rules to predict and interpret CD spectra, but a comprehensive and fundamental theory has not been described.

Theory

The phenomenon of circular dichroism is the differential absorption by a substance of left- and right circularly polarized light. There are two requirements for CD activity. The analyte must be optically active and must have a chromophore for the absorption of electromagnatic radiation.

The incident unpolarized light is first linearly polarized and subsequently divided into two circularly polarized components. A number of devices have been used for the production of linearly polarized light, such as birefringence polarizers, dichroic polarizers, and reflection polarizers. Reflection polarizers are limited because of inefficient reflection and the variation of the polarizing angle. Dichroic polarizers are the most commonly used for the polarization of visible light, but this polarizer is not widely used with CD spectropolarimeters because of the high absorbance and weak dichroism properties of the material in the ultraviolet region. Most modern instruments commonly use a birefringence polarizer such as

the Rochon polarizer which is made from crystals that exhibit a uniaxial optical axis. The Rochon polarizer (15) is made by crossing the optical axes of two prisms cut from a uniaxial crystal.

Plane polarized light, which consists of an electric wave orthogonal to a magnetic wave, is commonly depicted as a single wave (Figure 1). Plane polarized light can be resolved into circularly polarized components of opposite angular direction, i.e., rotating clockwise and counter clockwise respectively. The vector summations of the two in-phase components result in plane polarized light, where the amplitude of the wave is a function of the angle of the rotating vector coponents.

Fresnel postulated in 1825 that when the circularly polarized light beams pass through an optically active medium, which may be a solid, liquid, or gas, the refractive index for one circularly polarized component will be different from that for the other. The medium is said to be circularly birefringent and to have the property given by equation 2.1:

$$n_L - n_R \neq 0 \qquad (2.1)$$

Differences in refractive indices determine the differences in light speeds. Consequently, one of the two circularly polarized components of the linearly polarized light becomes retarded with respect to the other.

Plane-polarized light can be resolved into two circular components, as shown by Figure 2. Their vectors can be

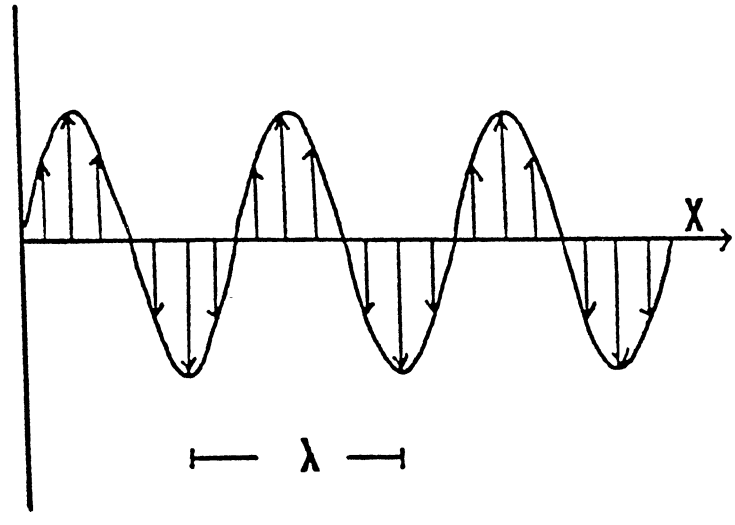


Figure 1. Wave Motion Propagated in the x Direction; λ is the Wavelength. The Arrows Denote the Electric Field Vector at a given Instant as the Light Wave Progresses along the x Axis

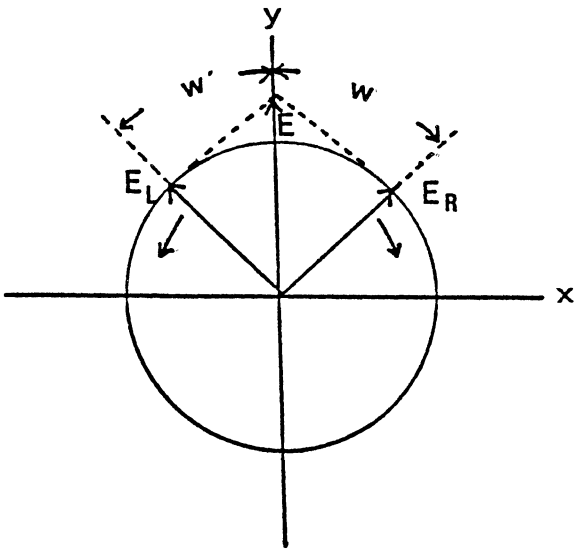


Figure 2. End View of the Electric Field Vector E as the Resultant of Two Rotating Vectors, E_R and E_L (plane-polarized light)

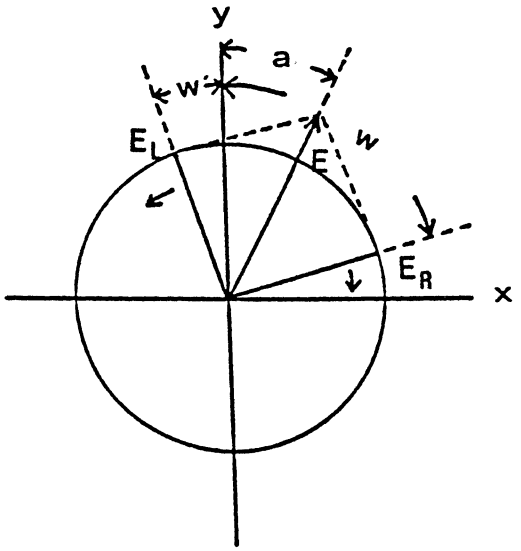


Figure 3. End View of the Electric Vectors on Passage of Plane-Polarized Light through an Optically Active Material (a is the observed rotation)

represented by E_L (left rotation) and E_R (right rotation). When plane-polarized light travels through an optically active material, vectors E_R and E_L will rotate at different speeds and at a given moment the angles with respect to the original plane will be different ($w \neq w'$). For Figure 3 compounds which allow E_R to travel faster than E_L establish the condition that $w > w'$ and are said to be dextrorotatory. The compound is levorotatory when the new plane of polarization is rotated through a negative angle ($w < w'$).

The angle of rotation can be expressed by the following relationship (equation 2.2)

$$\alpha = \frac{\pi (n_L - n_R)}{\lambda} \qquad (2.2)$$

Where α has units of radians per unit length, λ is wavelength of incident radiation in cm, and n_L and n_R are indices of refraction of left- and right- circularly polarized light respectively. The measurement of α versus wavelength produces an optical rotatory dispersion (ORD) spectrum (16,17). The property of α is dependent upon the solution concentration. To normalize α for comparison among solutions of different concentrations, the specific rotation [α] is defined by the expression (equation 2.3)

$$[\alpha] = \frac{1800}{\pi} \times \frac{\alpha}{c} \qquad (2.3)$$

where c is the concentration of solution (grams/ml) and $1800/\pi$ is the conversion factor needed to give the specific rotation in the new units of degrees/decimeter.

For comparisons among different compounds in solution of different concentrations, the more general molecular rotation [ϕ] term is defined, equation 2.4:

$$[\phi] = [\alpha] \times \frac{M}{100} \qquad (2.4)$$

where M is the molecular weight of compound.

In the region of an absorption band, the two circularly polarized components, in addition to suffering a differential retardation due to the circular birefringence of the medium, are also absorbed to different extents. In other words, the optically active medium has an unequal molar absorptivity ε for left and for right circularly polarized light. This difference in molar absorptivity (equation 2.5) is termed circular dichroism.

$$\Delta E = \varepsilon_L - \varepsilon_R \neq 0 \qquad (2.5)$$

In the optically active medium the two circularly polarized components not only are out of phase but are of unequal amplitude. Their resultant vector ε no longer oscillates around the circle, but rather traces out an ellipse as shown in Figure 4. The linearly polarized light beam has been converted into elliptically polarized light by the unequal absorption of its two circularly polarized components. Hence CD occurs only in the vicinity of an absorption band.

The resulting ellipse is characterized by the angle of ellipticity, ψ , which is given by the following equation 2.6.

$$\psi = \pi \times (A_L - A_R) / \lambda \qquad (2.6)$$

where A_L and A_R are the absorptivities for the left and

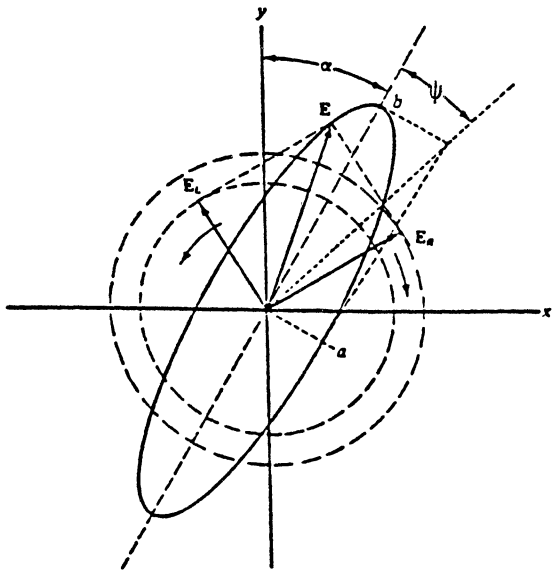


Figure 4. End View of Electric Field Vectors on Passage of Plane-Polarized Light through an Optically Active Material (the wavelength of light is near an absorption band)

right circularly polarized components respectively. The A values are related to the molar absorptivity (ε) and the molar concentration (c). The relationship is shown as equation 2.7.

$$A = \varepsilon bc \tag{2.7}$$

By analogy with specific rotation, the specific ellipticity [ψ] is defined by equation 2.8:

$$[\psi] = \frac{\psi}{bc} \tag{2.8}$$

where ψ is the angle in degree, b is the path length in decimeters, and c is the concentration of solution of in g/ml. Similarly, the molar ellipticity [θ] is defined by equation 2.9.

$$[\theta] = \frac{[\psi]\ M}{100} \tag{2.9}$$

The molecular ellipticity, analogous to the molar absorptivity, is defined by the following relation (equation 2.10):

$$[\theta] = \frac{\psi_{ob}\ M.W.}{100\ b\ c} \cong 3300(\varepsilon_L - \varepsilon_R) \tag{2.10}$$

where b is the path length in dm

c is the concentration of the absorbing medium in g/dl

M.W. is The molecular weight of the absorbing species

and ε_L and ε_R are the molar absorptivities of the solute for the left and right circularly components respectively.

Here a definition is adopted for [θ] that is more in accordance with the Beer-Lambert law for absorption spectroscopy (18).

The spectra typically show positive and/or negative peaks. The wavelengths of the positive and negative maxima will be referred to as λ^{+}_{max} or λ^{-}_{max}. These maxima and the wavelengths of crossover points (λ^{o}) are characteristics for each CD active compound and can be used for qualitative distinction. These crossover wavelengths are the wavelengths at which the sample medium is not circularly dichroic ($\varepsilon_L = \varepsilon_R$). All together these qualitative spectral characteristics are "fingerprints" for the compounds.

Achiral analytes should not be detectable by CD. Optical activity however can be induced by the complexation interaction of an achiral compound (guest) which contains the chromophore, with a chiral, non-absorbing molecule (host). Cyclodextrin sugars (19,20) are excellent hosts for a number of drugs and have been used to determine achiral drugs in complex mixtures. The cyclodextrin rings of six (alpha), seven (beta), or eight (gamma) alpha-D-glucose residues have internal diameters ranging from six to ten angstroms (Figure 5). Alpha-cyclodextrin with a 4.5Å diameter complexes with small mono- and di-substituted benzene derivatives. Beta-cyclodextrin, at 7.0Å diameter, is large enough to accommodate almost any intermediate sized organic molecule. Gamma-cyclodextrin can complex with large organic molecules. All of the cyclodextrins are about 6.7-7.0Å thick. The complexes formed are usually of 1:1 stoichiometry.

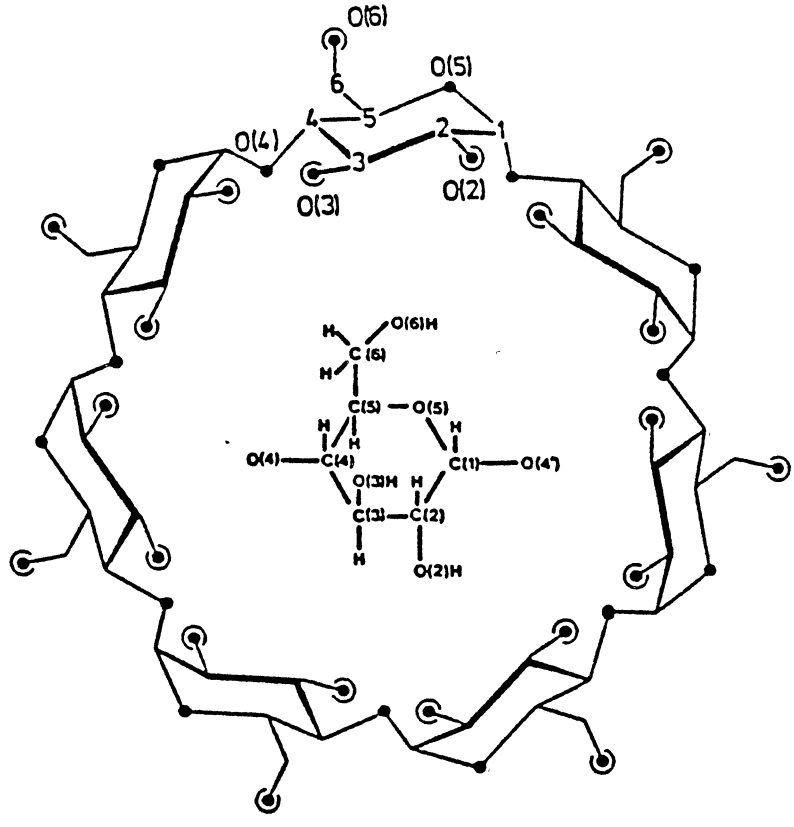


Figure 5. Molecular Structure of BCD

CD spectropolarimetry is not only useful in qualitative analysis but also in quantitative analysis in biologically active compounds such as pharmaceuticals.

Comparison between Chiroptical Methods

Linearly polarized light is used in the investigation of optically active molecules in order to obtain structural and stereochemical information. Three applications are polarimetry, optical rotatory dispersion, and circular dichroism. These are briefly discussed in turn.

Polarimetry is the oldest form of the chiroptical methods. In it the change in the direction of the plane of vibration of polarized light when it interacts with optically active materials is measured (21). In the early nineteenth century, rudimentary prisms and other devices for the production of polarized light were developed. The most common light sources for polarimetry are sodium vapor and mercury vapor lamps (22). The sodium lamp emits light of wavelengths 589 nm and 589.6 nm plus a little continuous background which can be largely eliminated with an appropriate filter. The mercury lamp emits light of several wavelengths with strong lines at 435.8, 491.6, 546.1, 577.0, and 579.1 nm. The proper choice of filters permits the isolation of each line.

Measurement of the angle of rotation of plane polarized light at the sodium-D emission line has been the standard test for chiral substances in the clinical and forensic

laboratory. This test is valuable in confirming the identity of an active enantiomer, as in the case of cocaine, but is not considered sufficient to uniquely identify an anonymous compound.

If the rotation of plane polarized light is measured as a function of wavelength, even more discrimination among drugs can be obtained. This technique is optical rotatory dispersion (ORD) (23). It was first reported by Biot and developed by Djerassi. Cotton effects, named for their discover, Aime Cotton, are observed in ORD spectra when a chromophore near a chiral center absorbs in the wavelength regions examined. This phenomenon allows for a greater distinction among chiral analytes than in UV spectrophotometry due to the fact that the spectra contain more information, e.g. negative, positive, and zero dispersions, so the spectra have more information than the UV spectra for the same compounds. However, these anomalous dispersions (24) are always superimposed on a rising or falling "plain" dispersion curve, making it difficult to distinguish small changes of rotation in this region.

CD measures the differential absorption of left- and right-handed circularly polarized light. The most significant difference between ORD and CD is that CD signals are observed only in the vicinity of an absorption band for a chiral molecule whereas ORD which involves measurement of a rotation that is theoretically finite everywhere. Figure 6 shows that the ORD curves and CD curves for a

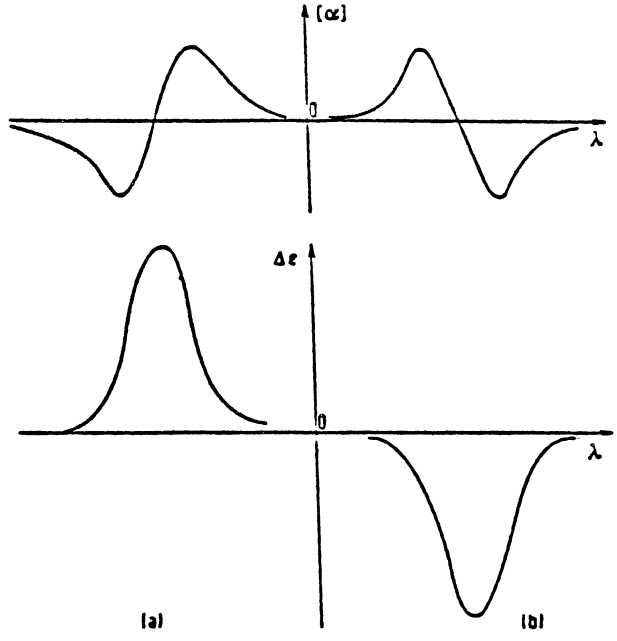


Figure 6. Optical Rotatory Disperson Curves (above) and CD Curves (below) for a Dextrorotatory Chromophore (a) and for a Levorotatory Chromophore (b)

(from reference 25)

dextrorotatory chromophore and for a levorotatory chromophore (25). Chromophores in the far UV contribrute to the sign and magnitude of the ORD signal at longer wavelengths. In addition, the rather broad appearance of the 'S'-shaped bands associated with the ORD Cotton effect often causes difficulty in separating the contributions from neighboring electronic transitions and in detecting vibrational fine structure.

The dispersive nature of ORD can contribute to the acquisition of information on the Cotton effects which might occur at wavelengths beyond the limits of the instrument by determining the sign and magnitude of the ORD "plain" curve at these limits. In ORD the capacity to measure rotations at various wavelengths can be useful for comparison purposes with data in the literature.

A related problem occurs with compounds of low optical activity, e.g., chiral hydrogen-deuterium compounds or substances of low optical purity. In these cases ORD measurements are necessary.

Although in principle CD and ORD provide the same structural information, the inherent simplicity of CD spectra over ORD curves recommends CD for the majority of applications. Circular dichroism also has a measurement advantage over optical rotatory dispersion. For a 1^{o} observation of optical rotation in ORD at 360 nm in a 1 dm cell, the instrument must measure the difference in indices of refraction of around 1 part in 10^{8}. Differences in molar

absorptivities on the other hand are significantly larger being 1 part in 10^6.

Relationship between UV and CD

The light beam used in UV-visible spectroscopy is essentially unpolarized. Use of linearly polarized light (sometimes less rigorously referred to as plane-polarized light) to investigate optically active (chiral) molecules is a powerful technique for obtaining structural and stereochemical information.

CD is analogous to absorption spectroscopy and the measured ellipticity can be used for quantitative analysis according to a modified Beer's law expression by equation 2.11:

$$\psi = \theta bc \qquad (2.11)$$

where ψ is the observed ellipticity (deg.)

θ is the molar ellipticity (deg. M^{-1})

b is the cell path length

and c is the molar concentration

The conventional Beer's law expression (Equation 2.12) is shown for comparison.

$$A = \varepsilon bc \qquad (2.12)$$

Both linear equations describe calibration curves whose slopes are equal to the molar values.

UV-visible Spectrophotometry

Brief History of Development

Spectroscopic science began during the eighteenth century (26). The first ideas concerning photometry were recorded in 1700 by L'Abbe Marie who primarily studied single elements. Valentin published his book on the application of the spectroscope to physiology and medicine in 1863. He also diagnosed and examined narcotic remedies and poisons spectroscopically. Brewster first described ultraviolet spectra in 1835. In 1885, Hartley used a comparatively crude spectroscope to painstakingly plot absorption spectra in the ultraviolet region for certain major alkaloids known at that time. Dobbie (27,28) investigated the relationships between ultraviolet spectral characteristics and chemical structure for a number of additional alkaloids. Subsequently, similar investigations of organic compounds numbering into the thousands have been made.

Castille and Ruppol described the application of UV absorption to the determination of caffeine in 1928. Other toxicological applications were discussed by Hans Fischer. Goldbaum's (29) procedure for the ultraviolet spectrophotometric determination of barbiturates in blood, published in 1948, represents the first practical application of the instrumentation to chemical toxicological analysis. Since then a number of compendia of ultraviolet and visible spectra for pharmaceuticals have been published (30,31).

Theory

The UV and visible regions of the electromagnetic spectrum are associated with electronic transitions in the interaction of light with chemical substances. The ultraviolet region can be subdivided into the far or vacuum ultraviolet (10 to 200 nm) and the near ultraviolet (200 to 400 nm). In the short wavelength region (10 to 200 nm) absorptions result from inner orbital electronic transitions requiring higher energy quanta, while the near UV and visible ranges are associated with lower energy, valence shell electronic transitions. The distinction between the near ultraviolet and the visible region is due to the fact that the human eye is sensitive in the visible region (400-800 nm). The increase in energy ΔE is expressed by the relation equation 2.13:

$$\Delta E = h\nu = h\frac{c}{\lambda} = h\bar{\nu}c \qquad (2.13)$$

This equation relates the energy absorbed in an electronic transition to the frequency (ν), wavelength (λ), and wavenumber ($\bar{\nu}$) of the radiation producing the transition. h is Planck's constant and c is the velocity of light in vacuum. The energy absorbed is dependent upon the energy difference between the ground state and the excited state. Generally speaking, tightly held electrons require more energetic photons to accomplish absorption than more loosely held (delocalized) electrons. Molecular absorption in the

ultraviolet and visible region depends upon the electronic structure of the molecules. Transitions in the ultraviolet range are mostly associated with conjugated systems.

The total energy of a molecule may be considered to be the sum of the contributions from electronic, vibrational, rotational, and translational energies. The change ΔE in the energy of a molecule upon absorption of electromagnetic radiation is given by equation 2.14:

$$\Delta E = \Delta E_{elec} + \Delta E_{vib} + \Delta E_{rot} + \Delta E_{trans} \qquad (2.14)$$

where ΔE_{elec} is the spacing between allowed electronic energy levels, and ΔE_{vib}, ΔE_{rot}, ΔE_{tran} are the spacings between allowed vibrational, rotational, and translational energy levels, respectively. The electronic energy is generally larger than the E_{rot} and E_{vib} and E_{trans}. ΔE_{trans} is usually extremely small and unimportant in all absorption regions. An electron in a molecule may be excited from any of the vibrational and rotational levels in the ground state to any of a large number of possible vibrational and rotational levels in a given excited state. Because a photon of slightly different energy corresponds to each of the many possible a transition, visible and UV molecular absorption spectra consist of many lines which altogether produce broad absorption bands with little resolution.

The absorption spectrum of a substance is obtained by measuring the proportion of the incident light transmitted through an absorbing medium. When a molecule absorbs

radiation, its energy is increased. The intensity of absorption may be expressed as transmittance (T), defined by equation 2.15:

$$T = \frac{I}{I_o} \tag{2.15}$$

where I_o is the intensity of the incident radiation, and I is the intensity of the radiation after passing through the absorbing medium. The relationship between the transmittance, the sample thickness (b), and the concentration of the absorbing species (c) is given by equation 2.16:

$$\log\left(\frac{1}{T}\right) = kbc = A \tag{2.16}$$

where k is a constant characteristic of the solute at a given wavelength and A is absorbance. When c is expressed in moles per liter and the path length through the sample is expressed in centimeters (b), the preceding expression becomes:

$$A = \varepsilon bc \text{ (Beer's law)} \tag{2.17}$$

The term ε is known as the molar absorptivity, formerly called the molar absorption coefficient. If the concentration (c) of the solute is defined in g per liter, the equation becomes:

$$A = abc \tag{2.18}$$

where a is the absorptivity and is thus related to the molar absorptivity by

$$\varepsilon = aM \tag{2.19}$$

Where M is the molecular weight of the absorbing species. ε

is wavelength dependent and is determined analytically or measured experimentally from a calibration curve which shows absorbance is a function of concentration.

Chromophore

It is a long recognized fact that many colored organic substances owe their color to absorption of light by one or more unsaturated linkages. These linkages or functional groups were first called chromophores by Witt in 1876.

Ultraviolet-visible radiation is absorbed in an electronic transition within the chromophore moiety rather than by the molecule as a whole. Chromophores are mostly covalent unsaturated groups, e.g. C=C, C=O, aromatic rings, etc. and can be either conjugated or unconjugated.

Transitions which occur in the far UV (Figure 7) are of very high energy and involve saturated molecules which have either only single bonds (σ^b) or single bonds and non-bonded electron pairs (σ^n). These electronic transitions are designated as $\sigma^* \longleftarrow \sigma^b$ and $\sigma^* \longleftarrow \sigma^n$ in molecular orbital or ligand field theories. Examples of these are the alkanes and the alkyl substituted functional groups such as amines, alcohols, respectively. A saturated group attached to a chromophore is called an auxochrome. When any of these groups and a chromophore are combined in the same molecule, the absorption bands are moved to longer wavelengths and show an increase in intensity. Excitations of electrons from nonbonded orbitals occur at longer

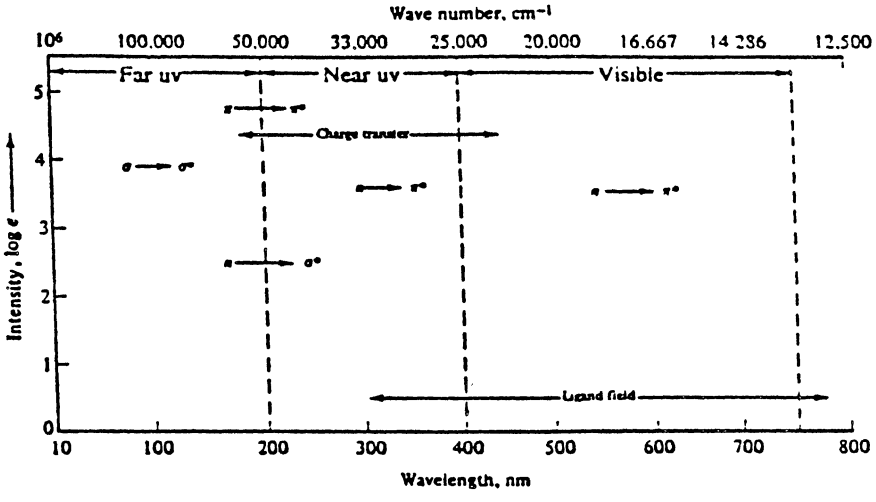


Figure 7. Ultraviolet and Visible Regions of the Spectrum and the Types of Absorption Bands (from reference 32)

wavelengths than those for the corresponding saturated hydrocarbons since non-bonded electrons are more loosely bound.

Chromophore groups containing both π^b and σ^n electrons can have 3 transitions: $\sigma^* \longleftarrow \sigma^n$, $\pi^* \longleftarrow \pi^b$, $\pi^* \longleftarrow \sigma^n$. The transitions are normally observed in the 270-290 nm range and are typically associated with aldehydes and ketones.

A molecule that contains more than one chromophore has an absorption spectrum which is approximately the sum of the separate transitions plus modifications which result from interactions between the chromophores. If the two chromophores are conjugated the electronic absorption spectrum is totally different from the sum of the bands for the isolated chromophores. In conjugated systems the π^b electrons are delocalized, which decreases the $\pi^* \longleftarrow \pi^b$ transition energy and increases the molar absorptivity as a result of a higher probability for the transition. The UV spectrum of morphine is shown Figure 8. The absorptions in the wavelength ranges 240-255 and 280-290 nm are attributed to $\pi^* \longleftarrow \pi^b$ transitions. In apomorphine (Figure 9), the aromatic rings probably contribute to the signals observed in excess of 300 nm as a $\pi^* \longleftarrow \sigma^n$ transition.

A shift to longer wavelength (lower energy) is called a bathochromic shift or red shift while a hypsochromic shift or blue shift is a shift to shorter wavelength (higher energy). An increase in the intensity of a band is called a

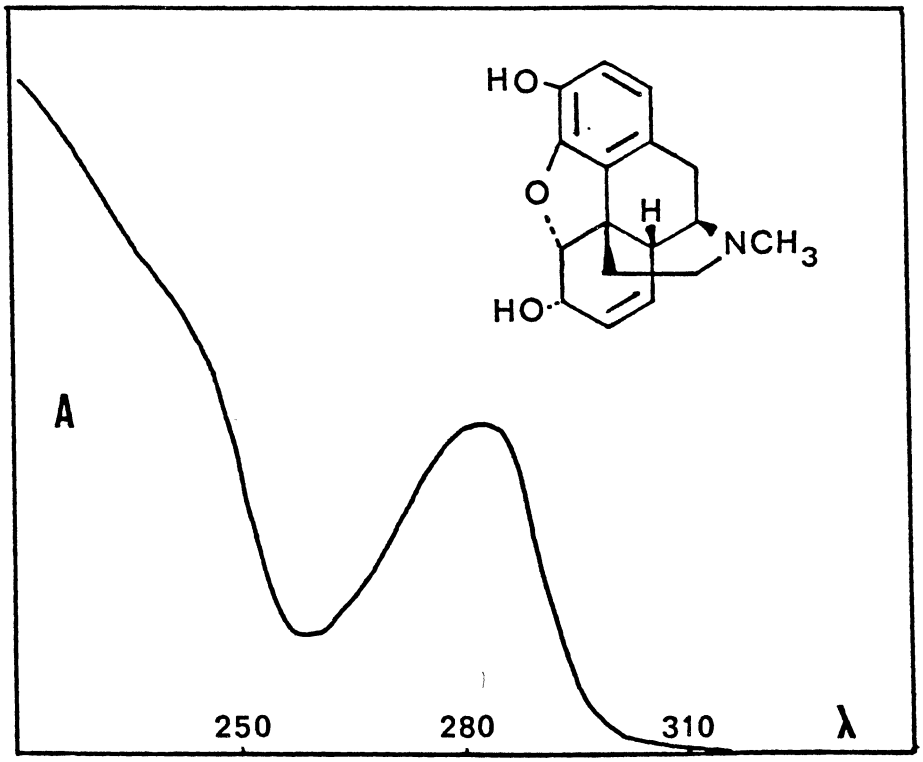


Figure 8. UV Spectrum for Morphine

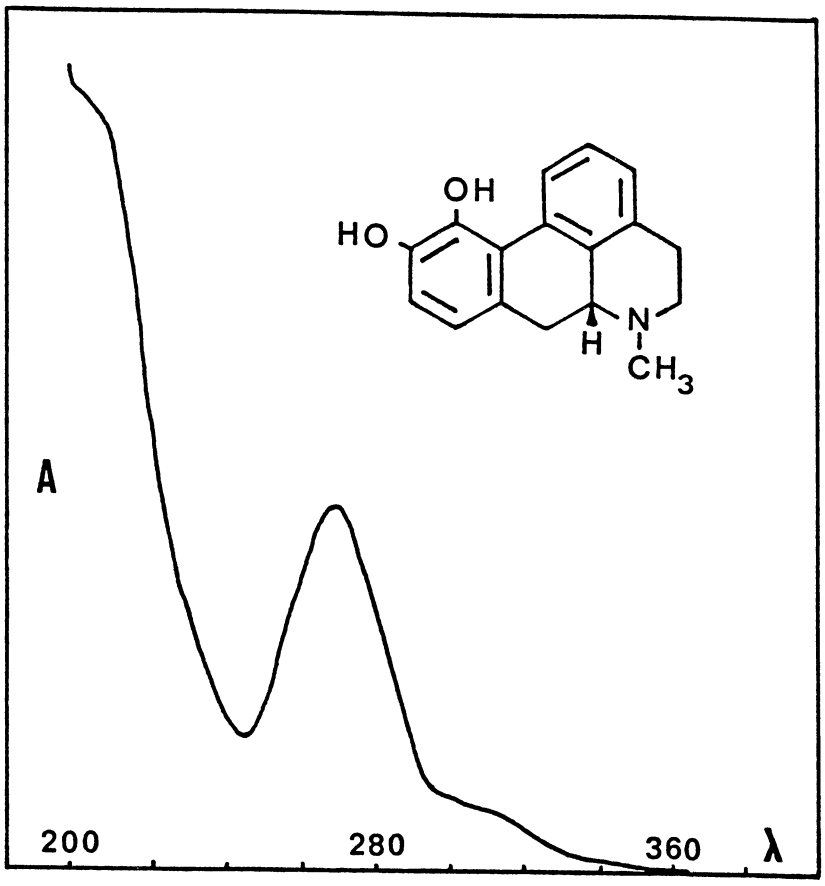


Figure 9. UV Spectrum for Apomorphine

hyperchromic effect, while a decrease is called a hypochromic effect. In summary, there are three ways in which chromophores can be linked together in a molecule (32): (1) when two chromophores are directly linked together, for example an allene (C=C=C) or ketene (C=C=O) group, the combined spectra will be very different from the individual C=C or C=O chromophores; (2) when two chromophores are conjugated, their characteristic absorption patterns retain their individuality except that $\pi^* \longleftarrow \pi^b$ and $\pi^* \longleftarrow \sigma^n$ transitions undergo a bathochromic (red) shift and show a hyperchromic effect; (3) when two chromophores are separated by more than one single bond, the π electrons are said to be isolated and the spectrum is practically the sum of those for each chromophore.

Solvent Effects

The wavelength and the intensity of absorption bands are both affected when a molecule is placed in a solvent environment and its spectrum is compared with that for the molecule in the gas phase. Electrostatic interactions between polar solvents and polar chromophores tend to stabilize both the nonbonding electronic ground states and the π^* excited states. This interaction causes the $\pi^* \longleftarrow \sigma^n$ transitions, which usually occur at lower energy than the $\pi^* \longleftarrow \pi^b$ transitions, to move to higher energy and $\pi^* \longleftarrow \pi^b$ transitions to move to lower energy. Thus the $\pi^* \longleftarrow \sigma^n$ and $\pi^* \longleftarrow \pi^b$ absorptions of polar chromophores move closer to

each other with increasing polarity of the solvent. The structural character and the position of absorption bands depend on the nature of the solvent. Polar solvents tend to shift the position of the bands and diminish their vibrational structure. Non-polar solvents produce little change in the bands compared to the gas phase spectra. The effect of solvent is directly related to the degree of interaction of solvent with the absorbing molecule.

Solvent effects on absorption spectra were first employed by Buraway (33) to characterize electronic transitions. He noted the presence of a low intensity, long wavelength band that moved to shorter wavelength upon changing the solvent from hexane to ethanol. McConnell (34) suggested the use of solvent effects for distinguishing the $\pi^* \longleftarrow \sigma^n$ and the $\pi^* \longleftarrow \pi^b$ transitions. Buraway defined two bands, the K band which shifts toward shorter wavelength, and the R band which shifts toward longer wavelength on solvent change toward polar. The K band refers to $\pi^* \longleftarrow \pi^b$ transitions in which ε is 10,000 or more. In one ring and biphenyl aromatic systems, two K bands are present, the E_1 and E_2 bands. The E_1 band is generally below 200 nm and the E_2 band above 200 nm. The R-band refers to $\pi^* \longleftarrow \sigma^n$ transitions in which ε is less than 100. This usually includes C=O and NO_2 group absorptions.

The $\pi^* \longleftarrow \pi^b$ transitions may be very intense (allowed) or weak (forbidden), but the $\pi^* \longleftarrow \sigma^n$ transitions are

generally forbidden and consequently of weak intensity. Solvent effects on transitions in the near ultra-violet spectrum of mesityl oxide (35) are given in Table I. It has been found that even $\sigma^* \longleftarrow \sigma^n$ transitions show solvent blue-shifts similar to $\pi^* \longleftarrow \sigma^n$ transitions. The effect of solvent on the carbonyl transition of acetone are shown in Table II (36). The solvent effects on the $\pi^* \longleftarrow \sigma^n$ transition of aliphatic carbonyl and nitro compounds have been investigated by Rao (37), and were found to be consistent with the decreasing order of the hydrogen-bonding ability of the solvents (water > methanol > ethanol > chloroform > hexane).

TABLE I

THE EFFECT OF SOLVENT ON THE UV ABSORBANCE PROPERTIES FOR MESITYL OXIDE

Solvent	λ_{max} (Å) ($\pi^* \leftarrow \pi$)	ε_{max}	λ_{max} (Å) ($\pi^* \leftarrow \sigma^n$)	ε_{max}
Water	2426	9,700	-[a]	-
Methanol	2368	11,000	3088	57
Isopropyl alcohol	2362	10,400	3112	51
Isooctane	2306	11,900	3210	38
Acetonitrile	2339	8,600	3140	33
Tetrafluoropropanol	2408	12,400	-[a]	-
n-Butyl alcohol	2372	10,200	3111	53
Ethylene glycol	2401	10,400	3074	61
95% ethanol	2371	10,500	3096	57
Ethanol	2364	10,600	3105	52
Dimethyl sulfoxide	-	-	3195	45
Chloroform (0.13M EtOH)	2379[b]	10,800	3148	53
Chloroform, pure	2376[b]	-	3154	-
Ethylene dichloride	2357	10,600	3184	47

[a] Submerged. [b] 0.01 cm cells required

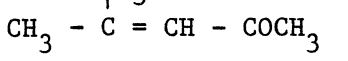


TABLE II

THE EFFECT OF SOLVENT ON THE $\pi^* \longleftarrow \sigma^n$ TRANSITION FOR ACETONE

Solvent	water	methanol	ethanol	chloroform	hexane
λ_{max}, nm	264.5	270	272	277	279

CHAPTER III

INSTRUMENTATION

CD Measurements

CD measurements were made using a JASCO-500A automatic recording spectropolarimeter fitted with a DP-500N data processor. The data processor provides for improve signal to noise (S/N) ratios by repeated scanning, time-scanning at fixed wavelength, time-dependent spectra with preset intervals, smoothing, derivative spectra, alternate measurement, and many other operations. Spectra can be viewed on the videomonitor before transferring to hard copy on the instrument recorder.

The wavelength range can be scanned from 180 to 800 nanometers, and the sensitivity scale varied from 0.1 to 50 milli degree per centimeter (m^{o}/cm). The instrument is purged with nitrogen gas bled from a liquid nitrogen reservoir for economic reasons. The flow rate is 2L/min. The presence of nitrogen prevents the production and accumulation of ozone from interaction of high intensity ultraviolet radiation with oxygen which has a deterioration effect on the optical mirrors. Measurements made at wavelengths below 200 nm require that the flow rate of nitrogen gas be increased to 3-5L/min.

The light source is a 450 watt high pressure Xe arc lamp. The lamp is water cooled (2L/min) to protect the instrument from the high temperatures of the arc. The instrument is operated at 23 amp lamp current, and 105 voltage. The optical system is shown in Figure 10.

Daily calibration of the ellipticity scale was made against a 0.05% (W/V) androsteron/1,4-dioxane solution. The ellipticity scale of the instrument is adjusted to read a peak height of 96.2 millimeters at 304 nm with the sensitivity set at 20 m^{o}/cm.

UV-vis Absorption Measurements

Absorption measurements were made on a HITACHI Model 100-80 UV-visible spectrophotometer. The light sources are deuterium (190-370 nm) and tungsten (325-870 nm) lamps which are automatically switched at 325 nm. The wavelength accuracy is 0 ± 0.2 nm. The absorbance range is from 0.000 to 3.000 absorbance units (A), and the transmission range is 0 to 100%. Base stability is 0.0004 A/hr at 340 nm.

Cuvettes for both of these spectrometers were made from quartz, and were available in a variety of pathlengths. Absorption measurements were made in the double beam difference mode, the solvent being used as reference. CD measurements were made in single beam mode, with baseline corrections made by subtraction on the DP-500N.

Weighings were made on two balances , a CAHN electrobalance (model 2000 RG) for small samples between

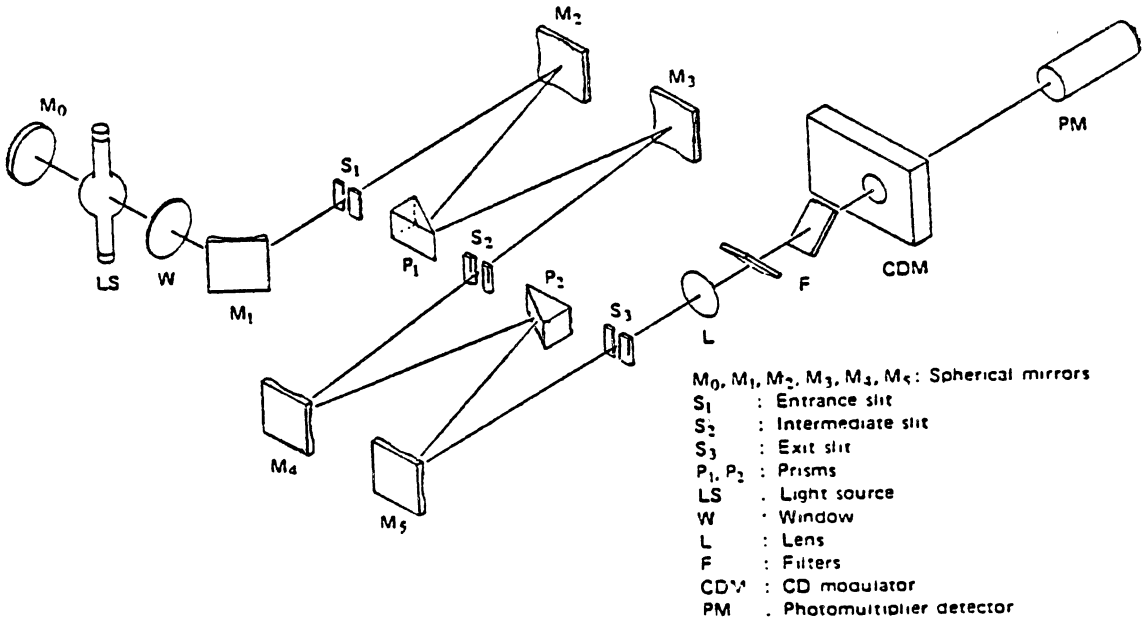


Figure 10. Optical System of the JASCO J-500A Circular Dichroism Spectropolarimeter Source: JASCO

0.001mg to 10.000mg and a Sartorius (Sartorius Model 2403) for large samples in excess of 10mg. All of the solvent extractions were done using an ultrasonic cleaner (Cole-Parmer), Model 8845-44.

CHAPTER IV

CHIRAL ANALYTE

Introduction

The determination of an anonymous analyte in a mixture without any separation of any kind was the original objective for investigating the application of CD to drug analysis. It is generally understood that CD focuses on the molecular property of optical activity, and it is the detection of that particular property which would be exploited in accomplishing the objective. It is not so commonly understood that simultaneous energy absorption is also a necessary prerequisite to CD activity. This latter property adds the quantitative dimension to the analysis of drugs. Whereas optical activity may appear to be the more important of the two prerequisites, which it is for drug discrimination, this property can be induced into otherwise achiral molecules. This will be described in more detail in later chapters. Absorption is essential to CD activity and can not be readily induced into a molecule. In searching for substances to which the detection and determination by CD may be applicable, the presence of a chromophore is more important than the presence of an apparent center of optical activity. The structure which on paper may appear to be

optically active, may exist as a racemic mixture so additional information as to its source is necessary. Nevertheless CD-induction into a racemate is still possible.

In this chapter we deal with representatives from those drug molecules which meet both requirements. We have especially chosen substances which are difficult to individually distinguish by UV absorption (38,39,40) and in some cases even by chromatography. Included in this collection are compounds taken from the tetracyclines (41), the penicillins (42) and cephalosporins; and the quinine and cinchonine alkaloids.

Experimental Section

Quinine hydrochloride, quinidine hydrochloride, cinchonine and cinchonidine free bases; cephalothin and cephalexin; tetracycline, doxycycline, minocycline, chlorotetracycline, demeclocycline, oxytetracycline were all obtained from Sigma Chemical Co., and used without purification. Other analytes included in the study were D-fructose (Fisher); D-penicillamine (Aldrich Chemical Co.); cloxacillin, dicloxacillin, methicillin, nafcillin, oxacillin, ampicillin trihydrate (Bristol Myers Co.). Reagent grade $CaCl_2$ $2H_2O$ was obtained from Mallinckrodt Inc.

A deficiency in much of the early work from this laboratory was the failure to recognize the effect of pH on the CD spectra of the drugs investigated in aqueous solutions. Maxima in UV absorption spectra are known to be

red or blue-shifted with pH change which is dependent upon the chromophore responsible for the absorption. In CD however, polarity changes can accompany a pH change. Accordingly all aqueous solutions were buffered to 9.8±0.05, 9.2±0.05, or 5.6±0.05 (pHydrion Buffers, Micro Essential Laboratory).

Result and Discussion

The molecular structures (Figure 11) and the UV absorption spectra (Figure 12) for the two quinine and cinchonine diastereoisomer pairs are identical. Distinction is not possible from these data. If pure, and not in a mixture, distinction can be made by polarimetry because rotations are opposite for the diastereoisomers (43). In mixtures this is not always possible because of chiral interferences. By CD the structural isomers can be uniquely identified either alone or in mixtures. Later the simultaneous determination of both in a binary mixture will be described (Chapter IX).

From the molecular structures (Figure 13) and the CD spectra for the tetracyclines (Figure 14) the sensitivity of the spectra to subtle structural modifications is clearly demonstrated. Whereas the class of compound may be identified from absorbance spectra, individual distinctions among the members are possible by CD. The confidence in making an individual identification can be enhanced by observing the pH dependence of the spectra, for example

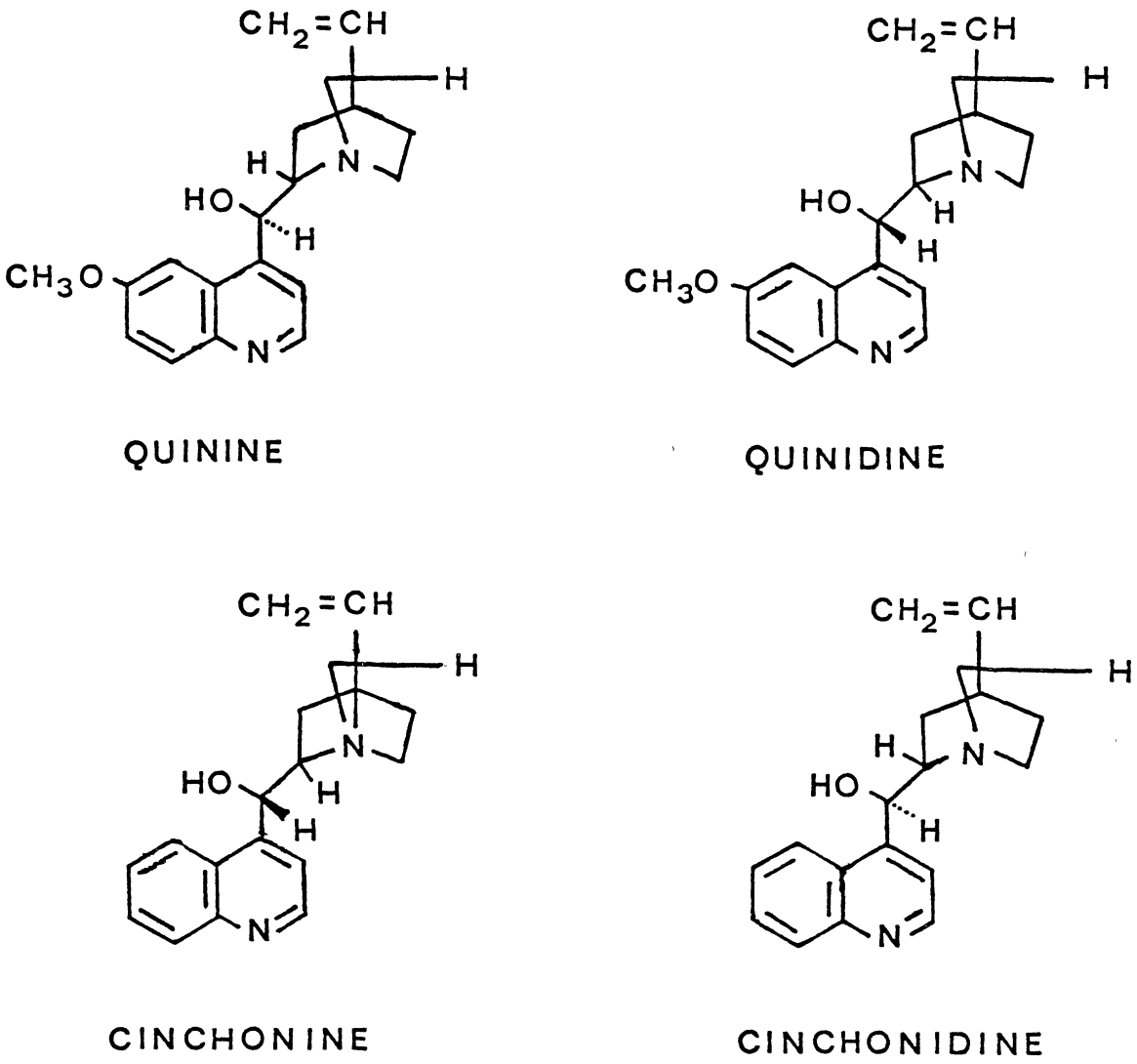


Figure 11. Molecular Structures for Quinine, Quinidine, Cinchonine, and Cinchonidine

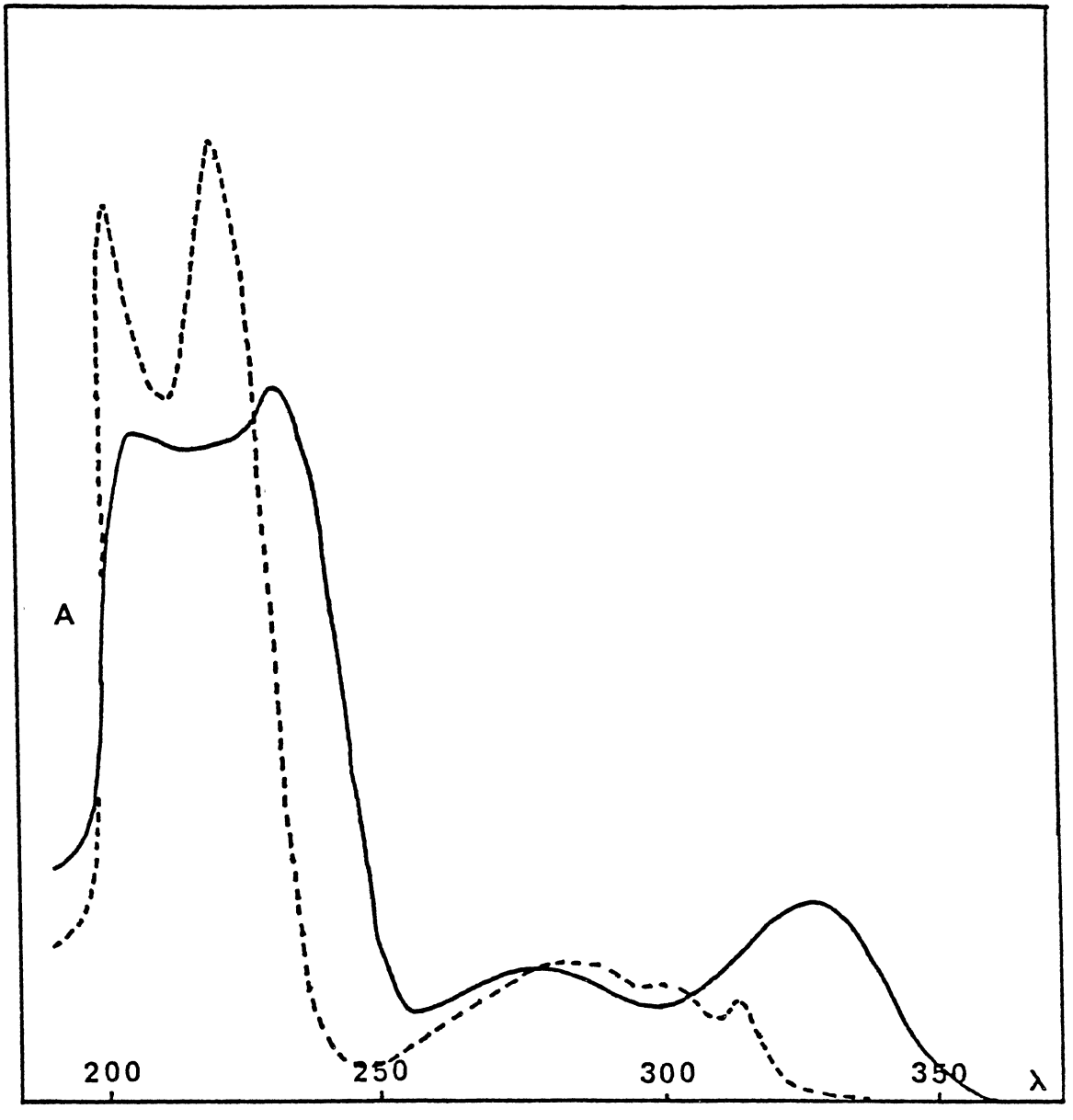
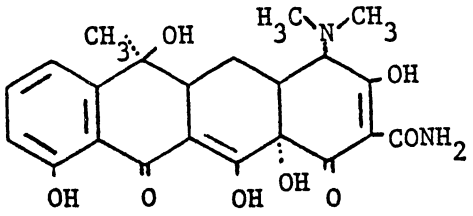
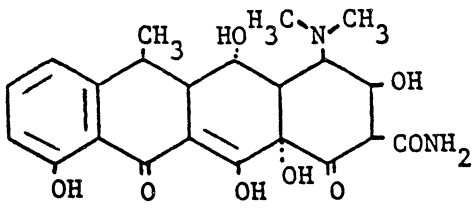


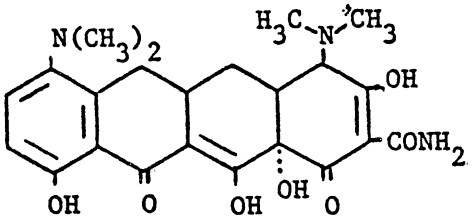
Figure 12. UV Spectra for the Two Quinine (——) and Cinchonine (----) Diastereoisomers at pH 8.0



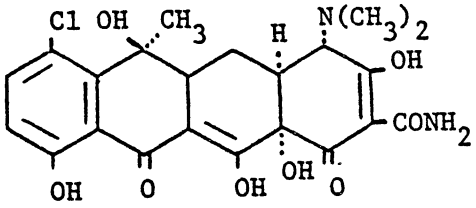
TETRACYCLINE



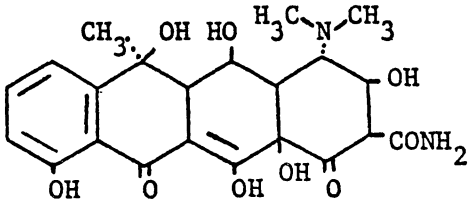
DOXYCYCLINE



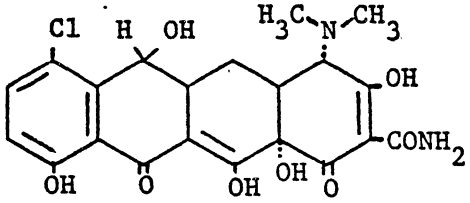
MINOCYCLINE



CHLORTETRACYCLINE



OXYTETRACYCLINE



DEMECLOCYCLINE

Figure 13. Molecular Structures for Tetracyclines

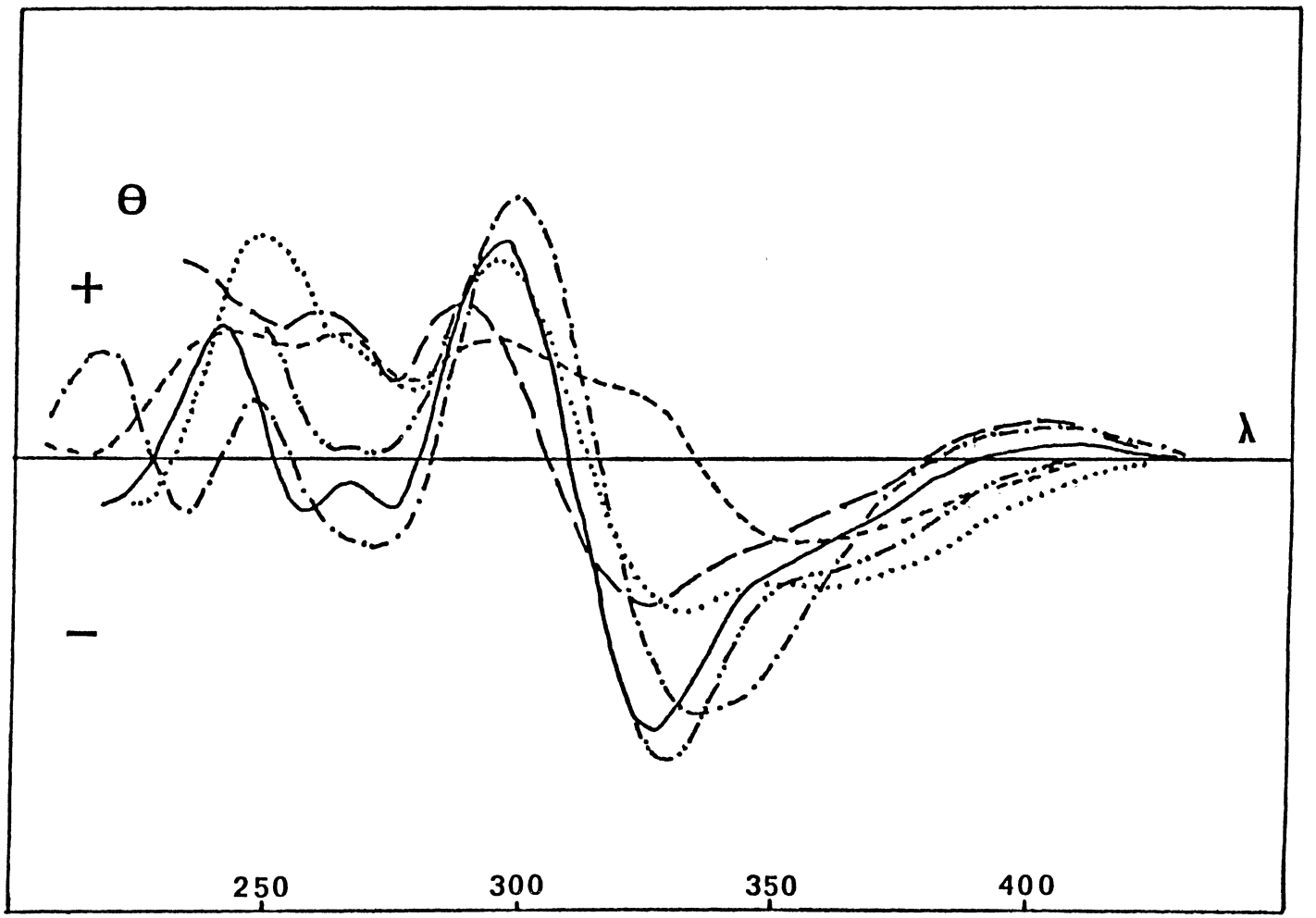


Figure 14. CD Spectra for Tetracycline (——), Deoxycycline (----), Minocycline (-·-·-), Oxycycline (— —), Chlorotetracycline (.....), Demeclocycline (—··—)

tetracycline in Figure 15. Prescription forms of the drug are usually unadulterated, so no quantitative assays were performed. Equilibrium constants were calculated from CD data for the complexation reactions (44,45) with Ca^{++} ion. Results are in good agreement with values obtained from other methods: log K = 3.27 (Ca - tetracycline), 3.08(Ca - chlorotetracycline) and 2.13 (Ca - oxytetracycline).

For the beta-lactams, that is, the penicillins and cephalosporins, obvious differences in CD spectra in pH 9.8 buffer were observed. Once again the drug classes are readily distinguishable from each other (Figure 16) by CD. Results for individual distinctions were not as rewarding as they were for the tetracyclines. Assay experiments were not performed.

The polarity of the CD spectrum is reversed from that for penicillin for the metabolic product penicillamine (Figure 17). Preliminary measurements were made to characterize the dramatic spectral changes which occur with time on the addition of Cu^{++} to the aqueous solution (Figure 18). Strong transient CD signals in the visible range were produced in what is presumably a complex reaction mechanism involving metal complexation and even reduction-oxidation steps. The problem was not pursued at the time.

A number of mono- and disaccharides were examined as possible common interferences to drug determinations using CD without separation steps. A very full study had been reported previously (46). Our results confirmed that the

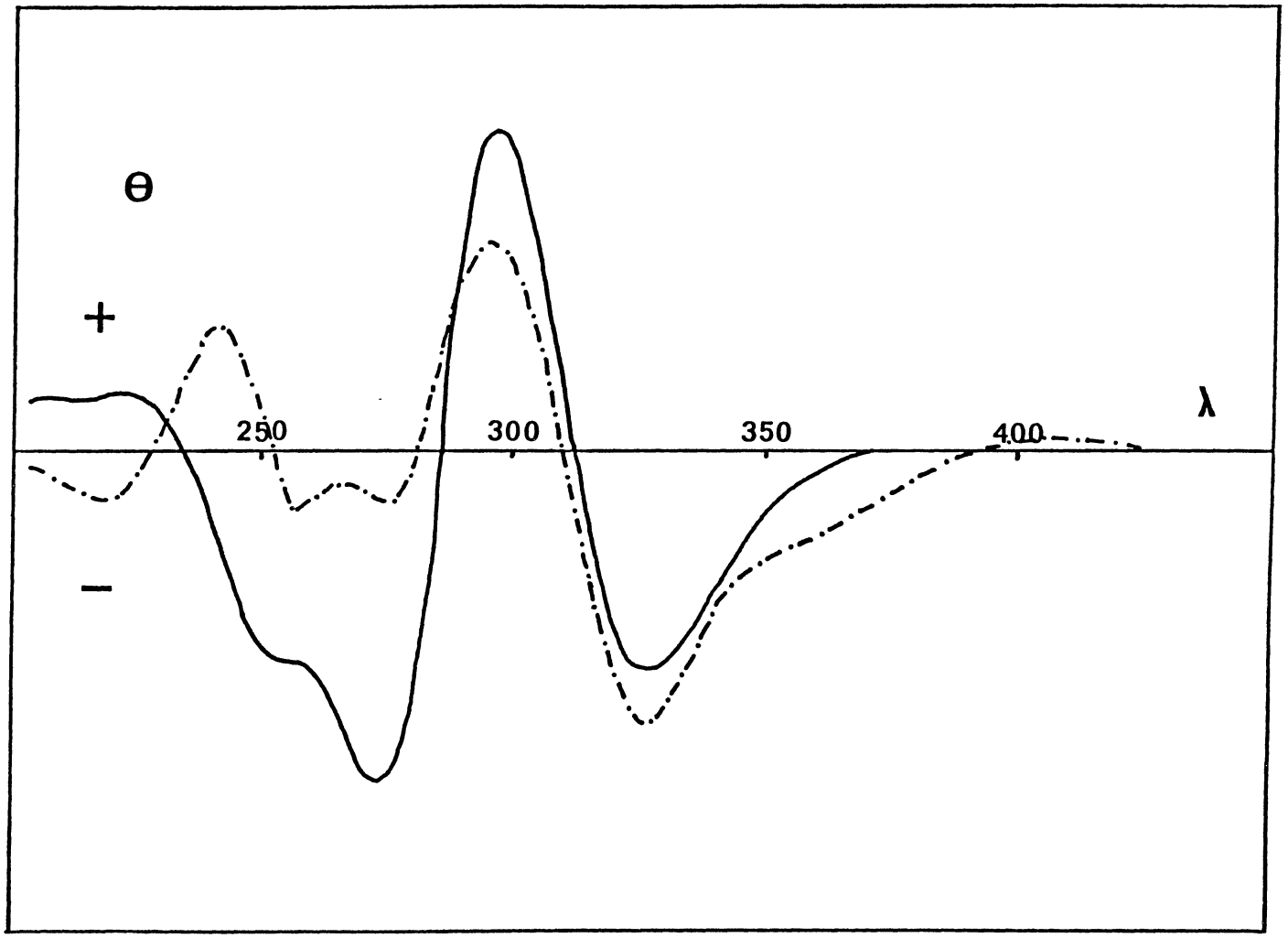


Figure 15. pH Dependence of CD Spectra for Tetracycline; pH 5.6 (——), pH 9.2 (-·-·-)

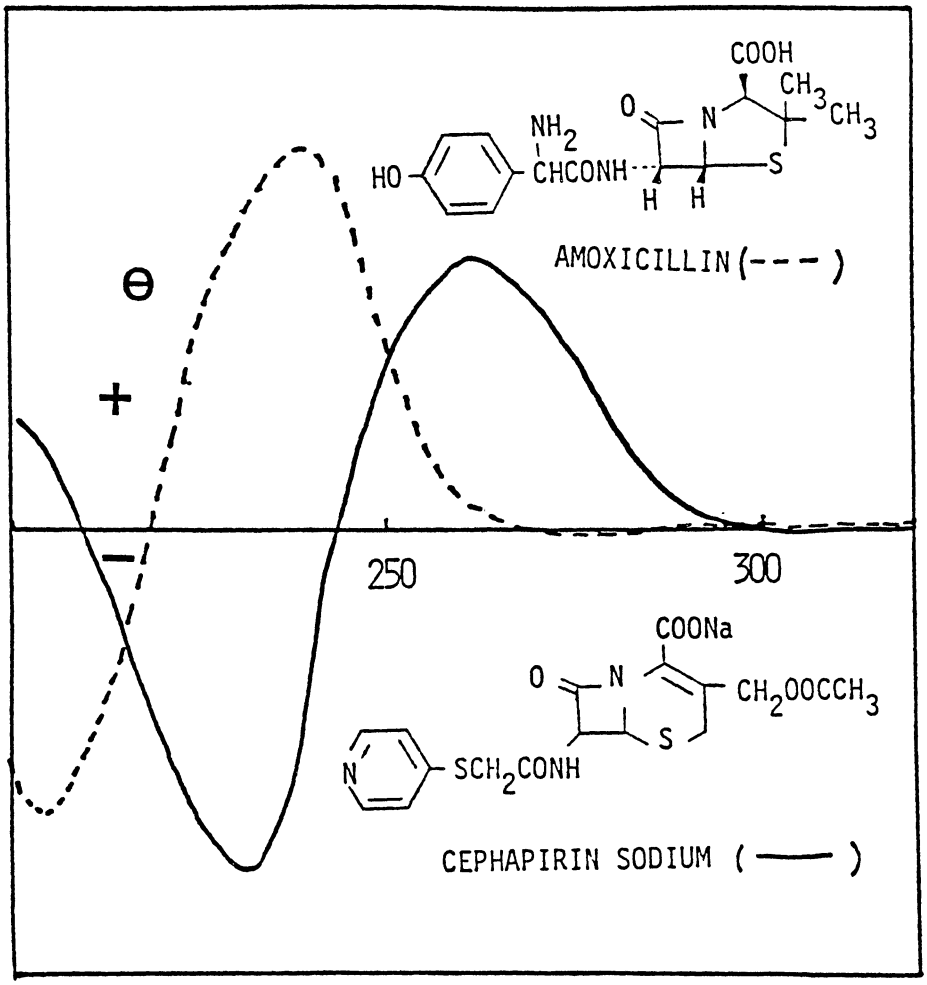


Figure 16. CD Spectra of β-Lactams; Amoxicillin and Cephapirin Sodium

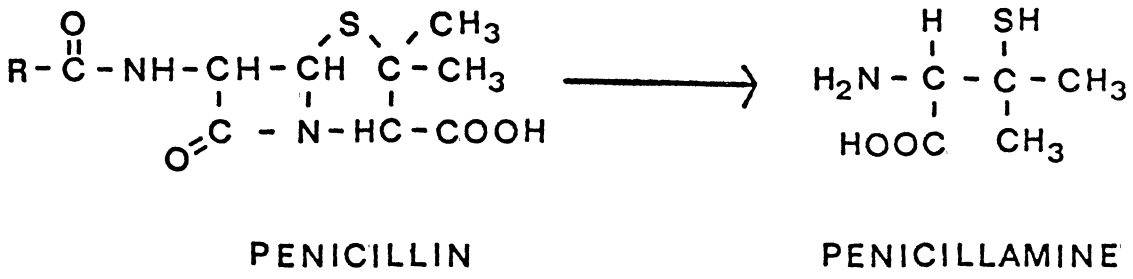


Figure 17. Molecular Structures for Penicillin and Penicillamine

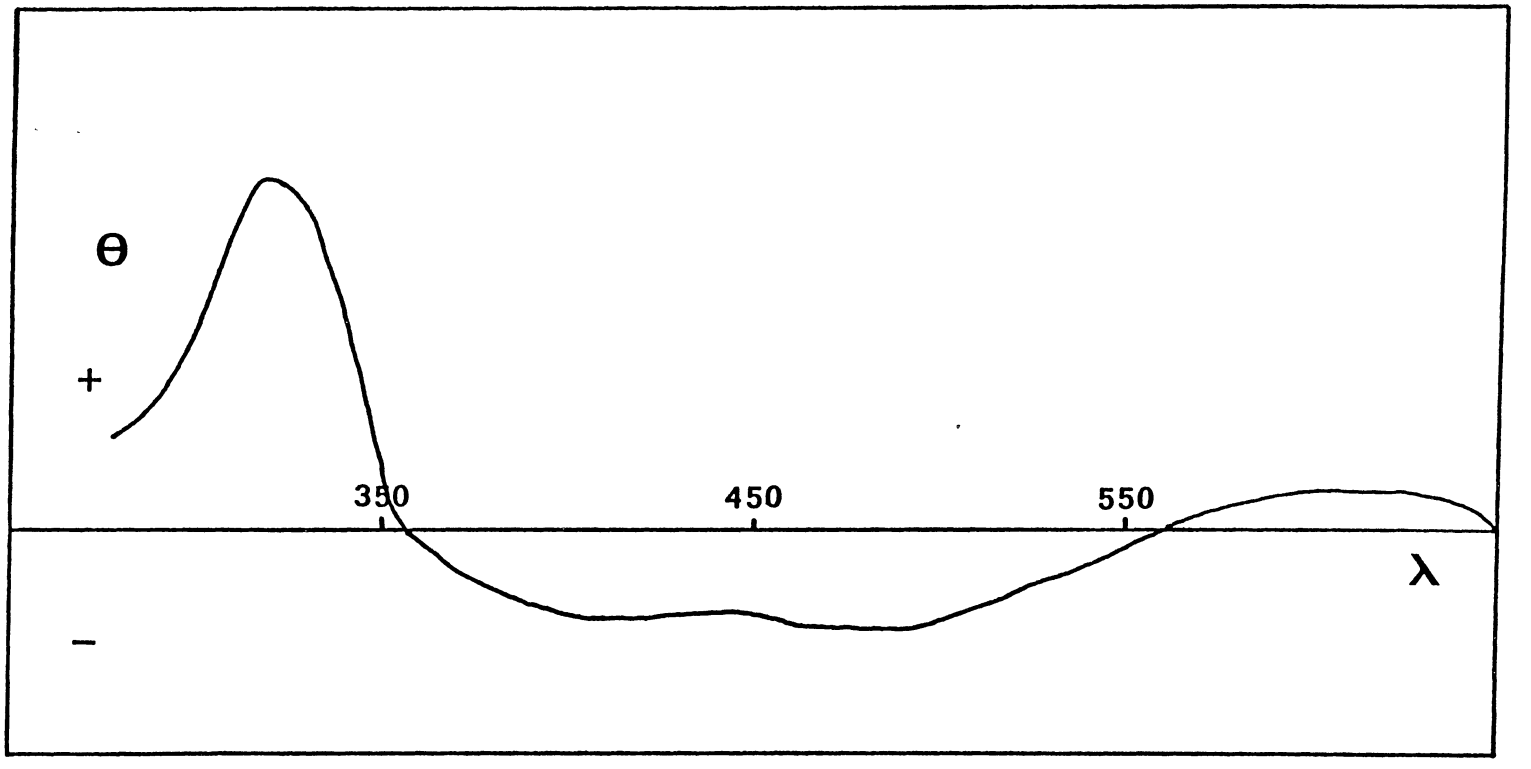


Figure 18. Effect of Cu^{++}-Amoxicillin Complexation on CD Spectrum

simple sugar whose presence would be a principal concern is D-fructose (Figure 19). The molar ellipticity value is comparatively small and approximately 40 minutes equilibrium time is required for complete mutarotation to have occurred. D-fructose is a common additive, being present in a number of soft drinks and as sweeteners in oral drug forms, as well as being a hydrolysis product of invert sugar and sucrose.

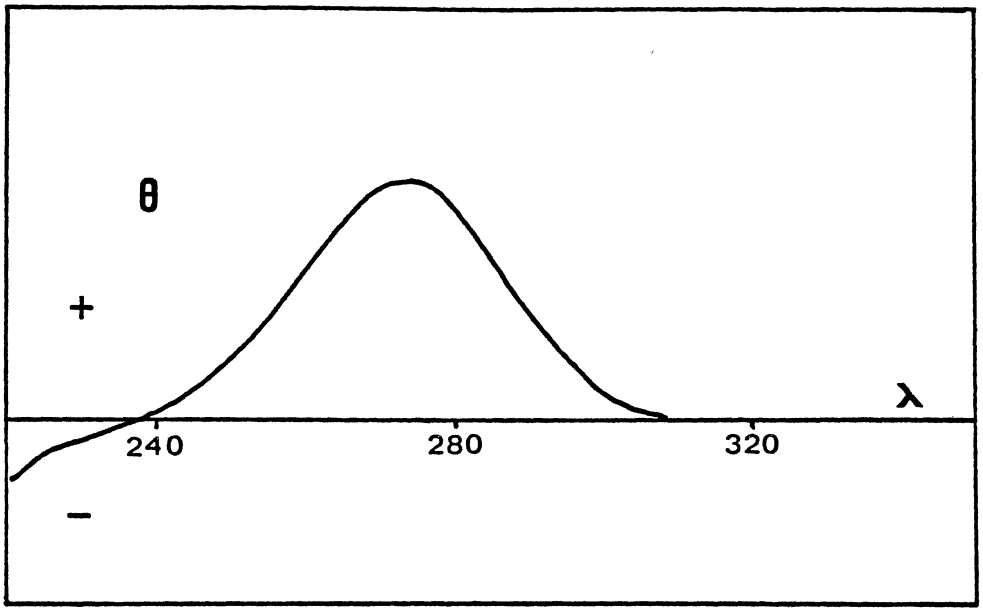


Figure 19. CD Spectra for D-Fructose

CHAPTER V

COMPLEXATION OF ACHIRAL DRUGS WITH CYCLODEXTRIN

Introduction

The ability of BCD to act as a host in complexing a wide variety of other molecules in aqueous solutions has been recognized for many years and recently reviewed (19,20). The nature of the interaction has been the subject of a number of thermodynamic studies (47,48). From this information the center of the cyclic oligosaccharide is known to be hydrophobic, so it is accessible to nonpolar, usually aromatic molecules. BCD and its aqueous solutions are stable, although the solubility is limited. It is available in relatively high purity and is inexpensive.

Chirality can be induced into an achiral molecule when it is complexed with BCD (49). Induced CD activity was previously reported (50,51) using first a cholesteric liquid crystalline solvent, which could not be exploited for quantitative studies, and second for L-cocaine and phenycycline (PCP) using BCD. If the guest (achiral molecule) contains a chromophore the complex will have a CD spectrum. In theory these spectra could be used for drug identification and as a method to determine the formation constants for the complexation equilibria.

Experimental Section

The BCD used in this work was obtained from Eastman Kodak and was used without further purification. The following achiral molecules, all of which contain at least one aromatic chromophore, were obtained in pure form from the suppliers listed. PCP (phencyclidine), PCPY (PCP Pyrrolidine), and PCM (PCP Morpholine) hydrochloride (Applied Science); beta-phenylethylamine and phenobarbital (Sigma Chemical Co.); meperidine hydrochloride (Sterling-Winthrop); and diazepam and dilantin (Drug Enforcement Administration).

The concentrations of the guest compounds were 4.5 to 4.7 x 10^{-3} M (PCP, PCPY, PCM), 9.5 to 9.6 x 10^{-4} M (beta-phenylethylamine), 1.3 to 1.4 x 10^{-3} M (phenobarbital), 8.0 to 9.0 x 10^{-4} M (meperidine), 8.7 to 8.8 x 10^{-3} M (diazepam), and 2.1 to 2.2 x 10^{-3} M (dilantin) to be consistent with a solution absorbance which is within the dynamic range of the CD instrument. The first five compounds were dissolved in distilled water. Diazepam was dissolved in 2 x 10^{-3} M hydrochloric acid, and dilantin and meperidine in 0.1 M sodium hydroxide because of their limited solubilities in water. BCD is stable in dilute base but is rapidly hydrolyzed in acid solutions which are more concentrated than 0.1 M.

For the calculations of the formation constants spectra were obtained for a series of solutions in which BCD was

added to a fixed concentration of guest up to a maximum host concentration of 10^{-2} M. Wherever possible BCD was in molar excess.

Results and Discussion

Figure 20 shows the induced CD spectra and molecular structure for the eight complexed analytes. With three exceptions the three $\pi^* \longleftarrow \pi^b$ aromatic transitions at wavelengths between 250 and 275 nm are well represented. The chirality of these bands is principally negative. There are different and statistically reproducible ratios for the peak maxima from compound to compound which, together with the variability in the sign of the 240 nm band, allow for a qualitative distinction among the numbers of this subgroup.

The spectrum for dilantin has a single negative Cotton band with a maximum at 235 nm. Dilantin and meperidine were complexed with the host in 0.1 M sodium hydroxide solution but only the spectrum of meperidine shows the typical aromatic transition. The apparent absence of these bands for dilantin is not necessarily attributable to the alkaline conditions because the bands persist for alkaline solutions of phenobarbital and beta-phenylethylamine as well as for meperidine. It must be assumed that either the rotational strengths for these transitions in dilantin are lower than the detection limit of the instrument at the limiting concentrations used, or the bands are concealed under the major band observed.

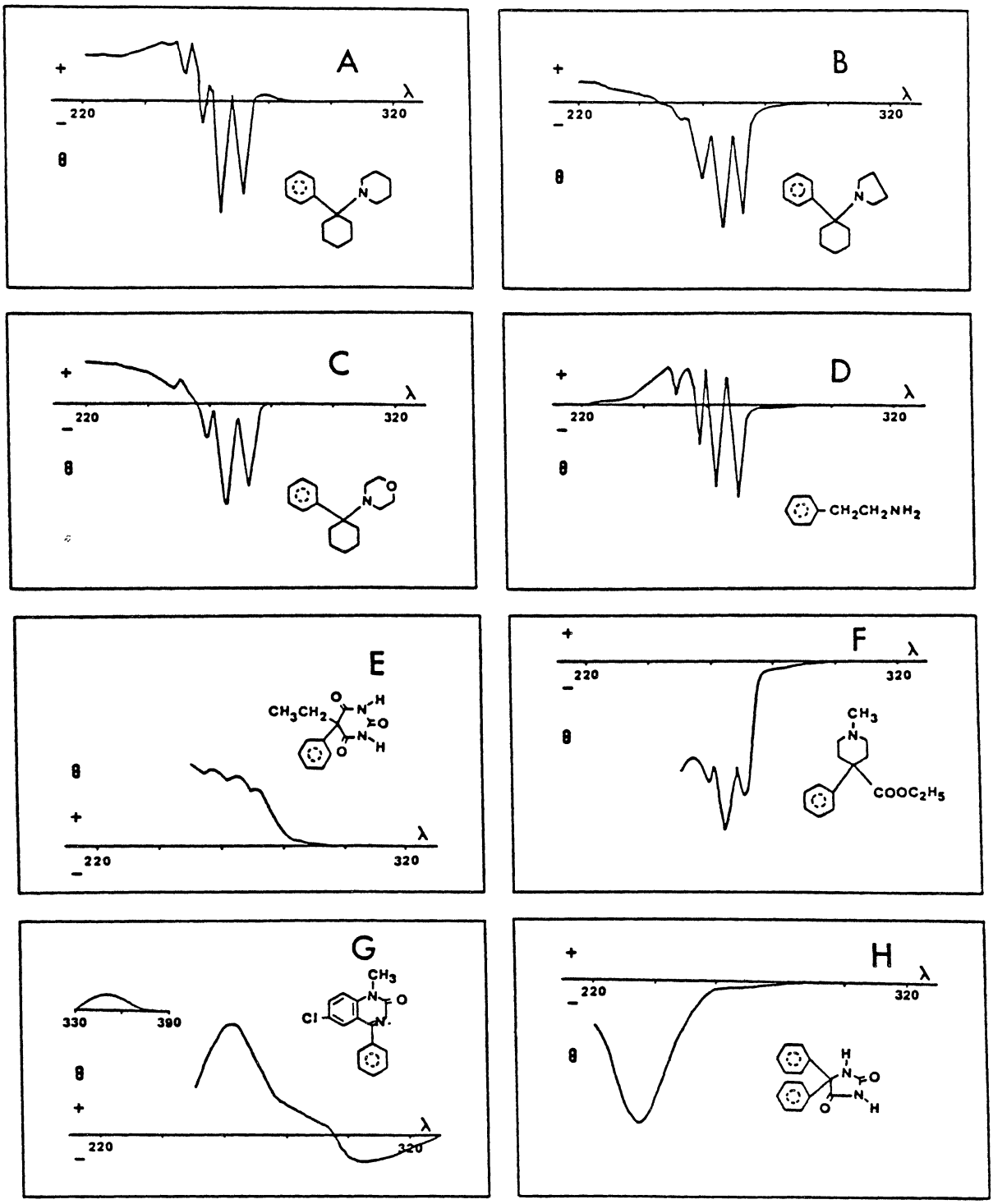


Figure 20. Induced CD Spectra for (A) PCP (B) PCPY (C) PCM (D) beta-Phenylethylamine (E) Phenobarbital (F) Meperidine (G) Diazepam, and (H) Dilantin

The other two exceptions to the general pattern are phenobarbital and diazepam. All three exceptions contain a carbonyl chromophore whose CD activity dominates the spectrum at wavelengths longer than 250 nm. A positive influence is induced on complexation for phenobarbital, while both positive and negative features are evident for diazepam.

Equilibria were treated as 1:1 complexation interactions between drug (D) and sugar (S)

$$D\ (\text{Drug}) + S\ (\text{Sugar}) \rightleftharpoons DS\ (\text{Complex})$$

$$K = \frac{[DS]}{[D]\,[S]} \tag{5.1}$$

Molar concentrations are related to the experimental ellipticities according to:

$$\psi = \theta_D[D] + \theta_{DS}[DS] \tag{5.2}$$

when θ_D is zero,

$$K = \frac{\psi/\theta_{DS}}{[C_D - \psi/\theta_{DS}]\,[C_S - \psi/\theta_{DS}]} \tag{5.3}$$

Where θ_D and θ_{DS} are the molar ellipticity coefficients for the free and complexed drug respectively. θ_{DS} is zero for achiral molecules. C_D and C_S are total analytical concentrations. Formation constants were calculated by a procedure attributed to Hildebrand, in which the derived equation (5.4) is solved in a converging iterative procedure (52).

$$\frac{b\,[D]\,[S]}{\psi_{DS} - \psi_D} = \frac{([D] + [S] - [DS])}{\theta_{DS} - \theta_D} + \frac{1}{K(\theta_{DS} - \theta_D)} \tag{5.4}$$

b is the cell path length in cm.

[D] [S] [DS] are the equilibrium concentrations of drug sugar and complex

ψ_{DS} is the observed ellipticity of complex

ψ_{D} is the observed ellipticity of drug

θ_{DS} is the molar ellipticity of complex

θ_{D} is the molar ellipticity of drug

Iteration is initiated by assuming [DS] = 0. The formation constant K and θ_{DS} are obtained from the intercept and slope respectively of the linear equation. Iteration is programmed to terminate when successive K values differ by unity.

Table III shows the formation constants for these compounds. Determinations were done in replicate and calculations were made at all wavelengths of maximum signal intensity. K values are overall averages. θ_{DS} values correspond with the wavelengths given in parentheses. A previous K value for PCP hydrochloride (50) was reported as very uncertain. This is in part due to the lower sensitivity of the older Cary 61 (CD) instrument used in that work, and in part due to the mathematical procedure employed which used a graphical convergence criterion.

The weakest interaction is observed between BCD and beta-phenylethylamine in terms of both the K value and the induced molar ellipticity. The value of θ_{DS} is similar to that calculated for DL-(alpha)-phenylethylamine and is almost one tenth of the θ_{D} value for either D-(alpha)-, or

TABLE III

FORMATION CONSTANTS AND INDUCED MOLAR ELLIPTICITIES FOR ACHIRAL COMPOUNDS WITH BCD

compound	K(SD)	θ_{DS}(SD)	λ, nm
PCP	1024(15)	-0.9(0.1)	271
PCPY	712(8)	-1.6(0.2)	271
		-1.8(0.2)	264
		+0.3(0.1)	235
PCM	460(5)	-1.6(0.1)	271
		-2.0(0.1)	265
β-phenylethylamine	82(5)	-0.9(0.1)	270
merperidine	134(7)	-1.9(0.2)	271
phenobarbital	1098(18)	+12.5(0.3)	273
		+16.0(0.3)	268
diazepam	83(3)	+12.8(0.2)	350
		-25.1(0.2)	307
		+151.8(0.3)	260
dilantin	144(5)	-426.7(0.5)	235

L-(alpha)-phenylethylamine alone in aqueous solution (Chapter VI). Beta-phenylethylamine might be representative therefore of the minimum affect of complexation on a CD spectrum assuming the side chain has no influence on the interaction.

The largest $^{\theta}_{DS}$ for a particular chromophore would be indicative of the greatest susceptibility to chirality induction. In this group θ_{DS} is greatest for diazepam, the value for dilantin describing a different electronic transition. Of the eight compounds investigated, diazepam has the most structural rigidity.

The eight analytes described here are all monosubstituted benzene derivatives. In another study, a few compounds in which the aromatic ring is substituted more than once is described in Chapter VI.

Commercially available Demerol Tablets (Winthrop) were assayed for meperidine. The typical capsule weight is around 125 mg and it contains 50 mg of meperidine. The tablet was dissolved in 0.10N NaOH and an aliquot of standard BCD stock solution was added. Correspondance with the prescribed amount was better than 98%. The technique might be suitably developed for quality control applications in the pharmaceutical industry. Any of these eight analytes would be accurately determined in a straightforward, simple procedure in a brief period of time. Other analytes could qualify after the equilibrium constant has been calculated.

CHAPTER VI

DRUG DISCRIMINATION BY CYCLODEXTRIN

Introduction

The content of the previous chapter concentrated on the chirality induced by BCD complexation on achiral drug molecules exclusively. It is erroneous to think that only achiral molecules are complexed by BCD, just as it is wrong to think that only drug molecules participate in the molecular association. In this chapter other complexations which might interfere in determinations are considered. Obviously, if BCD complexes with guest molecules indiscriminately, all compounds in a mixture could conceivably become CD-active and the original analytical objective of determination without separation is lost. The list of analytes includes chiral drugs, racemic mixtures, compounds with more highly substituted aromatic rings, and commonly encountered additives to commercial products. The results of the study would allow us to compare the inherent CD versus induced CD for chiral substances, the relative stabilities of the complexes formed between D- and L-enantiomers and BCD, and the effect of the structure of the guest on the magnitude of the induced Cotton effect.

Experimental Section

The fifteen guest molecules used in this study were: DL-methadone and L-isomethadone (Drug Enforcement Administration), quinine sulfate, quinidine hydrobromide, D-, L-, and DL-(alpha)-phenylethylamine, L-hyocyamine, atropine sulfate, acetylsalicylic acid, and caffeine hydrobromide (Fisher Chemical Co.), D- and DL-penicillamine (Aldrich Chemical Co.), mescaline, and psilocin (National Institute for Drug Abuse). The concentrations of the guest compounds were in the ranges 1.2 - 1.3 x 10^{-3} M (DL-methadone), 0.9-1.1 x 10^{-4} M (L-isomethadone), 5.4 - 5.5 x 10^{-4} M (quinine and quinidine), 7.0 - 7.1 x 10^{-4} M (D(+)-alpha-phenylethylamine, L(-)-alpha-phenylethylamine), 9.5 - 11.0 x 10^{-3} M (DL-(alpha)-phenylethylamine), 1.2 - 1.5 x 10^{-4} M (L-hyoscyamine), 2.6 - 2.9 x 10^{-4} M (atropine sulfate), 1.5 -1.7 x 10^{-3} M (acetylsalicylic acid), 1.4 - 1.6 x 10^{-3} M (D- and DL-penicillamine), 1.2 - 2.4 x 10^{-3} M (mescaline), 2.5 - 2.7 x 10^{-4} M (psilocin) to be consistent with a solution absorbance which is within the dynamic range of the CD instrument. All of the compounds were dissolved in distilled water. For the calculations of the formation constants, spectra were obtained for a series of solutions in which BCD was added to a fixed concentration of guest up to a maximum host concentration of 10^{-2} M. Wherever possible BCD was in molar excess. The compounds, with the exception of the two penicillamines, contain an aromatic and/or heterocyclic ring chromophore, and an occasional

carbonyl so the wavelength range of interest was from 220 - 350 nm.

Results and Discussion

The list of fifteen compounds includes seven chiral, four racemic, and four additional achiral molecules. The last four are aspirin, caffeine, mescaline, and psilocin, none which shows any evidence for chirality induction. Aspirin and caffeine therefore can be eliminated from the list of possible interferences in the direct determination of drugs using CD. For mescaline and psilocin the extent of aromatic substitition might prohibit a strong interaction with BCD, while the comparatively polar alkylamine substituent would be less favored by the hydrophobic center of the host. Of the remainder quinine, quinidine, D- and L-(alpha)-phenylethylamine and D- and DL-penicillamine show no CD evidence for complexation by BCD. For quinine and quinidine the aromatic ring is tri-substituted and again interaction with the host may be greatly restricted. The penicillamines have no phenyl substituent and are polar in aqueous media.

Figure 21 shows the CD spectra of the four chiral compounds. Spectra either altered or induced by the addition of BCD are given in Figure 22. All others show a gradual increase or decrease in signal intensity, with no sign inversion at the same wavelength maxima of the free drug, which is proportional to the concentration of added

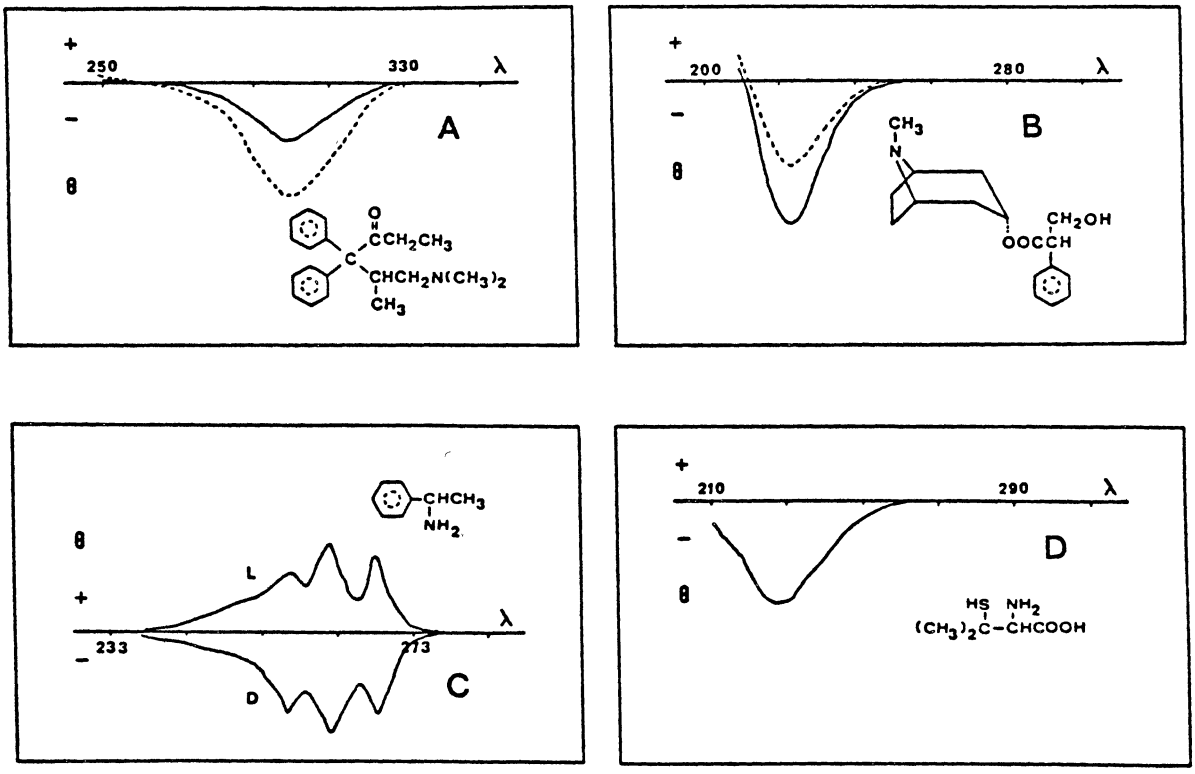


Figure 21. CD Spectra for (A) L-Isomethadone (B) L-Hyoscyamine (C) D- and L-(Alpha)-Phenylethylamine (D) D-Penicillamine (solid lines represent the inherent CD) (broken lines represent induced CD)

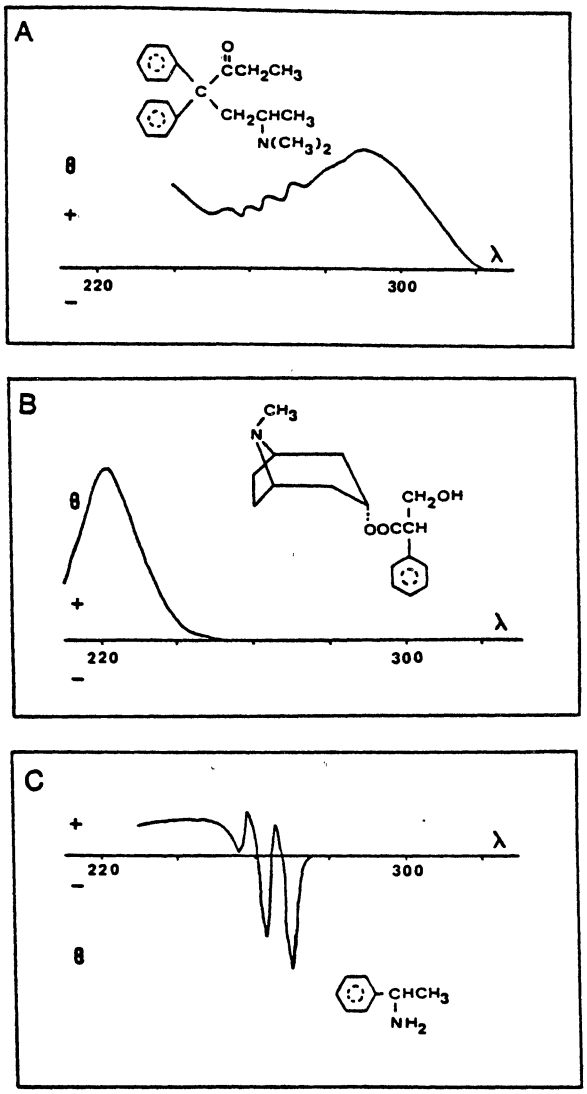


Figure 22. Induced CD Spectra for (A) DL-Methadone (B) Atropine, and (C) DL-(Alpha)-Phenylethylamine

BCD. The same is presumably true for the racemic mixtures for which neither enantiomer was available for wavelength maxima confirmation. CD induction was observed and is reported for DL-(alpha)-phenylethylamine but not for either enantiomer. In order to observe the induced signal for the racemate, the analyte concentration had to be almost 10^{-2} M which is about equal to the maximum concentration of BCD used. Under equivalent concentration conditions for either isomer the CD signal inherent to the chiral molecule was at least ten times greater than the anticipated induced signal which introduces significant error in the measured ellipticity change with BCD concentration. No satisfactory complexation constant could be calculated. As a general rule the induced ellipticities for the racemates appear to be in the positive direction.

Table IV shows the formation constants K calculated in the manner described in chapter V. The value for the complexation with DL-(alpha)-phenylethylamine compares favorably with that for the beta-phenylethylamine isomer, implying little to no effect upon the relative stabilities by minor structural changes in the non-interacting side chain. Although θ_{DS} values are of similar magnitude, they are of opposite sign. Other K values are uniformly three to four hundred. θ_{DS} values in contrast show no simple regular correlation with structural type. These should not be taken too literally however because the values might be associated with quite different electronic transitions.

TABLE IV

FORMATION CONSTANTS AND MOLAR ELLIPTICITY DATA FOR CHIRAL COMPOUNDS WITH BCD

compound	K	θ_D(λ, nm)	θ_{DS}(λ, nm)
DL-methadone[a]	337	0	9.8 (290)
DL-α-phenylethylamine[a]	66	0	0.6 (270)
atropine (DL-hyoscyamine)[a]	308	0	65.6 (222)
L-isomethadone	467	-263 (300)	-400 (300)
L-cocaine[c]	406	-55 (245)	+87 (245)
S-(-)-nicotine[c]	358	-60 (268)	-64 (268)
		-64 (264)	-71 (264)
L-hyoscyamine	308	-268 (222)	-12 (222)
D-hyoscyamine	169	+268 (222)	-71 (222)
quinine		-52 (336)	
		+21 (259)	
quinidine		+47 (336)	
		-25 (259)	
D-penicillamine		-19 (228)	
D-α-phenylethylamine		-2.9 (268)	
		-3.3 (262)	
		-2.3 (257)	
L-α-phenylethylamine[b]			

[a] K values calculated as averages for both enantiomers.

[b] θ_D values same as for the D-α-isomer but with positive signs.

[c] from reference 87

Calculations from the data for the racemic mixtures in actuality provide only average values for K and θ_{DS}. The number of instances where both enantiomers are available and the induced CD is substantial enough for an accurate distinction to be made between the two equilibria is very limited. Two cases in point here are the (alpha)-phenylethylamines and the penicillamines. This is true too of isomers of dopa and phenyl-substituted aminoacids. Other isomers of methadone and isomethadone were unobtainable. The only qualifiers we had access to at this time to compare enantiomeric differences in complexation were atropine (or DL-hyoscyamine) and L-hyoscyamine. Even here the data for the D-form must be derived and not measured directly.

Equation (5.1) must be modified to include the species involved in the competitive equilibria between both isomers and BCD, so that the experimental ellipticity consists of four terms (equation 6.2).

$$\psi = \theta_D[D] + \theta_{DS}[DS] + \theta_L[L] + \theta_{LS}[LS] \qquad (6.2)$$

In the solution of this equation it can not be assumed that θ_{DS} and θ_{LS} are equal either in magnitude or in sign, unlike the values for θ_D and θ_L. By treating L-hyoscyamine separately, the last two terms in equation (6.2) can be determined from previously calculated θ_{LS} and K values and ψ can be corrected to reflect only the contribution from the D-isomer. Values for the D-form are then derived by the prescribed iterative procedure.

The positive induced CD signal for atropine is therefore a consequence of a competitive enantiometric discrimination by BCD in which both isomers are complexed. For both complexed forms the molar ellipticity coefficient for the induced CD signal is of the same sign as the inherent CD, but both are numerically smaller.

Only five of fifteen potential guests produced CD evidence for complexation and only three of these were from a subset of eight achiral compounds. These odds are very good for the direct determination of particular achiral drugs in mixtures in which the other constituents are also achiral. All CD-active chiral compounds would theoretically interfere with the achiral analyte determinations. For example in a binary mixture of a CD-active and a CD-inactive compound, the former is easily determined with no interference. The latter might be determined by the addition of BCD and the ease with which this is accomplished depends upon the effect that complexation has on the spectrum of the former analyte.

Where CD induction by BCD occurs, the effect appears to be greatest when a carbonyl chromophore is present. Add to this observation the fact that carbonyl electronic transitions usually occur at wavelengths longer than 290 nm and out of range of the aromatic transitions, then suitable target analytes for future study can be identified.

The ultimate conclusion to this aspect of the work is that BCD is a discriminating host, a property which can be

exploited in this analytical method as well as in others such as chromatography.

CHAPTER VII

CYCLODEXTRIN COMPLEXATION OF BARBITURATES IN AQUEOUS SOLUTION

Introduction

This study involves a complexation reaction in which an otherwise CD-inactive molecule can be made CD-active and the analyte included in the library of those substances which can be determined by CD. In order to perform a determination, the equilibrium constant for the association reaction must first be calculated. This is conveniently done using CD and the procedure is described. The inactive non-absorbing host molecule used is BCD. The choice of barbiturates as analytes illustrates where CD could be used in the direct determination of pharmaceuticals. Separation steps are not required.

Experimental Section

Barbituric acid and the seven 5,5'-disubstituted analogs, allo-, amo-, buta-, hexa-, pento-, pheno-, and seco- were obtained from Sigma Chemical Co. The 5,5'-diethyl barbital was obtained from Mallinckrodt Inc. The pharmaceutical dispensary product seconal sodium (Eli Lilly) was obtained in capsular form from the O.S.U. Student Health

Center. All of the standard chemicals were used without further purification. Because of the acid-base properties of the analytes and the effect of pH on the respective spectra, all of the aqueous solutions were buffered at 9.8±0.05 pH (pHydrion buffers, Micro Essential Laboratory).

Concentrations of the stock barbiturate solutions were prepared to be in the range 0.98 - 2.2 x 10^{-3} M. The concentration of BCD was incremented from 1.0 x 10^{-3} M to 1.0 x 10^{-2} M in a series of additions of the solid material so that BCD was always in excess compared with the barbiturates. With the host in excess, the complexation of the guest is maximized. Induced CD signals were observed at wavelengths in excess of 250 nm. Below 250 nm the S/N ratio was so low that the data were of no quantitative value.

Formation constants were calculated from spectral data taken at 15°C, 25°C, and 35°C, respectively for amo-, buta-, and pentobarbital. Temperatures were controlled to ±0.1°C using water circulated around the cuvette from an external thermostat (Haake, Model A81).

Results and Discussion

The general structural formula for the barbital analogs is shown in Figure 23. All are 5,5'-disubstituted derivatives of barbituric acid and only hexabarbital has further substitution, namely a methyl group on the nitrogen in position one. The logarithms of first acid ionization constants for all analogs, except barbituric acid, are in

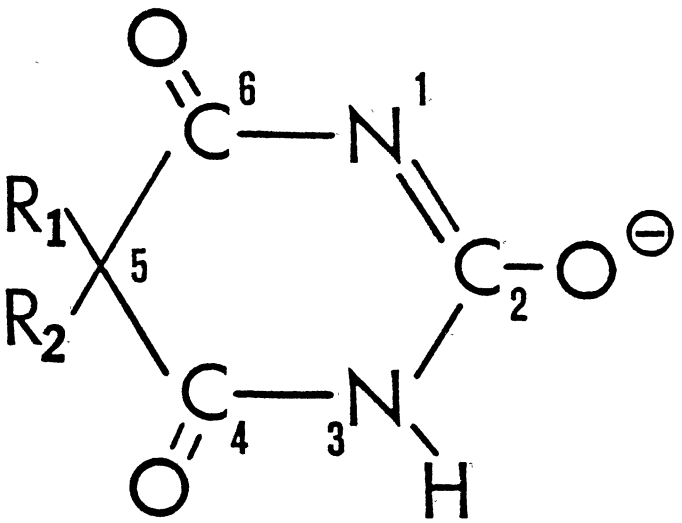


Figure 23. General Structural Formula for the Barbitals

the range 7 - 8.5, so the only species in pH 9.8 buffer is the enolized monoanion. The pK value for the ionization of the second proton is in excess of 13 (53). Induced CD spectra are shown in Figure 24 for secobarbital and phenobarbital. Spectra for all the compounds, except phenobarbital, are the same as the spectrum of secobarbital. The spectrum of phenobarbital shows the normal triplet for the aromatic ring $\pi^* \longleftarrow \pi^b$ transition. It is possible to distinguish phenobarbital from all the others; however distinction among the others by induced CD is not possible. No CD spectrum is obtained for barbituric acid either under these experimental conditions or in acid media. UV-vis spectra of secobarbital and phenobarbital are shown in Figure 25.

The resultant K values are given in Table V. Previous results for the complexation of phenobarbital with BCD are 4090 in pH 9.2 (49,54) and 1098 in water (55). Since the calculation of K is very sensitive to small changes in θ_{DS}, the differences are presumably due to instrument improvements. High pH change effected loss of signal intensity.

If a nominal subtraction is made for the contributions from the aromatic transitions to the CD spectrum for phenobarbital, the resultant spectrum is analogous to that for the other derivatives. This and the fact that the BCD core is hydrophobic might support the suggestion that one of the substituents on position 5 is bonded to BCD on

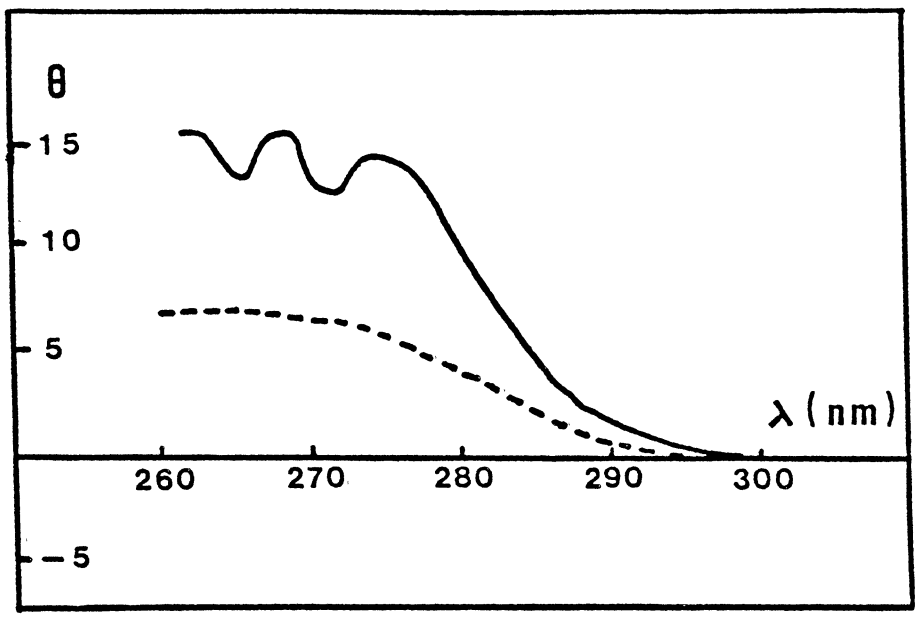


Figure 24. Induced CD Spectra for Phenobarbital (——) and Secobarbital (----) in Aqueous pH 9.8 Buffered BCD Solution

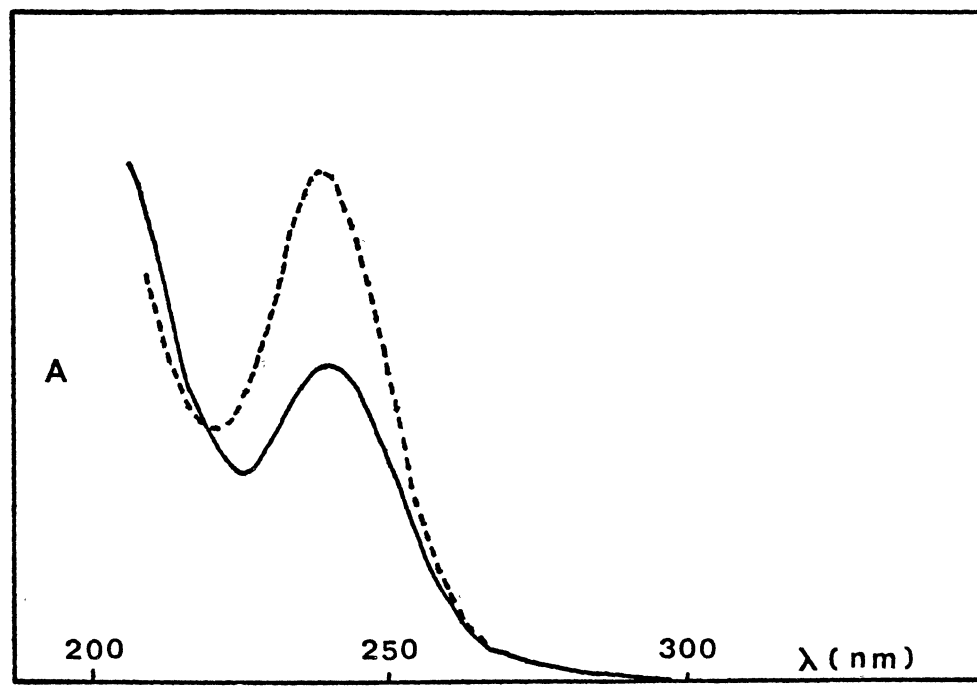


Figure 25. UV Spectra for Secobarbital (----) and Phenobarbital (——) in pH 9.2 Buffer

TABLE V

FORMATION CONSTANTS FOR BCD-BARBITAL COMPLEXES

compound	R_1	R_2	K	θ_{DS}
barbital	CH_3CH_2	CH_3CH_2	29	10.6
butabarbital	CH_3CH_2	$CH_3CH_2CH(CH_3)$	155	8.7
pentobarbital	CH_3CH_2	$CH_3CH_2CH_2CH(CH_3)$	318	7.6
amobarbital	CH_3CH_2	$CH_3CH(CH_3)CH_2CH_2$	554	18.8
phenobarbital	CH_3CH_2	C_6H_5	135	15.5
allobarbital	$CH_2=CHCH_2$	$CH_2=CHCH_2$	22	6.2
secobarbital	$CH_2=CHCH_2$	$CH_3CH_2CH_2CH(CH_3)$	229	6.9
hexobarbital	CH_3	C_6H_{11}	156	27.1
barbituric acid	H	H	0	0

complexation. The suggestion is further substantiated by the observation that the barbituric acid monoanion is not complexed or the K value is too small to measure. From Table V, when the R_1 group remains the same (e.g. CH_3CH_2-) the K values increase and θ_{DS} values decrease with the increase in length of the R_2 group. K values are increased ten times from barbital to pentobarbital and from allobarbital to secobarbital.

K values are logarithmically related to ΔG^o the standard free energy change for the complexation process. These alone do not allow for a thermodynamic interpretation of the interaction. Enthalpy changes were calculated for butabarbital, pentobarbital, amobarbital by calculating K as a function of temperature. These compounds have the same R_1 groups, so it is possible to investigate the effects of increasing the chain length in R_2. The thermodynamic parameters (ΔG^o , ΔH, and ΔS at 25^oC) are shown Table VI. K values were measured at three temperatures. θ_{DS} values were not fixed but were solved for independently in the solution of equation 4.4 at each temperature. Good correspondences in θ_{DS} were obtained for each analyte at all three temperatures. The value of $-\Delta H$ is calculated using $d(\ln K)/d(1/T) \times R$. The entropy change was calculated from the Gibbs equation $\Delta S = \Delta H - \Delta G/T$ (56).

All complexations are exothermic as previously observed and have been interpreted in terms of an extensive solvent rearrangement to a more ordered system which includes

TABLE VI

THERMODYNAMIC PARAMETERS FOR BCD-BARBITAL COMPLEXES

T, ^{o}C	K	ΔG^{o}, kcal/mol	ΔH, kcal/mol	ΔS, cal/(mol.deg)
		Butabarbital		
15	193			
25	155	-2.99	-5.30	-7.7
35	107			
		Pentobarbital		
15	505			
25	318	-3.41	-6.52	-10.4
35	246			
		Amobarbital		
15	719			
25	554	-3.74	-4.02	-0.9
35	461			

perhaps the release of "high energy" water coordinated in the center of cyclic structure. A negative entropy change supports this interpretation. Bearing in mind the hydrophobic character of the complexation site on the host, the preference of BCD for pentobarbital over butabarbital may reflect a greater contribution to the solvent reorganization from the longer R_2. Surprisingly, perhaps the stabilities of the cyclic R_2 derivatives are intermediate in comparison. The θ_{DS} values are, however, substantially greater which might explain the historically earlier recognition of aromatic BCD complexes. The range in K values suggests that BCD, in the solid state for example, might chromatographically separate a series of aliphatic alkyl anions.

In the analytical application, seconal sodium in secobarbital was determined by CD. Two sampling techniques were used. One capsule (Eli Lilly, F 40) of weight around 200 mg, has 100 mg of seconal sodium. In the first procedure an entire capsule (~ 50 % seconal) was dissolved in 10 ml of pH 9.8 buffer. After centrifugation 0.1 ml portions of this were diluted to 10 ml with a standard buffer-BCD solution, and used for CD spectral measurements. The assay was within $\pm$0.4 % of the prescribed amount. In the second procedure individually weighed samples (~2 mg) were taken and dissolved in a 10 ml portion of the standard buffer-BCD stock solution. The assay was within $\pm$1.0 % from the prescription amount.

It has been demonstrated that CD can effectively be used to measure the formation constants for complexation reactions and the procedure is simple because the spectra are relatively uncomplicated. The method can also be used in quantitative applications which may be of value as a quality control procedure.

CHAPTER VIII

DETERMINATION OF NICOTINE IN TOBACCO

Introduction

In this work we have characterized the CD spectrum of S-(-)-nicotine in 0.05 N methanolic-KOH solutions and have determined the total nicotine alkaloid content of a few cigarettes and smokeless tobaccos. These experiments are preliminary to later studies of the nicotine content in tobacco smoke, in pesticides, and in biological fluids, and to a full analogous study of the cannabinols in marihuana (Chapter IX).

Determination of nicotine is very important in the tobacco induatry and in the area of toxicology. Many methods have been developed for this purpose including chromatography, mass spectrometry, UV-vis spectrophotometry, and polarimetry.

An earlier CD study of nicotine-type alkaloids was done for the purposes of a preferred conformation study (57). Molar ellipticities of nicotine and related analogs were reported. The evidence suggests that the analogs are indistinguishable from nicotine by CD. Results are reported in this work, therefore, as total nicotine.

Experimental Section

S-(-)-nicotine, as the liquid free base, was obtained from Eastman Kodak and used without further purification. CD spectra for the standard were measured for the analyte dissolved in 0.05 N methanolic (Fisher Sci. Co.)-KOH (Baker Chemical Co.). Extractions from tobacco leaves were made using the same solvent system. Samples of smokeless tobaccos and cigarettes were chosen at random from local convenience stores.

Sample weights used for the smokeless tobacco study were about 200 mg. Whole cigarettes were used whose average net weight (filters removed where appropriate) were in the range of 550-850 mg. All determinations were made in triplicate. For 0.05 N methanolic-KOH extractions the leaves were shaken for 1 hour in an ultrasonic cleaner (58). The extraction efficiency was compared against the results obtained after a 15 hour distillation experiment using a Soxhlet extractor. After the sample had been centrifuged to remove suspended material, the supernatant solution was transferred directly to the cell and the spectrum run. A stock standard solution was approximately 4×10^{-2} M. The standard was dissolved and diluted with 0.05N methanolic-KOH.

Prior to the extraction step samples were placed for 48 hours in a humidifier in order to obtain a more uniform moisture content. An excellent correspondence between the ultrasonic procedure and the Soxhlet procedure was achieved

after the leaves had been allowed to equilibrate in an environment of 81 % relative humidity maintained by a saturated solution of ammonium sulfate in a sealed desiccator. In contrast when P_2O_5 was used as the desiccant to thoroughly dry the specimens the extraction efficiency was about 10 % of that accomplished by Soxhlet extraction.

Results and Discussion

The CD spectrum for S-(-)-nicotine in 0.05 N methanolic-KOH and the molecular structure are given in Figure 26. CD spectral characteristics for nicotine in 0.05 N methanolic-KOH and other solvents (59) are included as Table VII. The wavelengths of the negative maxima, λ^-_{max}, correspond reasonably well with the anticipated absorption maxima for a substituted aromatic ring chromophore. Cross-over points and the positive Cotton band characteristics are in good agreement with an earlier study (57). The molar ellipticity coefficients[θ] were calculated from the slopes of the corresponding correlation curves of the experimentally observed ellipticities, ψ_{exp} plotted against the molar concentration of nicotine. The molar ellipticity [θ] is defined in this way to conform with the analogous molar absorptivity since CD is correctly identified as a modification of absorption spectrophotometry. This definition is not to be confused with molecular ellipticity for which values of [θ] are one hundred times greater (Chapter III). Correlation

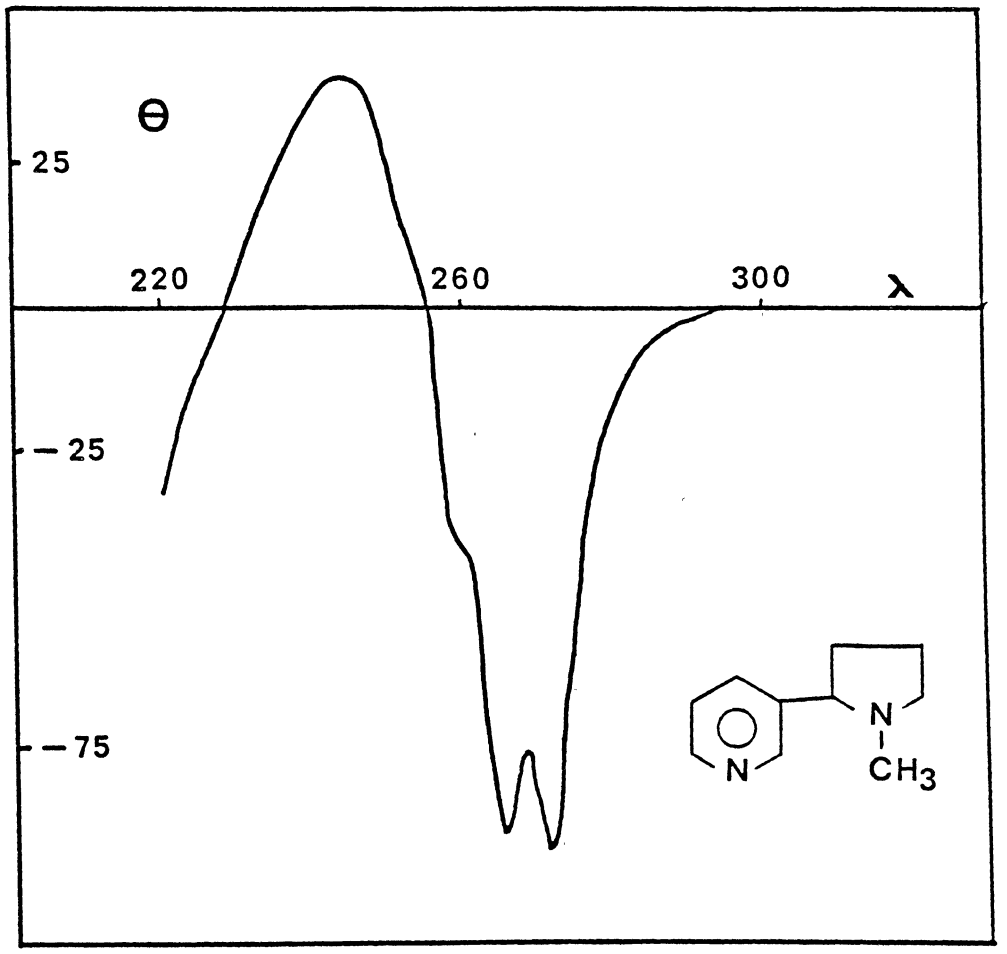


Figure 26. Molecular Structure and CD Spectrum for S-(-)-Nicotine in Methanolic-KOH

TABLE VII

CD SPECTRAL CHARACTERISTICS FOR NICOTINE

Solvent	λ^{o}(nm)	$\lambda^{+}_{max}(\theta)$	λ^{o}	$\lambda^{-}_{max}(\theta)$	$\lambda^{-}_{max}(\theta)$
2-propanol	222	236(41.0)	244	255(-77.8)	261(-87.6)
pH 7.2 buffer	220	236(34.2)	247	264(-63.3)	267(-62.5)
pH 8.2 buffer	220	240(25.0)	249	264(-64.4)	268(-60.0)
methanolic-KOH	229	243(37.0)	255	265(-91.5)	271.5(-92.2)

coefficients for the data for the three maxima are $\pm$0.999.

The instrumental limitations of the technique set the linear dynamic range for a 1 cm sample cell at an upper concentration limit of 10^{-3} M and a minimum detectable concentration around 10^{-6} M, dependent upon the wavelength chosen for quantitation. This figure is well within the predictable range for the determination of nicotine in either tobacco smoke or in biological specimens. The limit could be further improved by expanding the sensitivity scale of the instrument. The present figures were calculated from data measured at a sensitivity of 2 m^{o}/cm ellipticity. An additional ten fold extrapolation is easily accessible.

For tobacco, extraction with a strong basic solution, even an alcoholic-KOH solution is recommended (60). 0.05 N methanolic-KOH was used for the extraction of nicotine from the chopped tobacco leaves because after equilibrating the specimen for water content at 81 % relative humidity, the moist pliable leaf was susceptible to efficient extraction. Multiple extractions, performed simultaneously, effectively reduces the time for determinations to less than 10 minutes per sample. An earlier attempt which used 2-propanol (61) produced a brittle leaf from which extraction was very inefficient.

An advantage which is inherent to the CD technique over UV spectrophotometry is illustrated in Figure 27 and 28. Superimposed on the UV spectra for nicotine is the UV spectrum for the 0.05 N methanolic-KOH extract of Skoal

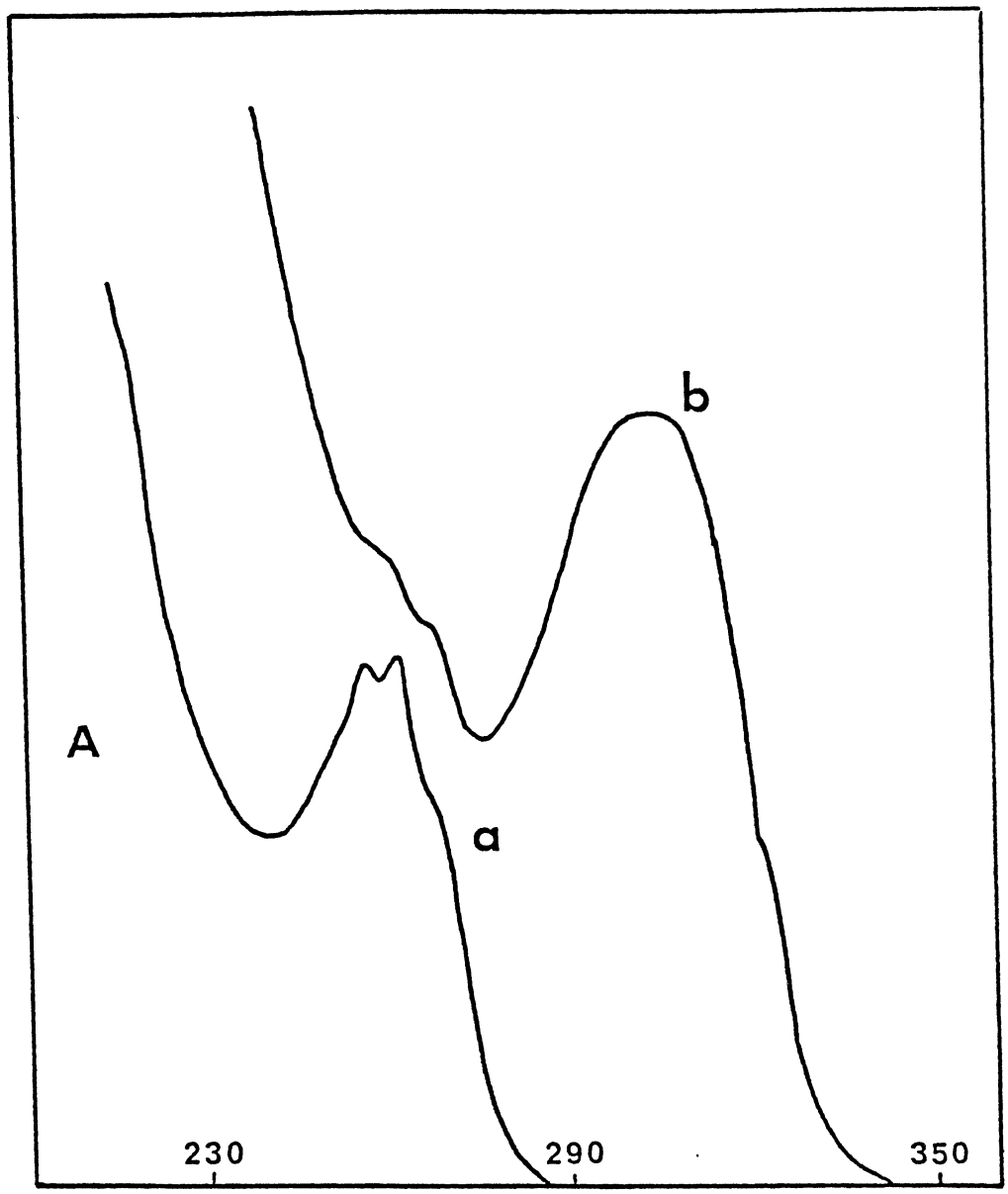


Figure 27. UV Spectra for (a) Nicotine (b) Skoal Extract

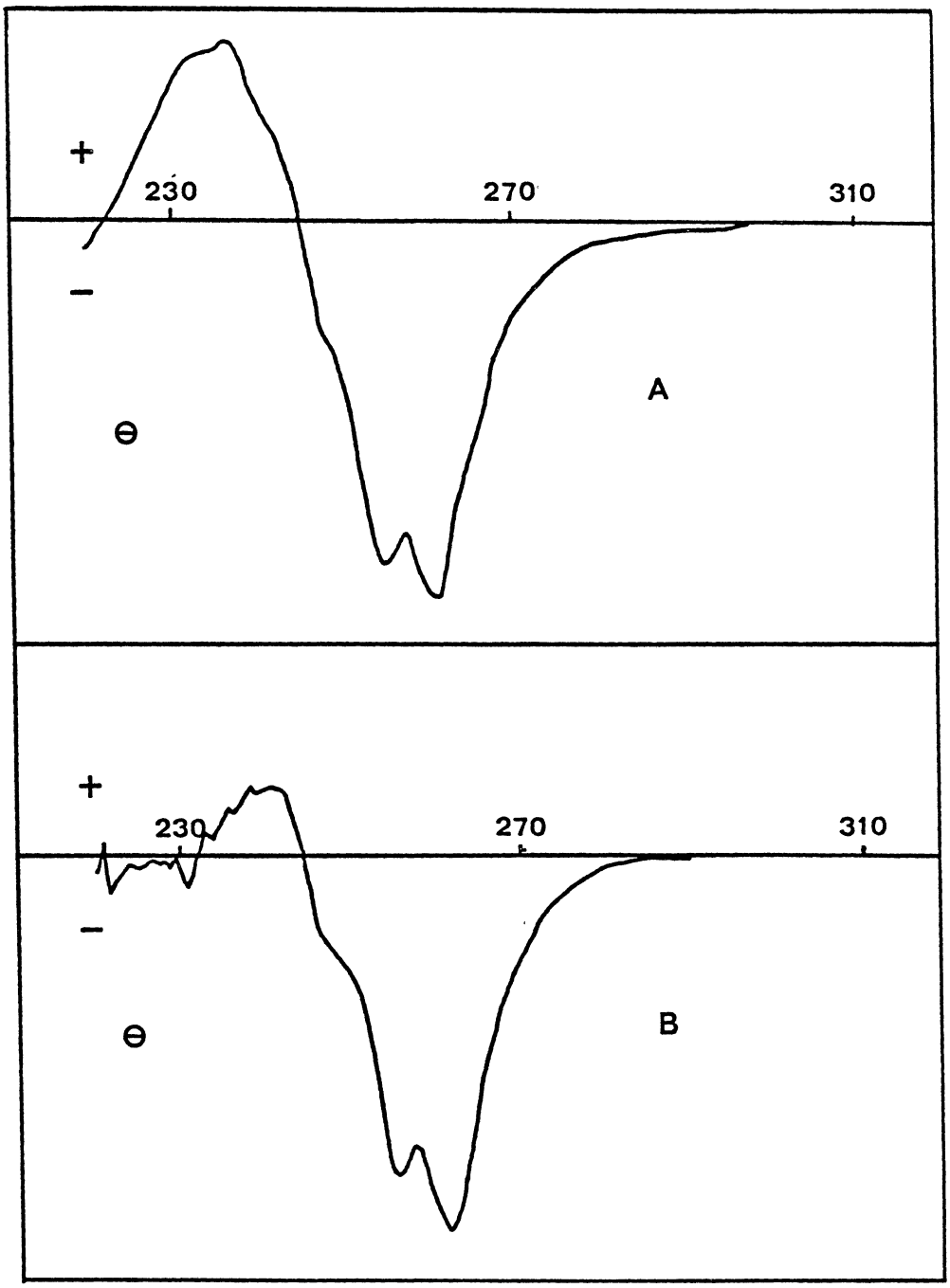


Figure 28. CD Spectra for (A) Nicotine (B) Skoal Extract

smokeless tobacco, Figure 27. In contrast, in the CD spectral comparison made for the identical solutions (Figure 28), nicotine appears as the "only" CD active component in the matrix and its determination can be made directly. Data at the positive maximum were not used for analysis because of the obvious deterioration in the signal to noise ratio.

Table VIII shows the data for nicotine determinations in cigarettes and smokeless tobaccos. Values are averages calculated from the data taken at the wavelengths of the negative maxima, and are reported as percent by weight. Only the 81 % RH values were determined by CD detection. Results reported for the dry specimens were calculated after measuring the moisture lost on standing over P_2O_5 until constant weight was obtained. Results are the average of at least three independent determinations. Considering the inhomogeneous distribution of nicotine within the chopped leaf blends, the reproducibility is $\pm$0.2%.

Most determinations of nicotine content in the many forms of tobacco reported in the literature and in annual reports by the Federal Trade Commission (62) deal with the content in smoke. Only a few references to the determination of nicotine contents in chopped tobacco leaf are available (63,64). Direct comparisons among the many methods are difficult because of the variety of blends and smoking conditions. For cigarettes, good agreement is obtained with earlier literature results for chopped leaves where values on the order of 2 %(W/W) were reported (63,64).

TABLE VIII

NICOTINE CONTENT OF COMMERCIAL TOBACCO FORMS

BRAND[R]	MANUFACTURER (description)	METH-KOH (81% RH)[a]	METH-KOH (dry)
NOW	Reynolds Tob. Co. (F, S, 85mm)[b]	1.5	1.7
BARCLAY	Brown Williamson Corp. (F,S,M, 85 mm)[b]	1.5	1.7
CAMEL	Reynolds Tob. Co. (R, NF, S, 70mm)[b]	1.6	1.7
MARLBORO	Phillip Morris Inc. (F, S, 85 mm)[b]	1.4	1.6
ULTRALIGHT	Gary Tob. Co. (F, S, 85 mm)[b]	1.3	1.5
SKOAL	U. S. Tob. Co. (WG)[b]	1.3	2.2
HAWKEN	Conwood Tob. Co. (WG)[b]	0.4	0.5
KODIAK	Conwood Tob. Co. (WG)[b]	1.0	2.0
SILVERCREEK	Silvercreek Tob. Co. (WG)[b]	1.7	2.6

a. Results expressed as % nicotine (W/W).

b. F=filter; S=softpack; M=menthol; R=regular; NF=no filter; WG=wintergreen

Neither flavor nor sweeteners currently added to products to make them more palatable interfere with the direct CD detection although these may have been co-extracted. For example, the compounds responsible for the very dominant band from 290-325 nm in the absorption spectrum (Figure 27) are CD transparent and non-interfering.

In summary it has been demonstrated that CD can be used very effectively for the determination of nicotine contents in chopped tobacco leaves after a simple and direct single extraction into 0.05 N methanolic-KOH. The method is both economical and time effective because internal standards are not required, detection is free from interferences, and instrument calibration is necessary only on a daily basis at most. Calibration curve can be to achieve detection limits on the level of tens of nanograms per mL. However, the method does not distinguish among nicotine and its structural analogs and results must be reported as total nicotine. The analogs generally account for less than 5 % of the total nicotine fraction, and the lack of discrimination is therefore of little consequence except when the analogs are detrimental to the quality of the product.

CHAPTER IX

DETERMINATION OF CANNABINOIDS

Introduction

Marihuana consumption in the United States has increased dramatically in the last decade. Numerous methods have been used and are still in use for the identification of cannabinoids in marihuana (65). For a qualitative method, a number of color spot tests are used (66,67). For a quantitative method, gas chromatography (68), liquid chromatography (69), GC/MS (70,71) are most often the methods of choice. Sample preparation usually involves solvent extraction, volume reduction, and derivatization of the thermally unstable analogs or metabolites. Other more sensitive methods are employed in the analyses of biological specimens (72).

The great majority of the compounds extracted from the manicured leaf of marihuana have been identified. The list contains several dozen structurally similar compounds with the same chemical nucleus. These are generally termed "cannabinoids". The prevailing consensus of opinion is that the sole pharmacologically active ingredient is trans-delta 9-tetrahydrocannabinol (THC). Usually however an analysis encompasses more than just the determination of this one

analyte. Phenotype ratios of cannabidiol (CBD) to THC, or CBD to THC plus cannabinol (CBN) have been defined and correlated with both the potency and the geographical region of origin of the native plants (73). Plants high in THC content comprise the phenotype I and are considered "drug" type marihuana; those relatively low in THC are phenotype II and referred to as "fiber" marihuana.

From these remarks it would appear that the problems associated with marihuana analyses are satisfactorily resolved but some still exist. For example the clinically accepted EMIT test (74) is not without controversy, because of possible false positives, and subsequent confirmation is recommended. Another is that if fewer than five spots are developed in TLC screening tests, confirmation by GC/MS is frequently required (75). One of the objects of this work was to develop a new rapid quantitative screening test for marihuana and cannabinoids in which circular dichroism (CD) is the method of choice. The method was expected to distinguish among different plants after a simple solvent extraction, and to determine the amounts of THC and other chiral analytes without the need for a chromatographic separation of any kind. Precedent for this kind of application is found in the recent work on the determination of nicotine in chopped tobacco leaves, Chapter VIII (59). A review of the identities of possible interfering chiral analogs of THC (76) suggests that there may be as many as five. Of those five, GC studies indicate that four may be

present in sufficient abundance to complicate the analysis. With these favorable odds, CD would be measurably superior to absorption spectrophotometry if separation is not a prerequisite to determination, and more rapid than GC/MS in quantitative work in routine repetitive analyses.

The four chiral substances of particular interest based upon the GC identifications are THC, CBD, and their respective aromatically substituted carboxylic acid derivatives, especially the 4'-substituent, THCA and CBDA. The acids are thermally unstable and must be derivatized before they can be identified as such in GC. Decarboxylation produces the parent THC and CBD molecules. A metabolic pathway believed to occur in both the living plant and on subsequent ageing after harvesting is THCA to THC to CBN. CBN is achiral and therefore not amenable to CD detection, but at the same time does not interfere with the other determination. Most often the separate determination of the weight percent of THCA and other acids is excluded in routine GC analyses, and the original amount of THC is estimated by summing the results for THC and CBN.

Experimental Section

Drug standards for delta 8- and delta 9-THC and CBD were obtained from Sigma Chemical Co. The THC derivatives are available only in an alcoholic solution (100 mg/mL). Standards for the carboxylic acid analogs are not available in sufficient quantities for spectral calibration purposes

at this time. The quantitative segment of the present work is limited therefore to a study of only THC and CBD.

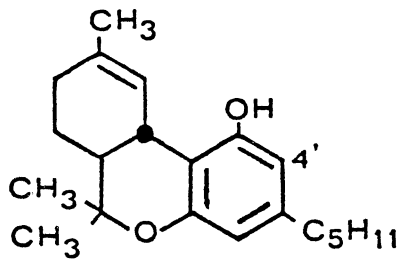
Marihuana sample were obtained from State Agencies. Since the samples had been confiscated, their precise history was unknown. The samples varied in age and levels of dryness. Sampling was done by choosing only certain parts of the plants in some cases, e. g. only flowering tops, and in others, by simply chopping entire plants.

Solvent extractions were made by shaking the specimens, which weighed anywhere from 20 to 500 mg in 20 mL of chloroform (Fisher Chemical Co.) for 30 minutes at room temperature in an ultrasonic cleaner. Although other non-polar solvents have been employed, chloroform is the extraction solvent most frequently recommended for GC studies. Completeness of the extraction was confirmed by comparing the CD spectra for these solutions with spectra for the extracts obtained after refluxing over a period of hours. Separation of the acid fraction from the remainder was done by extracting twice into equal aliquots of 1.0 M NaOH. The organic phase was diluted by equal aliquots of 0.05 N methanolic-KOH. The same procedure was followed for the other freshly chopped green leafy materials used in this study, i.e. marjoram, parsley, oregano, bayleaf, dillweed, sage, cumin, basil, chervil, thyme, tobacco, and hop. Appropriate dilutions of the extracts were made in order to keep absorptions within the dynamic range of the CD instrument.

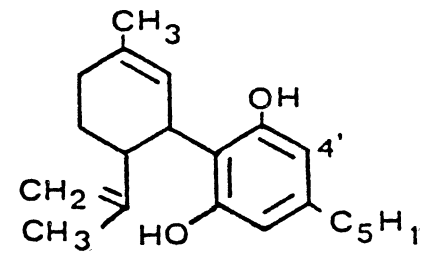
Results and discussion

The molecular structures for trans-delta 9-THC and CBD are shown in Figure 29. The acid analogs are substituted in position 4' with the -COOH functional group. UV spectra for both compounds show relatively weak absorptions with maxima at approximately 273 and 282 nm. Other analogs absorb similarly in the same wavelength range. A second strong absorption band is observed around 200 - 220 nm. These absorptions are typical of the aromatic chromophore. The spectrum of CBD in chloroform shows a fairly intense signal, while THC has a very weak band at the same sensitivity. The addition of an equal aliquot of 0.05 N methanloic-KOH enhances the CD signals for both compounds. A conceivable interpretation for the red shift and increased ellipticity in basic medium is that the phenolic group at position 5 is fully ionized. Analogous spectral changes are observed for morphine (77) and other phenolic opiates. The interpretation is supported by the observation that the spectral change is reversible on acidification and back extraction into chloroform.

Spectra for the standards for delta 9-THC and CBD standard are shown in figure 30, Cotton bands occur in the spectral range of the aromatic absorptions. The bands are of opposite polarity. The molar ellipticities at the appropriate wavelength maxima, measured from the slopes of the calibration curves, are -129.3 (293 nm) and -89.5 (282.5



trans-Δ^9-THC



CBD

Figure 29. Molecular Structures for Trans-delta 9-THC and CBD

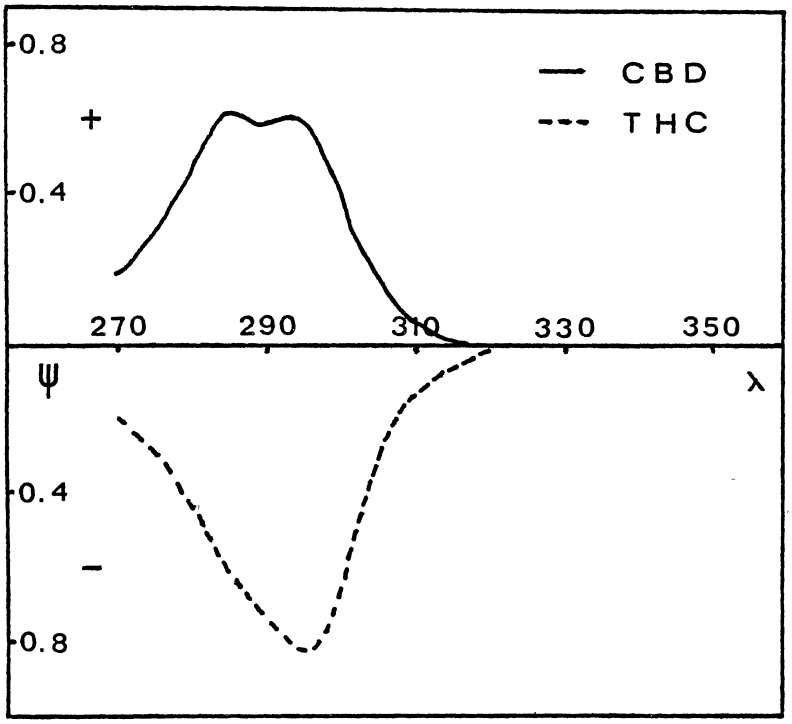


Figure 30. Standard CD Spectra for THC (6.36×10^{-5}M) and CBD (1.05×10^{-4}M) in a 50:50 Mixture of Chloroform and 0.05N KOH in Methanol

nm)and +56.9 (293 nm) and +57.9 (283.5 nm) respectively.

The curve A in Figure 31 is a spectrum typical of the unseparated chloroform extracts of marihuana. Curve B is the spectrum for the aqueous 1.0 M NaOH extraction from chloroform believed to contain only the fully ionized carboxylic acid derivatives since THC and CBD and essentially insoluble in aqueous solution. Curve C is the CD spectrum for the residual chloroform extract to which an equal aliquot of methanolic-KOH has been added. Data from the spectra for these solutions were analyzed as binary mixtures of THC and CBD. Curve D is the spectrum for all four analytes in a 50:50 mixture of chloroform and methanolic-KOH. All four spectra were obtained for the same sample of marihuana in order to exactly compare the solvent effects. Curve D shows the enhancement effect produced by adding 0.05 N methanolic-KOH. In this procedure, THC and CBD are essentially insoluble in aqueous solution, so a complete separation is effected.

Compared to marihuana, the CD spectra of the other leafy materials are relatively uninteresting (Figure 32, 33, and 34). Hops were included in the study because of the reputed morphological similarity to marihuana. Only those five plants which are CD active under the extraction conditions used are shown. The spectrum for nicotine (Figure 33) extracted from a fresh green tobacco leaf is almost exactly that already reported for extracts from commercial cigarette and smokeless tobacco (59). For hops,

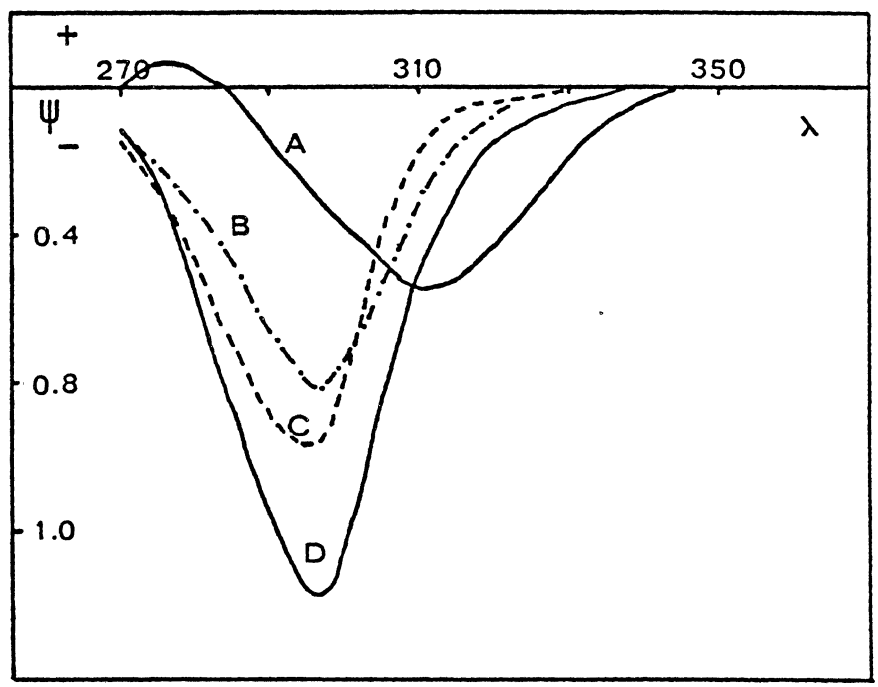


Figure 31. CD Spectra for Marihuana Extracts: (A) THC, CBD, THCA, and CBDA in Chloroform (B) THC and CBD in Chloroform and Methanolic-KOH after Extraction of THCA and CBDA (C) THCA and CBDA in Aqueous 1.0M NaOH (D) THC, CBD, THCA, and CBDA in a 50:50 Mixture of Chloroform and Methanolic-KOH

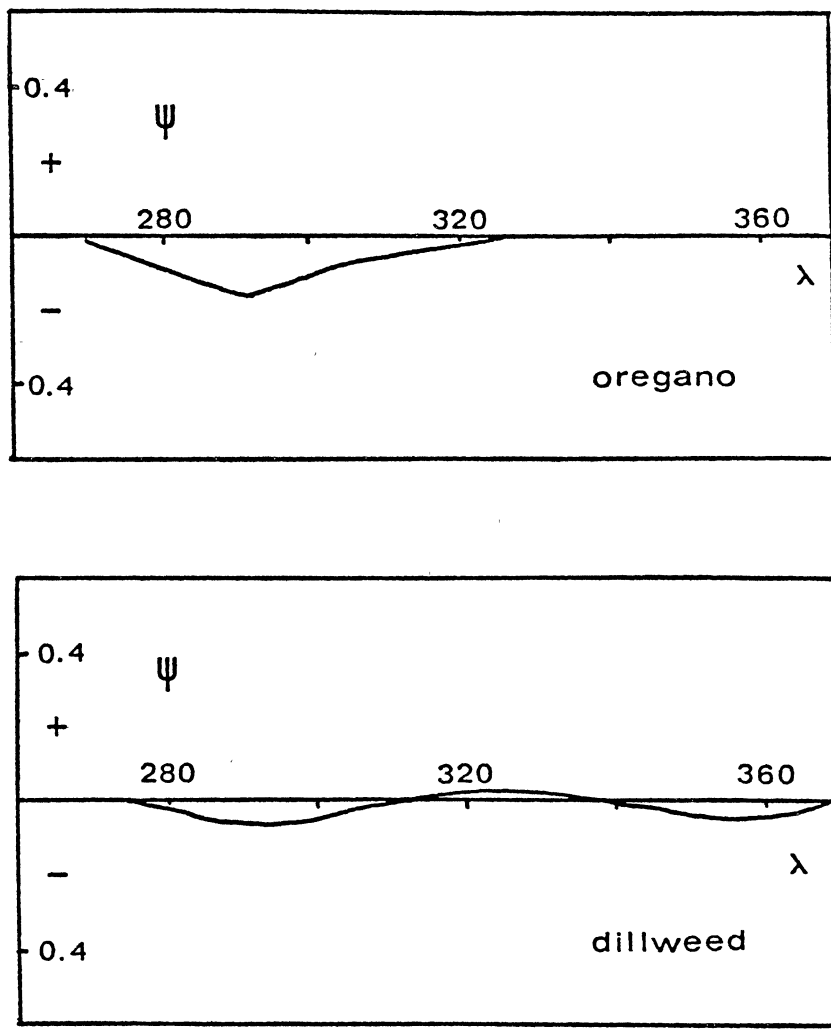


Figure 32. CD Spectra for Oregano (0.1123g) and Dillweed (0.0926g) after Extraction into Chloroform and the Addition of an Equal Volume Aliquot of 0.05N KOH in Methanol

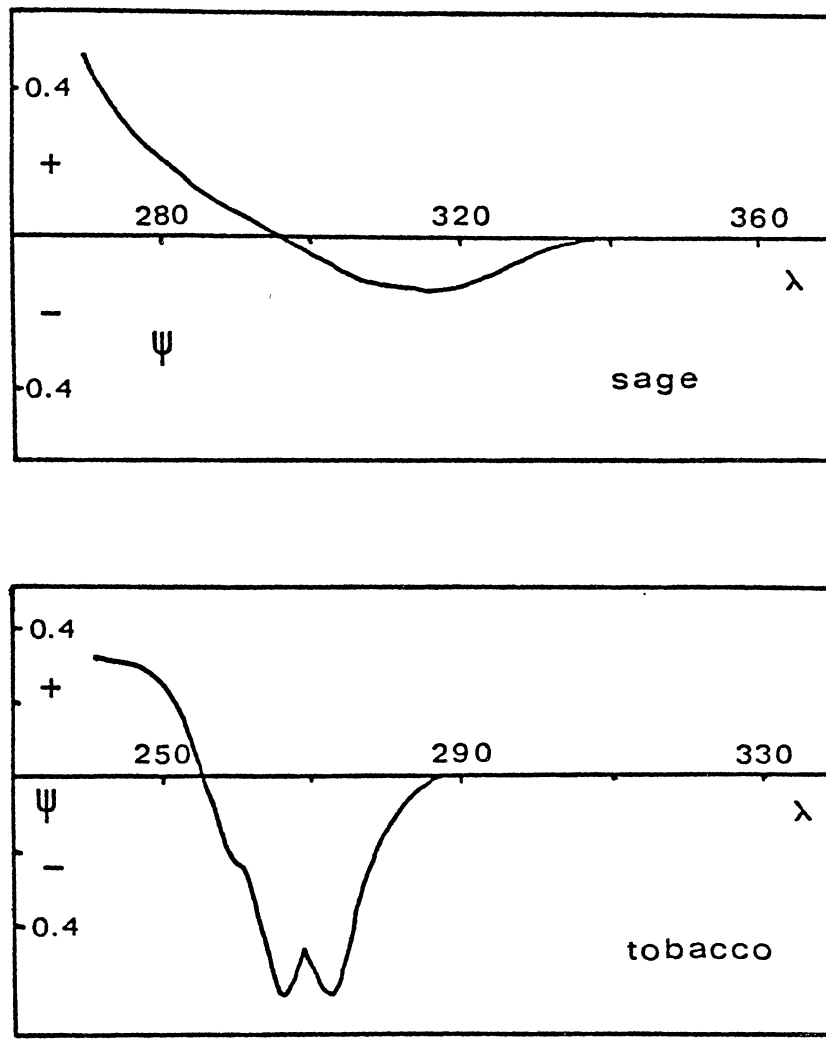


Figure 33. CD Spectra for Sage (0.1061g) and Tobacco (0.3615g) after Extraction into Chloroform and the Addition of an Equal Volume Aliquot of 0.05N KOH in Methanol

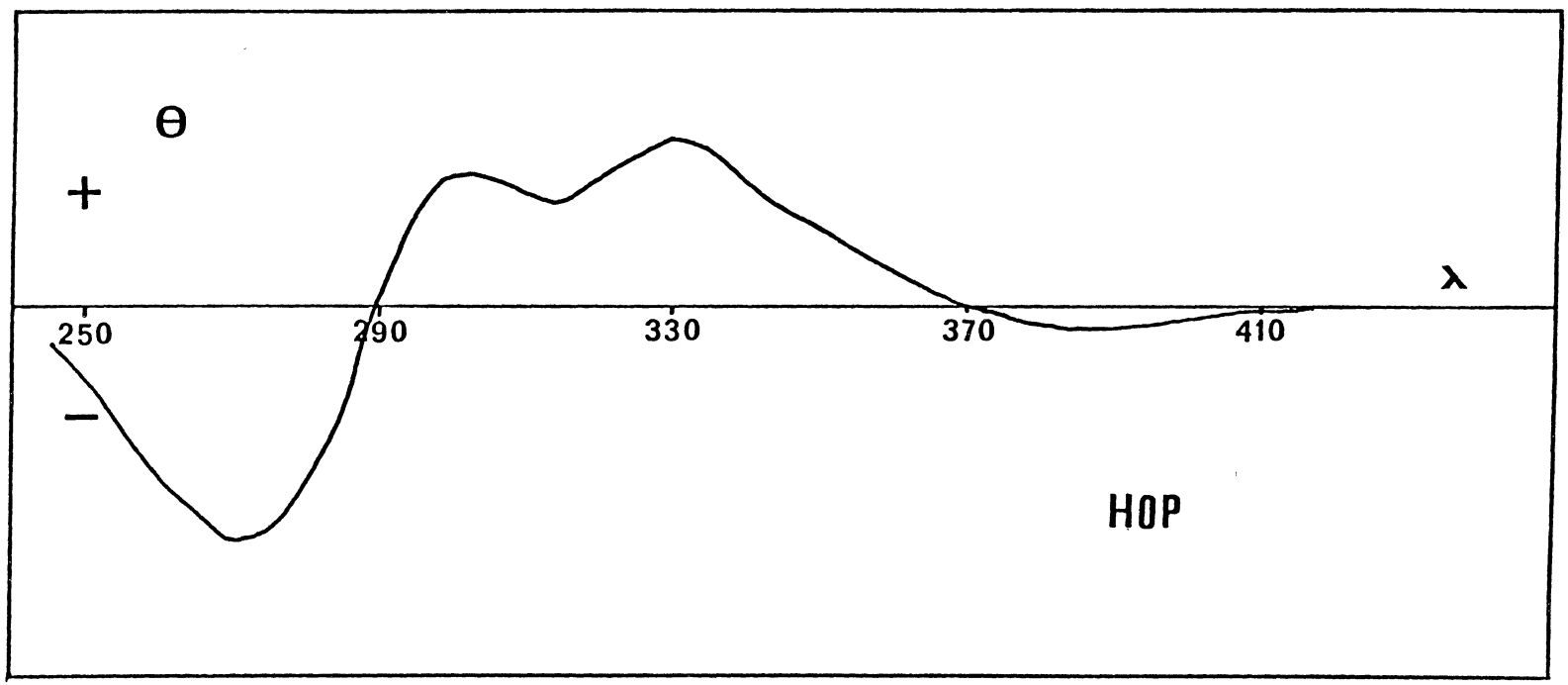


Figure 34. CD Spectrum for Hops (0.0492g) after Extraction into Chloroform and the Addition of an Equal Volume Aliquot of 0.05N KOH in Methanol

extracts from fresh leaves yielded no CD spectrum. The spectrum in Figure 34 obtained from the extract from the berries. The spectrum for hops is very distinct and offers the future development possibility for quality control in the beer manufacturing process. Differences among these spectra and that for marihuana are so great that conformation of the latter would be elementary. Only sage shows any possibility of being mistaken for marihuana. The first objective of the study is satisfied.

Without satisfactory information to the contrary, we have proceeded with the quantitative work on the assumption that four cannabinoid analytes are extracted, and that only THC and CBD remain in the chloroform phase after extraction. In the mathematical procedure used for multicomponent analyses, ellipticity data were taken at every nanometer over the absorption range in the spectra for both the standard solutions and for the extracts. A least squares fit of the spectra for the unknowns to a weighted algebraic sum of the spectra of the standards was made in a simple microcomputer program which minimized the coefficients in the solution of a set of linear equations.

Standard spectra, normalized to 1.0 M, are stored on a library disk and recalled by way of a search program when required. Each library entry is typically the average of almost twenty independent measurements. The simultaneous determination was first tested on two prepared mixtures of known composition, where the ratios of THC to CBD were 0.27

and 2.7 respectively. When analyzed as binary mixtures, relative errors were less than 1% for both components. When treated as either CBD alone or as THC alone, these being the analytes in excess, relative errors were 54% and 18% respectively. Confidence in the mathematical procedure is obtained from the very good correspondence between the experimental and calculated results for the mixtures. Results for seven marihuana specimens are given in Table IX. Variations among the results from the same specimens reflect the uncertainty in withdrawing uniform representative samples.

Since the prior history of the samples is unknown, an exact comparison with earlier results from other methods is not possible. Figures around a few percent for both constituents is however consistent with previous results. In spite of this apparently good comparison, it should be emphasized that whereas the GC results often report a total THC and CBD, which includes that produced by thermal decarboxylation of the acid analogs, the CD results do not include these analytes. The almost equivalent experimental ellipticity values observed for the chloroform methanolic-KOH and the aqueous extract, Figure 31, implies a total weight percentage for the two acids somewhat similar to that for THCA and CBDA. However it should be borne in mind that the experimental ellipticity is the product of the molar ellipticity coefficient and the analyte concentration, which separately are not necessarily equivalent to the

TABLE IX

DETERMINATIONS OF CANNABINOIDS IN MARIHUANA

Specimen	Organ	THC (wt.%)	CBD (wt.%)
Plant 1	leaves	0.67	0.75
	(chopped)	1.35	0.49
		1.48	0.42
Plant 2	leaves	0.59	0.40
	(chopped)	0.89	0.12
		2.18	1.06
Plant 3	seeds	~0	~0
Plant 4	flowering tops	2.48	2.90
		2.00	0.83
		2.10	2.32
Plant 5	flowering tops	2.31	0.94
		2.01	0.25
		1.27	0.05
Plant 6	whole plant	1.54	~0
	(chopped)	3.98	~0
		1.07	~0
rolled	whole plant	1.28	~0
cigarette	(chopped)	1.27	~0

values for THC and CBD. Furthermore it assumes that the Cotton effects are of opposite sign for THCA and CBDA, which may not be the case.

Results from gas chromatography have indicated lower total weight percentages for THCA and CBDA compared to THC and CBD which, if true for the present specimens, would imply larger values for the molar ellipticity coefficients for the acid forms. Assuming an arbitrary 1 % total weight for the acids in the original leaf, and using the experimental ellipticity maximum observed for an aqueous NaOH extract, the estimated molar ellipticity for the combined acid forms is -839. The calculation assumes negative ellipticities for both THCA and CBDA. In actual fact then a positive confirmatory test for marihuana which might use CD is the recognition of the presence of THCA and CBDA. Using the given, very approximate, figure for the molar ellipticity for these acids, one can calculate a minimum detectable concentration on the order of 25 ng/mL, which may will be in the range of detection for marihuana in a saliva swab or perhaps even in the breath of a recent user. No interferences are to be expected from proteins which would normally show Cotton bands at wavelengths less than 235 nm.

Without any quantitative information on the achiral analyte CBN available from CD determinations, phenotype ratios, as presently defined, can not be obtained from this work. The value of this ratio is open to question however

in that the GC procedure converts underivatized THCA and CBDA to THC and CBD, so the ratio does not accurately describe the composition that exists in the plant. Smoking however may effect the same conversion. THCA and CBDA can be determined by GC after derivatization if a standard is available, but not as rapidly as it could be done by CD. Attempts are being made in our laboratory to isolate and separate sufficient quantities of the acids. The ratios of THC to CBD that we have observed suggest that the plants are "drug" quality and perhaps grown south of the arbitrary 30° N latitude. The CD results also distribution in the various plant organs, showing flowering tops to be the most abundant reserve.

This work is another illustration of the selectivity potential of CD to identify particular chiral analytes in very complex mixture without the need for elaborate separation and standardization procedures. One could expect that a multitude of CD active, naturally occurring, substances are extracted from a plant, but the high relative abundance, and the high relative molar ellipticity coefficients, of nicotine in tobacco (59), and of the cannabinoids in this case, produce the necessary signal intensity for analytical determination.

CHAPTER X

DETERMINATION OF OPIUM ALKALOIDS

Introduction

Opium is the dried exudate of unripe seed capsules of the opium poppy (papaver somniferum) and is a complex mixture containing around 25 different alkaloids. Of the 25 alkaloids only 5 (morphine, codeine, thebaine, papaverine, noscapine) are present in significant quantities in crude opium (78). Among the 5 major components papaverine is not optically active and not determinable. A pharmaceutical concentrate commonly prescribed as a narcotic or analgesic is pantopon in which the alkaloid ratios are similar to what they are in opium. Identification and determination are frequently required in forensic and pharmaceutical laboratories. There are many methods for the identification and determination of opium; for example chemical spot tests, thin-layer chromatography (79), and gas (80) and liquid chromatography (81, 82). Chemical spot tests and TLC are at best of marginal qualitative value. GC methods require a degree of derivatization before they are quantitatively effective. The object of this study was to apply the multicomponent analysis program used successfully in the study of the cannabinoids to more than a binary mixture of

components (83). Once again separation was not to be a prerequisite to determination.

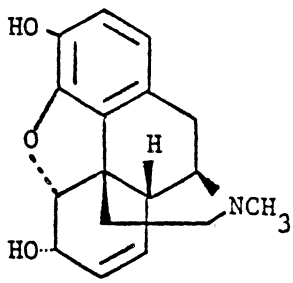
Experimental Section

Morphine sulfate, codeine, thebaine, and powdered Turkish opium were obtained from Mallinckrodt Inc. Pantopon was a gift from Hoffmann-LaRoche. Noscapine was obtained from Sigma Chemical Co. All of the standard chemicals were used without further purification. Because of the acid-base properties of the analytes (especially morphine) and the effect of pH on the respective spectra, all of the solutions were buffered at 9.2 $\pm$ 0.05 pH (phydrion Buffers, Micro Essential Laboratory).

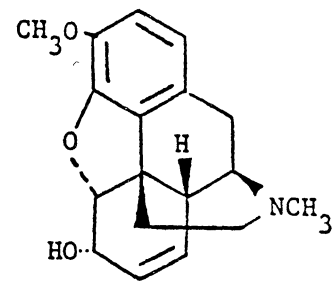
Extractions from opium and pantopon into buffer were done using ultrasonic agitation for 30 min. Insoluble materials were removed by centrifugation prior to collection of the spectral data. Sample sizes for opium and pantopon were 1.0 - 2.0 mg. Solvent aliquots used were 10 mL. Some difficulty was experienced with noscapine because of its limited solubility in base. Acid buffers could have been used, but the advantage of a positive signal for only morphine in basic buffer would both facilitate and improve the analysis.

Results and Discussion

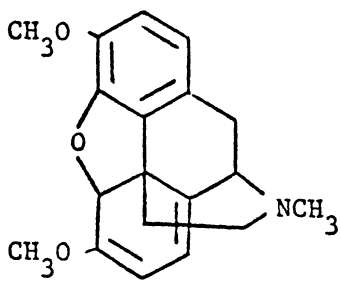
The molecular structures for morphine, codeine, thebaine, and noscapine are given in Figure 35. CD spectra



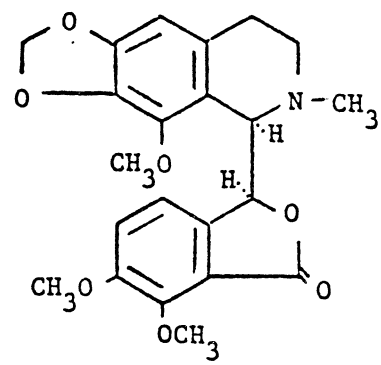
MORPHINE



CODEINE



THEBAINE



NOSCAPINE

Figure 35. Molecular Structures for Morphine, Codeine, Thebaine, and Noscapine

are given for morphine and codeine (Figure 36), and CD and UV spectra for thebaine are given in Figure 37. The spectrum of noscapine in water is shown in Figure 38. The θ values for the compounds at the respective band maxima are -10.88(286 nm) and 27.85 (302 nm) for morphine; -85.43 (286 nm) for codeine; -490.55 (286 nm) for thebaine; and -80.82 (315 nm) for noscapine. The value of thebaine is very large compared to the other components. Figure 39 shows the effect of pH on the UV and CD spectra for morphine. None of the other analytes exhibit any pH dependence for the CD spectra making morphine quite unique in this mixture. The negative and positive bands at wavelengths longer than 290 nm are particularly useful in mixture analyses.

As a preliminary precaution, analyses were made on prepared binary and ternary mixtures. Morphine and codeine were used in the binary mixture study and thebaine was added to these in the ternary mixture study. Results are shown in Table X. Good correspondences were obtained between the experimental and calculated results for these mixtures. In the ternary mixture solutions, concentrations were chosen which were consistent with literature reports for the various alkaloids in opium. Relative error on these determinations are larger than for the binary mixtures because the thebaine concentration is 20 times less than the concentration of morphine. The fact that thebaine is even detectable at this level is due to the relatively large molar ellipticity value. The CD spectra for the opium

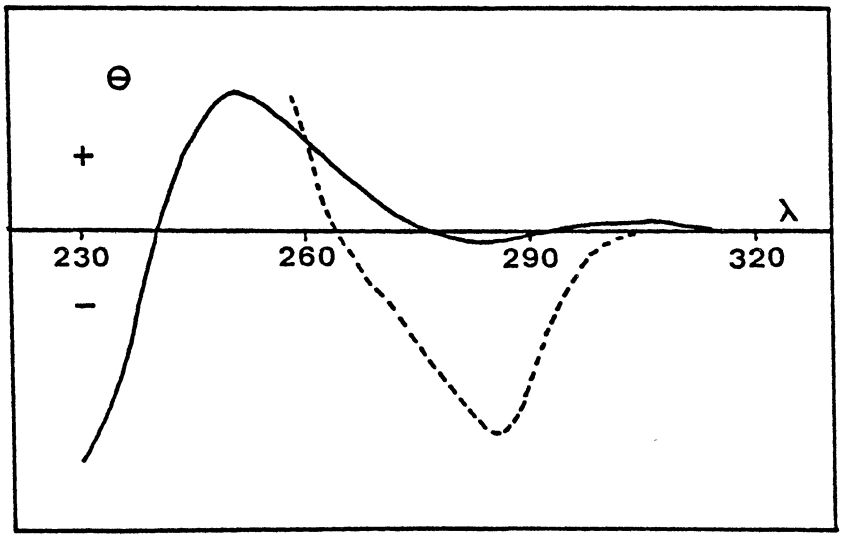


Figure 36. CD Spectra for Morphine (——) and Codeine (----) in pH 9.2 Buffer

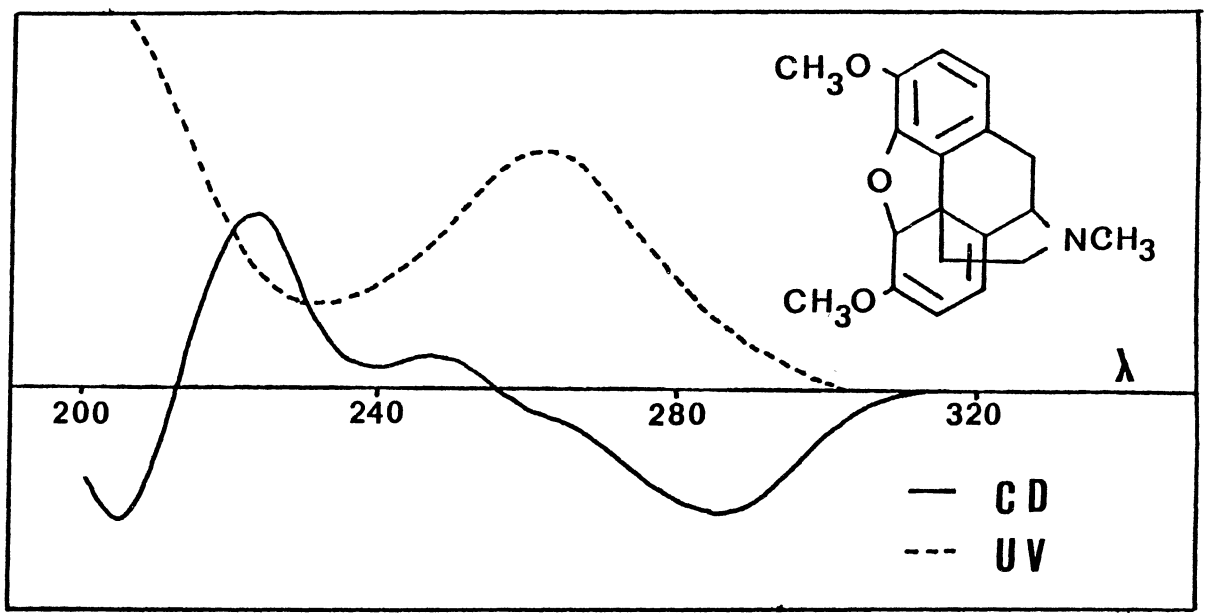


Figure 37. CD and UV Spectra for Thebaine

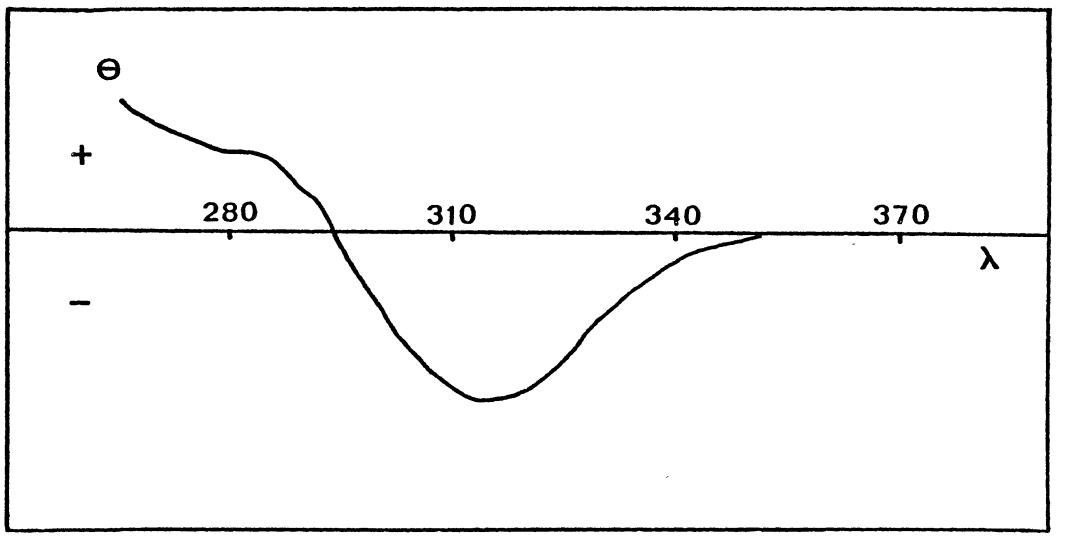


Figure 38. CD Spectrum of Noscapine in Water

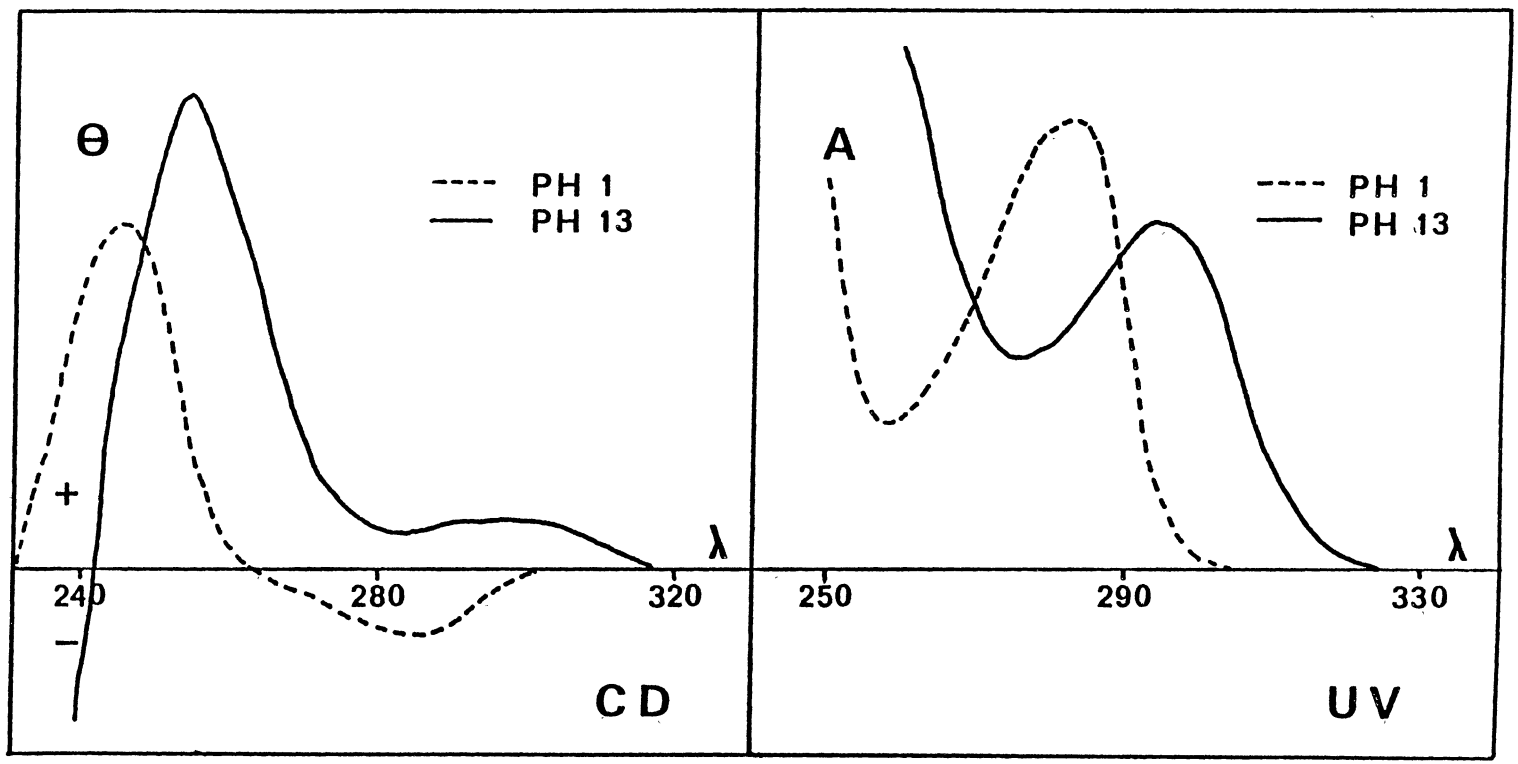


Figure 39. Effect of pH on the UV and CD Spectra for Morphine

TABLE X

DETERMINATION OF BINARY AND TERNARY MIXTURES FOR OPIATES

Sample	Morphine		Codeine		Thebaine	
	concentration(M)	error(%)	concentration(M)	error(%)	concentration(M)	error(%)
MC1	2.80×10^{-4}	0.11	2.80×10^{-4}	0.07		
MC2	3.36×10^{-4}	0.83	2.24×10^{-4}	0.18		
MC3	2.24×10^{-4}	-0.22	3.36×10^{-4}	0.21		
MC4	1.12×10^{-4}	-0.09	4.48×10^{-4}	-0.18		
MCT1	2.42×10^{-4}	-0.66	7.92×10^{-5}	-0.11	1.03×10^{-5}	8.04
MCT2	2.30×10^{-4}	-0.52	8.80×10^{-5}	3.81	9.83×10^{-6}	2.25

extract and for pantopon are shown in Figure 40. Table XI shows the results for the determinations of opium and pantopon as four component mixtures. Results for opium are within the statistical ranges reported for these analytes and obtained by other methods. Results for pantopon were in excellent agreement with the figures provided by the manufacturer.

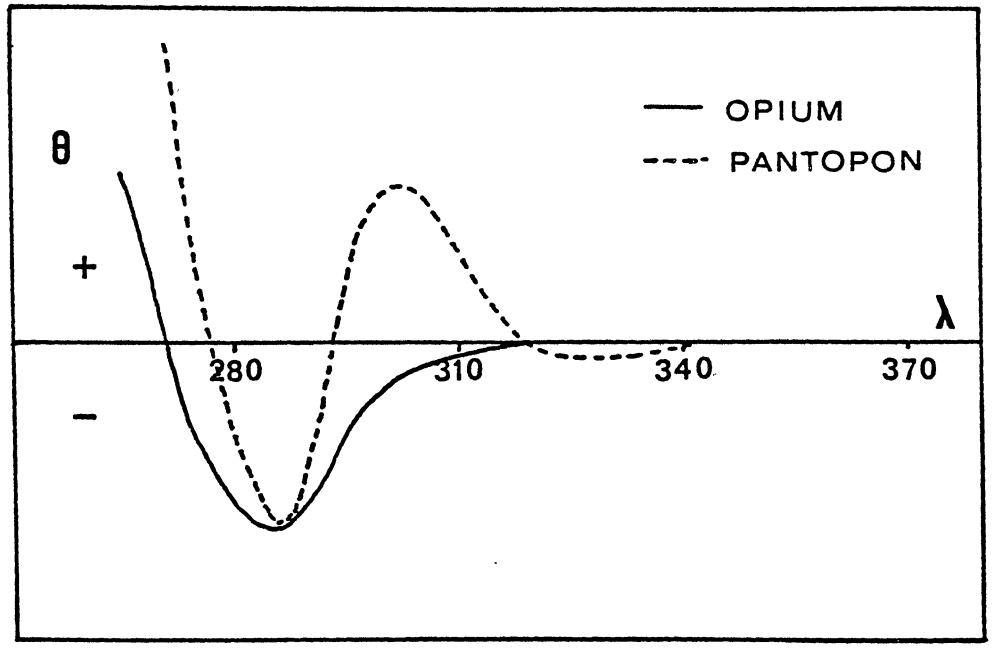


Figure 40. CD Spectra for Opium and Pantopon

TABLE XI

DETERMINATION OF OPIUM AND PANTOPON

component		pantopon	opium
Morphine	concentration(M)	1.94×10^{-4}	4.77×10^{-5}
	% of alkaloids(wt.)	46.0	9.5
Codeine	concentration(M)	4.84×10^{-5}	-
	% of alkaloids(wt.)	12.1	-
Thebaine	concentration(M)	-	1.57×10^{-4}
	% of alkaloids(wt.)	-	3.4
Noscapine	concentration(M)	3.31×10^{-5}	8.59×10^{-5}
	% of alkaloids(wt.)	11.4	2.5

CHAPTER XI

DISTINCTIONS BETWEEN ENANTIOMERS AND DIASTEREOISOMERS

Introduction

Enantiomeric forms (optical isomers) of the same molecule have identical melting and boiling points, the same indices of refraction, and the same infrared spectra. They do exhibit different solubilities and subtle differences in ^{13}C nmr spectra, but the only easy physical way to distinguish between enantiomers is to oberve their different behavior toward an incident beam of plane polarized light. Enantiomeric forms rotate the plane of linearly-polarized light to equal amounts but in opposite directions. They are known to show different behavior when they interact chemically with other chiral substances (84). When they occur together in equal amounts a racemic mixture is produced which exhibits no light rotation. Resolution of this mixture can be done either physically or chemically. Separation may not be 100% complete, in which case enantiomeric enrichment is achieved. The same term also describes the consequence of a stereoselective synthesis in which one enantiomer is produced in excess. Analytical methods are needed to determine the degree of enrichment (85,86,87). That is one of the goals of the work described

in this chapter. The illustrative example chosen is mixtures of atropine (DL-hyoscyamine) and L-hyoscyamine.

Diastereoisomers are also often difficult to distinguish by physical methods based on spectroscopy. They can be identified by polarimetry if they are known to be pure and free from chiral interferences. CD offers a spectral resolution to this problem. Quinine and cinchonine alkaloids and pilo- and isopilocarpine are used as examples.

Experimental Section

Quinine hydrochloride, quinidine hydrochloride, cinchonine, cinchonidine, atropine, L-hyoscyamine , and pilocarpine nitrate were obtained from Sigma Chemical Co. Isopilocarpine nitrate were obtained from Aldrich Chemical Co. All of the standard chemicals were used without further purification. Because of the effect of pH on the analytes, all of the solutions were buffered at 8.0 $\pm$ 0.05 pH and 8.2 $\pm$ 0.05 pH (PHydrion Buffers, Micro Essential Laboratory).

Results and Discussion

The UV and CD spectra of the naturally occurring L-hyoscyamine are shown in Figure 41. Atropine is DL-hyoscyamine and is formed during the extraction of L-hyoscyamine. A combination of UV absorbance and CD can be used to assay atropine and determine the excess of L-hyoscyamine. This was tested for a number of prepared mixtures of the drugs in which the weight of the total

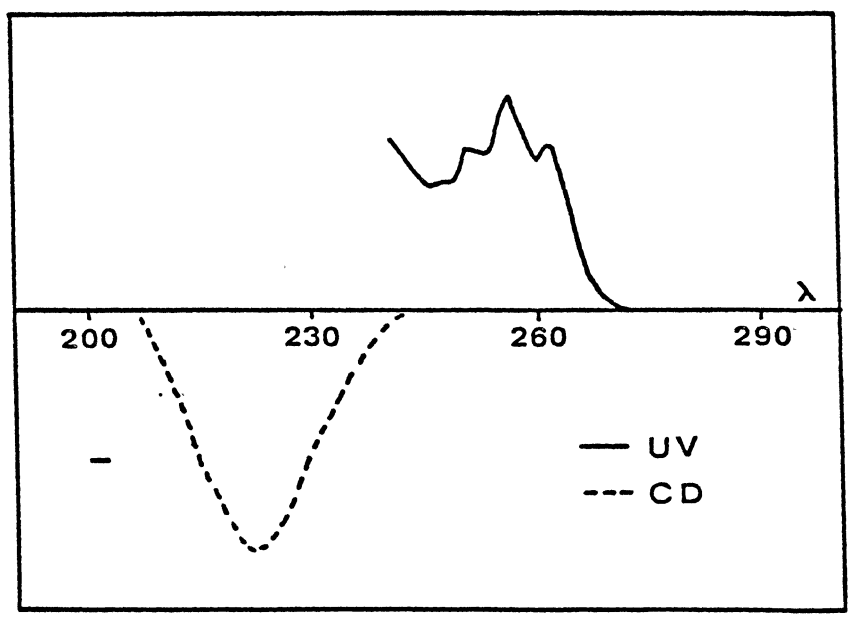


Figure 41. UV and CD Spectra for L-Hyoscyamine

alkaloid was calculated from data taken at the absorbance maximum (257 nm), and the excess amount of the L-hyoscyamine was determined from the known molar ellipticity value at 222 nm. The correspondences between the results were good, Table XII.

The spectra for quinine, quinidine, cinchonine, and cinchonidine in aqueous pH 8.0 buffer are shown in Figures 42 and 43. The spectra for the diasteroisomeric pairs are of opposite sign making their distinction very easy. Sufficient spectral changes exist to enable one to distinguish all four isomer, in spite of the very small structural differences (Chapter IV). Prepared mixtures of quinine and quinidine can be determined using the program described in Chapter IX. The results are given in Table XIII. The method used for atropine and hyoscyamine could not be used since molar absorbance values are not the same for quinine and quinidine. However since both analytes contribute to the CD signals, the simultaneous determination can be made using only CD data. The molar ellipticities for quinine (-65.2) and quinidine (55.2) at 333 nm are not numerically equal, so a 50:50 prepared mixture has a net CD spectrum.

The molecular structure of pilocarpine is shown in Figure 44. The pilocarpines are used as medications. Only pilocarpine is particularly effective as an eyewash. Their distinction is important for pharmaceutical laboratories. CD spectra are shown in Figure 45. Because the absorptions

TABLE XII

DATA FOR L-HYOSCYAMINE AND ATROPINE SIMULTANEOUS DETERMINATIONS

	Atropine actual concentration(M)	Atropine calc. concentration(M)	error %	L-hyoscyamine actual concentration(M)	L-hyoscyamine calc. concentration(M)	error %
#1	1.53×10^{-3}	1.56×10^{-3}	2.4	3.54×10^{-4}	3.74×10^{-4}	5.6
#2	1.55×10^{-3}	1.54×10^{-3}	-0.5	3.54×10^{-4}	3.70×10^{-4}	4.5
#3	1.68×10^{-3}	1.70×10^{-3}	1.1	3.10×10^{-4}	2.97×10^{-4}	-4.0
#4	1.81×10^{-3}	1.83×10^{-3}	1.3	2.65×10^{-4}	2.54×10^{-4}	-4.2
#5	1.42×10^{-3}	1.47×10^{-3}	3.7	3.98×10^{-4}	3.72×10^{-4}	-6.7
#6	5.17×10^{-4}	5.28×10^{-4}	2.2	7.08×10^{-4}	6.69×10^{-4}	-5.5
#7	1.03×10^{-3}	1.10×10^{-3}	6.9	5.31×10^{-4}	5.08×10^{-4}	-4.2

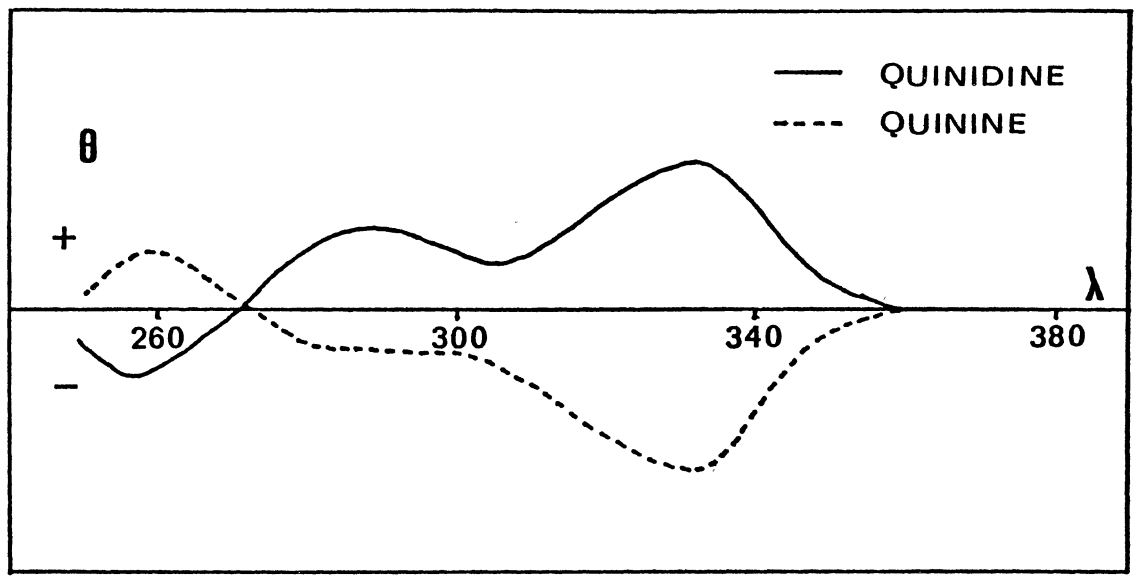


Figure 42. CD Spectra for Quinine and Quinidine

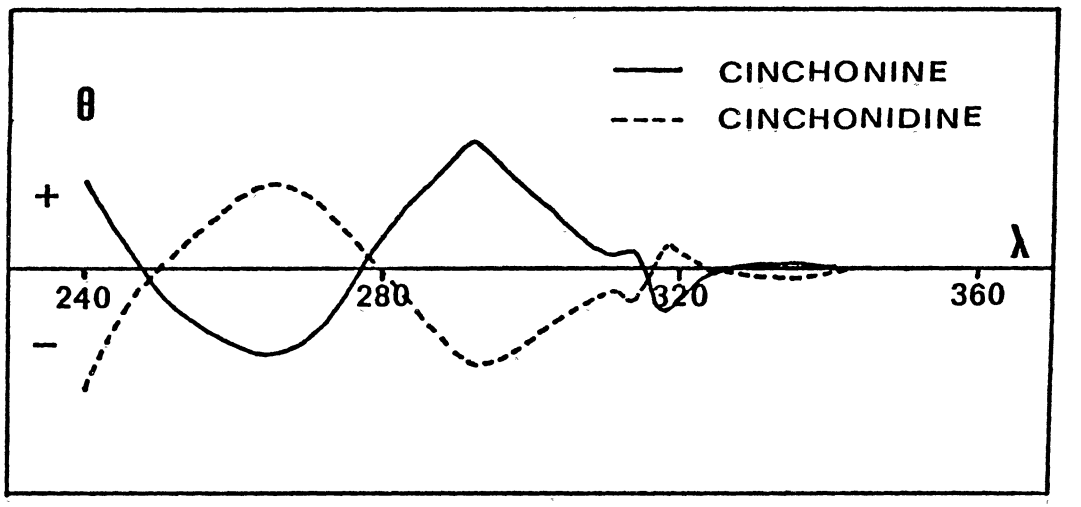


Figure 43. CD Spectra for Cinchonine and Cinchonidine

TABLE XIII

SIMULTANEOUS DETERMINATIONS FOR MIXTURES OF QUININE AND QUINIDINE

		ratio	calculated concentration(M)	actual concentration(M)	error
#1	quinine	40	1.25×10^{-4}	1.26×10^{-4}	1.1
	quinidine	60	1.87×10^{-4}	1.85×10^{-4}	-1.0
#2	quinine	20	6.23×10^{-5}	6.36×10^{-5}	2.0
	quinidine	80	2.49×10^{-4}	2.45×10^{-4}	-2.1
#3	quinine	50	1.45×10^{-4}	1.47×10^{-4}	1.8
	quinidine	50	1.45×10^{-4}	1.51×10^{-4}	4.4
#4	quinine	60	1.74×10^{-4}	1.70×10^{-4}	2.4
	quinidine	40	1.16×10^{-4}	1.14×10^{-4}	1.6
#5	quinine	80	2.49×10^{-4}	2.37×10^{-4}	-5.0
	quinidine	20	6.23×10^{-5}	6.36×10^{-4}	2.1

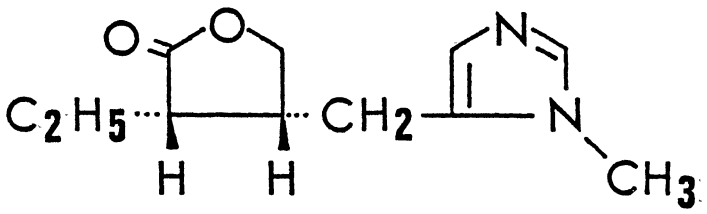


Figure 44. Molecular Structure for Pilocarpine

which occur in the middle UV range (~220 nm) are so intense, the weak CD signals have a poor signal to noise ratio making quantitative information difficult to obtain. Their qualitative distinction is apparent from the opposite polarities. Enantiomers and diastereoisomers are very easy to distinguish by CD, and determinations of enantiomeric excesses are possible.

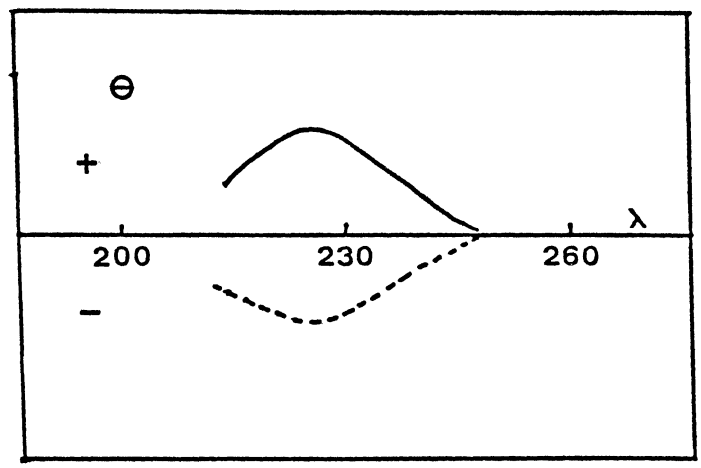


Figure 45. CD Spectra for Pilocarpine (———)
and Isopilocarpine (----)

CHAPTER XII

CONCLUSION

In the past two decades remarkable progress has been made in the development of methods for use in the determination of drugs. Ultraviolet-visible spectrophotometry is a very popular method for use in drug analysis. However, quantitative analysis of a mixture is often impossible without some sample preparation which usually includes separation steps. CD spectropolarimetry is particularly useful for their identification and determination. Since CD is only specific to chiral substances, separations and other work-up procedures are often unnecessary, increasing the speed and ease of the analysis. In choosing CD as a method of determination one can usually ignore the identity and composition of achiral components in the mixture for quality control.

The breadth of application can be increased by inducing chirality on complexation within a chiral substrate, for example, beta-cyclodextrin. However, one has to be aware of a potential disadvantage that all or nearly all of the components in a mixture could undergo chirality induction, in which case the original specificity advantage is lost.

In this work, analysis of mixtures of CD active

compounds extracted from pharmaceutical products, and from plant materials such as tobacco, marihuana, and opium, were investigated and multicomponent analyses were successfully concluded by mathematically fitting experimental data in computer simulations. It was assumed that other compounds were either not CD active or had molar ellipticities so very small as to be beyond the detection limit of the instrumentation. This is one of the remaining criticisms of the technique which requires further investigation.

In future developments of CD as an incisive analytical tool, the singularity of the spectrum of a single extract from very complex mixtures must be investigated. The CD method, being a modification of UV absorbance, could be used as a selective detector for HPLC. Many of the interpretative problems in liquid chromatography could be simplified by using a detector which is selective to a limited number of the eluted species and is blind to the others. A direct comparison could be made between the spectrum for unseparated extract and the spectra for the separated components. One on one spectral correlations would indicate no separation is necessary if the analyte is the one of interest. Instances could therefore be identified where it can be assured that chromatography is unnecessary. This would be of value in laborious repetitive tasks. Simplifications may also occur in that derivatizations may not be necessary in some instances. Quick whole spectral comparisons with standards could be

used for rapid screening of mixtures without chromatography. By comparison, many methods have been applied to the rapid screening of pure substances.

The successes of any or all of these propositions requires that an in-depth study of the application of CD as a detector be thoroughly examined. The gain of single wavelength CD detection over single wavelength UV detection is marginal. In fact an almost equivalent result could be obtained using a polarimeter as a detector. Decisions on technical developments toward diode array CD detection and miniaturization toward on-line automation would be dependent upon the results of these preliminary studies.

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