ANALYTICAL APPLICATIONS OF CIRCULAR DICHROISM IN PHARMACEUTICAL DETERMINATIONS

By

KATHY ANN SWALLOWS

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Central State University

Edmond, Oklahoma

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Dean of the Graduate College

PREFACE

CD is generally recognized as a technique employed for elucidation of stereochemical configuration. This application of CD is dependent upon the ability to develop model systems upon which emphircal rules can be formulated. At this time there is no comprehensive theory for assigning configurations and this deficient limits the effectiveness of CD as a structural probe. It is unfortunate that the historical application of CD is inhibiting the development of this technique as an analytical tool. The primary objective of this study is to demonstrate the advantages of employing CD detection for analysis of pharmaceutical and related substances. It is hoped that the investigations described in this work, combined with future work in the area, will help to demonstrate that CD is a modern analytical detector with unique characteristics that are well suited to the analysis of pharmaceutical and clinical samples.

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CHAPTER I

INTRODUCTION

Reliable analytical techniques are necessary tools in today's forensic, clinical, and pharmaceutical laboratories. The public's growing health consciousness has prompted the development of "shopping mall" cholesterol testing. The use of performance enhancing drugs and drugs of abuse by athletes has made drug testing a necessary part of most athletic events and programs. Unfortunately, abuse of drugs is not limited to the elite athlete. As of 1983, drug abuse cost more than thirty three million dollars in lost productivity alone (1). As a result, many companies require drug screening of all new employees and of employees in key areas where safety is an issue.

Drug screening is not new. Toxicologists have had to deal with such tests since 1968 when opiate abuse during the Vietnam war prompted the military to start screening for such substances (2). However, the recent trend towards large scale health and drug screening requires the development of quick, simple, cost effective screening methods that are specific and accurate under all conditions, every time they are employed. The need for such techniques has prompted many new developments in analytical methods as applied to drug and biomolecule determinations.

The needs of the pharmaceutical industry are also stimulating development of new methods for drug determination. The FDA requires a battery of tests in order to insure the safety and clinical efficacy of a drug. A pharmaceutical firm must submit a full description of the methods used in manufacturing the drug, including results that demonstrate the identity, strength, quality, and purity of the substance. This entails analysis of all starting

products, analytical monitoring of all the components during the manufacturing process, and quality control testing of the final dosage form before it is released for testing or marketing. The need for analytical determinations does not end here but continues into the field of the clinical chemist who is usually responsible for monitoring therapeutic agents during pharmacokinetic and toxicity studies, not only in the pharmaceutical lab but in hospital settings as well. Such determinations are difficult because of the complex sample matrices encountered, i.e. urine and blood serum. Such analyses must meet the same requirements dictated for the drug screening programs and thus represent a major challenge to the analytical chemist .

Many analytical techniques are employed in pharmaceutical determinations. All have relative advantages and disadvantages. Selection of a given method is usually based upon simplicity, sensitivity, and selectivity for the given analyte or analytes. Thermal analysis methods such as thermogravimetry, differential thermal analysis, and differential scanning calorimetry are used mainly to evaluate the thermal decompositon of pharmaceutical formulations, but they are also useful in the quantitation of impurities such as inorganics and volatile solvents. There have been some reports of qualitative identification of major components by thermal analysis, but little in the way of quantitation of such substances (3).

Immunoassay techniques rely on the interaction of an antibody with a specific antigen-labeled antigen pair. Detection is usually based on the radioactivity, fluorescence, or enzyme activity of the labeled antigen. Immunoassay kits are available commercially for a number of drugs. The primary advantage of such kits is their specificity for a given substance, however, cross-reactions with other molecules are not uncommon. A recent review by Smith and Joseph (4) is thorough and informative.

Flow injection analysis (FIA) is also employed for pharmaceutical determinations. FIA involves the injection of a sample into a flowing, non-segmented stream of reagent or carrier liquid. Controlled mixing of the sample and carrier or reagent occurs before reaching the detector. UV-visible, fluorescence, phosphorescence, chemiluminiscence,

electrochemical, and enzymatic detectors are commonly employed. The pharmaceutical applications of FIA have been reviewed by Calatayud (5).

"Traditional" analytical methods still have a foothold in pharmaceutical analysis. Massart and co-workers (6) have discussed ways to improve multiple component pharmaceutical preparations by ultraviolet spectrophotometry. Electrochemical methods such as polarography (7, 8) and voltammetry (8) are capable of determining very small quantities of pharmaceutical substances in a variety of matrices, making them valuable techniques for trace analysis. However, the recent trend in almost any type of analytical determination is to couple these methods with some type of chromatography. The chromatographic step provides added selectivity by separating the components before they reach the detector.

Chromatographic techniques constitute the largest percentage of modern pharmaceutical analyses. Thin layer chromatography (TLC) is the oldest form of this technique. Technological advances in other forms of chromatography have made it obsolete as a quantitative tool, but it is still used as a screening tool for narcotics, local anaesthetics, hallucinogens, and amphetamines (9). Liquid chromatography (LC), coupled with UV, fluorescence, or electrochemical detectors has become the method of choice for most determinations. The applications of LC in drug analysis have been extensively reviewed elsewhere (9, 10, 11, 12). However, there are some technological advances that have widened the applicability of LC even further. The development of photodiode array detectors combined with computerized spectral comparison has increased the potential of HPLC/UV as a screening method (13). Other advances include the development of microbore columns (14), column switching techniques, and the development of LC/MS interfaces (15).

The latter development is of importance because mass spectrometry (MS) is still the definitive technique for identification of drug substances. Because of technical incompatibilities between LC and MS, gas chromatography/MS (GC/MS) has been the

more common analytical procedure. GC/MS is recommended by the National Institute of Drug Abuse when confirmation of positive results from other screens is needed (14). However, the development of LC/MS interfaces may lead to changes in this recommendation. GC/MS techniques used in pharmaceutical determinations are well documented and have been extensively reviewed (9, 10).

Another form of chromatography has been developed recently that would compliment both LC and GC techniques. Supercritical fluid chromatography (SFC) combines many of the advantages of GC and HPLC into one technique. SFC has proven to be useful in determinations of polar compounds that are difficult to do using GC and it is easier to interface SFC with MS than it is to interface HPLC. Other advantages and applications have been discussed by Games and co-workers (16).

The growing sophistication in instrumental techniques has been paralleled by a growing understanding of how, why, and where drug substances work. This knowledge has generated a new type of analytical question which requires more specialized tools to answer. It has been known for many years that some pharmaceuticals are actually synthesized and dispensed as enantiomeric mixtures and that each enantiomer may have a different physiological effect because of differing metabolic pathways or pharmacokinetics. However, the relative enantiomer concentration in such drugs was not an issue until the late 1970's when chiral stationary phases were developed for HPLC. Prior to this development, enantiomeric ratio determinations were not easily accomplished; consequently the Food and Drug Administration (FDA) did not require any information on enantiomeric concentration, pharmacokinetics, side effects, or clinical efficacy. However, in 1988 the FDA issued a set of guidelines covering the submission of New Drug Applications (NDA) which required that the structure and relative concentrations of both enantiomers be reported if both were present in the drug substance (17).

The FDA guidelines encouraged the development of chiroptical techniques as detectors for HPLC. Traditionally, such techniques have been used to examine the stereochemistry

of substances. Recent applications of chiroptical detectors have focused on the same type of determinations. Yeung et. al. (18), and researchers at Jasco, Ltd. of Japan (19) have proposed the use of UV and circular dichroism (CD) detectors, connected in series, for following possible conformation changes in proteins during chromatographic separations. The UV detector is used for quantitation, while the CD is used for qualitative identification of the proteins and impurities. Meinard and co-workers (20) coupled UV detection with polarimetry, in order to identify enantiomers as they came off the column. The above examples constitute qualitative applications of chiroptical techniques. Little has been reported on the quantitation of chiral substances by such methods. However, Yeung et. al. (21) has explored the quantitative applications of CD by using fluorescence detected CD as an HPLC detector with good results.

Yeung, Meinard and the others have all acknowledged the relative selectivity that CD detectors possess. This selectivity is due to the structural requirements for CD activity, namely the presence of a chiral center and a chromophore in close proximity to each other. Many pharmaceuticals possess the required structural components and are generally well suited to detection by CD. The inherent selectivity of CD in many cases is so great that a prior chromatographic separation step is unnecessary and because CD is an absorbance measurement, samples can be quantitatively determined using the same methods employed in normal UV spectrophotometry. CD therefore has merits as a stand-alone analytical technique for determination of pharmaceuticals and especially for determinations of enantiomeric concentration.

The analytes and samples discussed in the following pages are representative of the analytical problems encountered by pharmaceutical and clinical laboratories. The resulting collection of determinations demonstrates a number of the advantages and a few of the possible applications of CD detection. Combining these investigations with past and future work should demonstrate that CD is a modern analytical detector with unique characteristics that make it well suited to the analysis of pharmaceutical and clinical samples.

CHAPTER II

HISTORICAL AND THEORETICAL BACKGROUND OF CIRCULAR DICHROISM

History

Optical activity was first observed over 175 years ago when the French astronomer Arago was investigating the interactions between quartz plates and polarized light (22, 23) by observing solar radiation through Iceland spar (24). One year later, in 1812, Biot demonstrated that the solutions of some organic compounds also rotated a beam of polarized light (23, 25), thus proving that optical activity was not limited to crystalline substances. In 1817 Biot and Fresnel independently observed that the rotatory power of a substance increased as the wavelength decreased (22). This phenomenon is now recognized as optical rotatory dispersion (ORD). Another manifestation of optical activity was recognized in 1846 when Haedinger observed a difference in the absorption of left and right circularly polarized light in amethyst quartz crystals (22), a phenomenon now identified as circular dichroism (CD).

The first clues to the physical basis of optical activity were provided by Louis Pasteur. In 1848 Pasteur discovered the hemihedrism of tartrate crystals (26). These isomorphic crystals exhibited tetrahedral facets that were oriented rightward or leftward with respect to the main crystal surfaces. Solutions of the separated crystals rotated a polarized light beam to the left or right depending on the orientation of the original tetrahedral facets. He concluded that the macroscopic asymmetry of the crystals was connected to the asymmetry

of the individual molecules. Based on this conclusion Pasteur suggested that molecules fit into two categories: those molecules that are superposable mirror images and those that are nonsuperposable mirror images (27). He believed that those molecules which were nonsuperposable mirror images would exhibit optical activity (23).

Pasteur was the first to suggest that optical activity was caused by a dissymmetric grouping of atoms in a helical or tetrahedral configuration (27). It wasn't until 1874 that van't Hoff and Le Bel (28) independently proposed the existence of an asymmetric carbon atom. Van't Hoff correctly proposed a tetrahedral configuration while Le Bel suggested a square pyramid geometry. The introduction of this concept made it possible to associate the optical activity of a compound with a definite carbon atom in the molecule. It then became apparent that two dimensional formulae did not adequately describe an organic molecule, and studies of the three dimensional structure of molecules became necessary. Seventy-five years would pass before Djerassi and Blout would employ the same chiroptical methods that lead to this conclusion as tools in elucidating the stereochemistry of organic molecules (29, 30).

Early investigations of chiroptical methods concentrated on discovering the physical causes and relationships of optical activity. Aime Cotton, another French physicist, used solutions of copper and chromium tartrates to investigate, in detail,both CD and ORD (22). He suggested that the CD curves of optically active compounds resulted from a difference in the absorbance of left and right circularly polarized light.

The first physical theory of optical activity was proposed by Drude in 1896. He postulated that the charged particles in a dissymmetric structure were constrained to move in a helical path (27) and that optical activity resulted from the interaction of electromagnetic radiation with this helical electronic motion. In 1916 Gray attempted to combine the geometrical models of van't Hoff and Le Bel with the physical model proposed by Drude (27). He considered the effect of a wave of plane polarized light on a hypothetical molecule consisting of a central atom with four different atoms bonded to it, each located at the

apices of a tetrahedron (24). This model, proposed independently by Gray and by Born and Oseen, used coupled oscillators and polarizabilities to describe optical activity (23, 24, 27). A simplification of Born's theories was proposed by Kuhn in 1930. By working out a special case of Born's theory, Kuhn was able to represent rotatory power as a first-order effect rather than a third order effect (24). Two of Kuhn's contemporaries, Mitchell and Lowry, also studied the optical activity of organic compounds in hopes of understanding the basic theories involved in this phenomenon (23).

Rosenfeld was the first to attempt a quantum mechanical approach to interpret optical activity (22, 23). Rosenfeld's quantum theory was expanded by Condon et. al. and by Kirkwood (26). Condon considered the effect of a static asymmetric field of a molecule on an electron in an otherwise symmetric chromophore. Condon's model is known as the one-electron theory in order to distinguish his model from the earlier coupled oscillator theories. Kirkwood introduced the asymmetric dynamic coupling of electrons with the electronic transitions of other electrons (26). Starting in 1960, Tinco refined and reformulated the quantum theory of optical activity by including both the static and dynamic coupling effects (26). Additional refinements are outlined and referenced by Crabbe (25) and Charney (26).

All of the above quantum mechanical theories were based on perturbation theory. Most are considered to be semi-classical treatments because the electromagnetic field was treated classically rather then being quantized. Unfortunately, most of these theories required a number of simplifications and assumptions, thus limiting their practical application. As yet there is still no comprehensive theory that allows the predetermination of the sign and magnitude of molecular optical activity.

Theory

An optically active substance is defined by IUPAC (31) as one which interacts differently with left and right circularly polarized light. Two types of optically active media are recognized: the inherently dissymmetrical molecule and the inherently symmetrical, but asymmetrically perturbed molecule (22). The first group is characterized by the absence of a center or plane of symmetry in the molecule, such as in hexahelicene. The latter group requires the presence of a chromophore in close proximity to an asymmetric (i.e. chiral) carbon atom. Enantiomers and diastereomers are typical of this class and constitute the type of optically active compounds discussed in subsequent chapters.

The varied spectroscopic responses of optically active compounds can best be explained by first examining the form of the electromagnetic radiation employed. According to the transverse wave model, a monochromatic light beam consists of time dependent electric and magnetic fields oriented at right angles to each other and perpendicular to the direction of propagation. Figure 1 is a schematic representation of the electric field. The associated magnetic field can be ignored during this discussion.

The monochromatic light generally employed in most spectroscopic techniques consists of a number of electric fields vibrating in many different planes perpendicular to the direction of propagation, Figure 2a. Conversely, the linearly polarized light beam employed in chiroptical techniques has an electric field vibrating in only one direction, Figure 2b, and can be represented as the vector sum of the two circularly polarized components, Figure 3. The electric field vectors of the left and right circularly polarized components trace out a left-handed and right handed helix, respectively, as the wave propagates along a given axis. If the electric field vector is allowed to propagate in time, but not with distance, a counterclockwise or clockwise circle results as illustrated in Figure 2c and d.

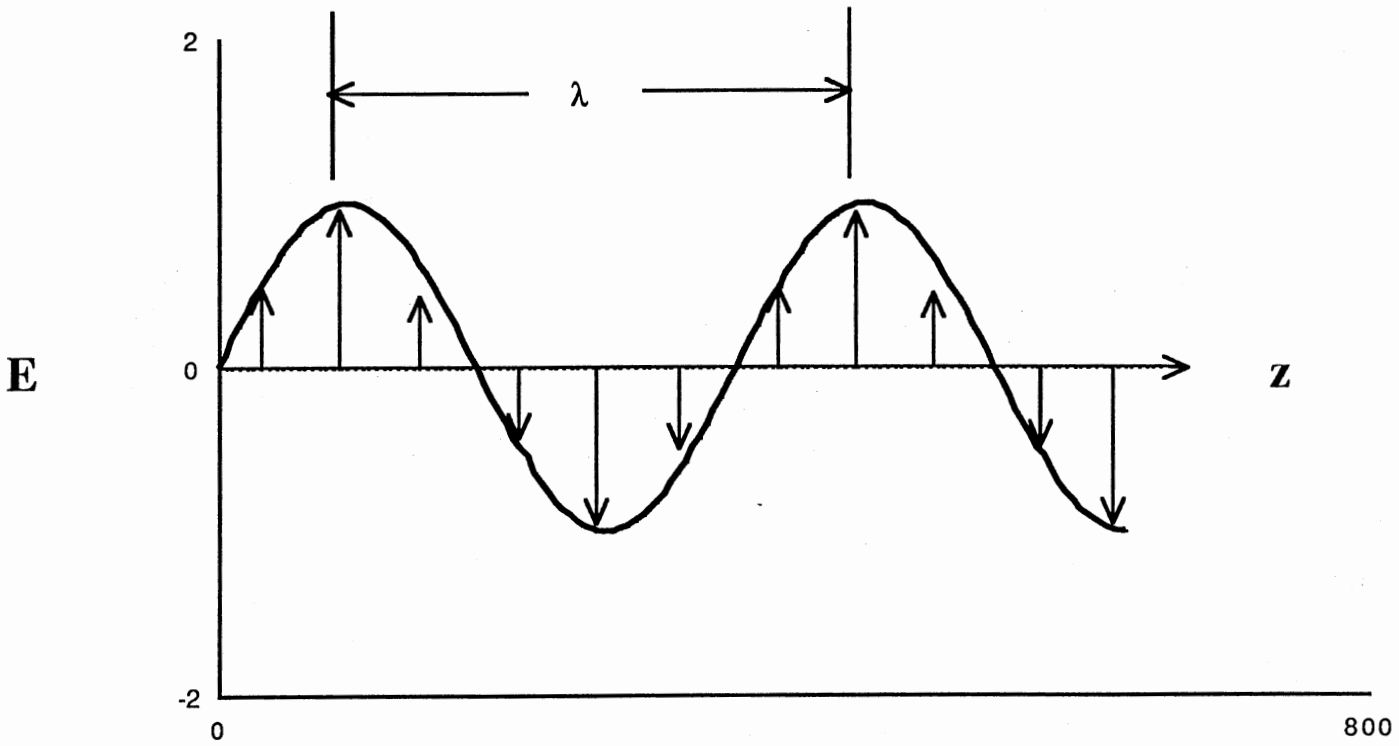


Figure 1. Transverse wave representation of the electric field associated with a monochromatic light beam. The arrows represent the magnitude of the oscillating electric field. The distance between cycles is related to the wavelength, λ, of the radiation.

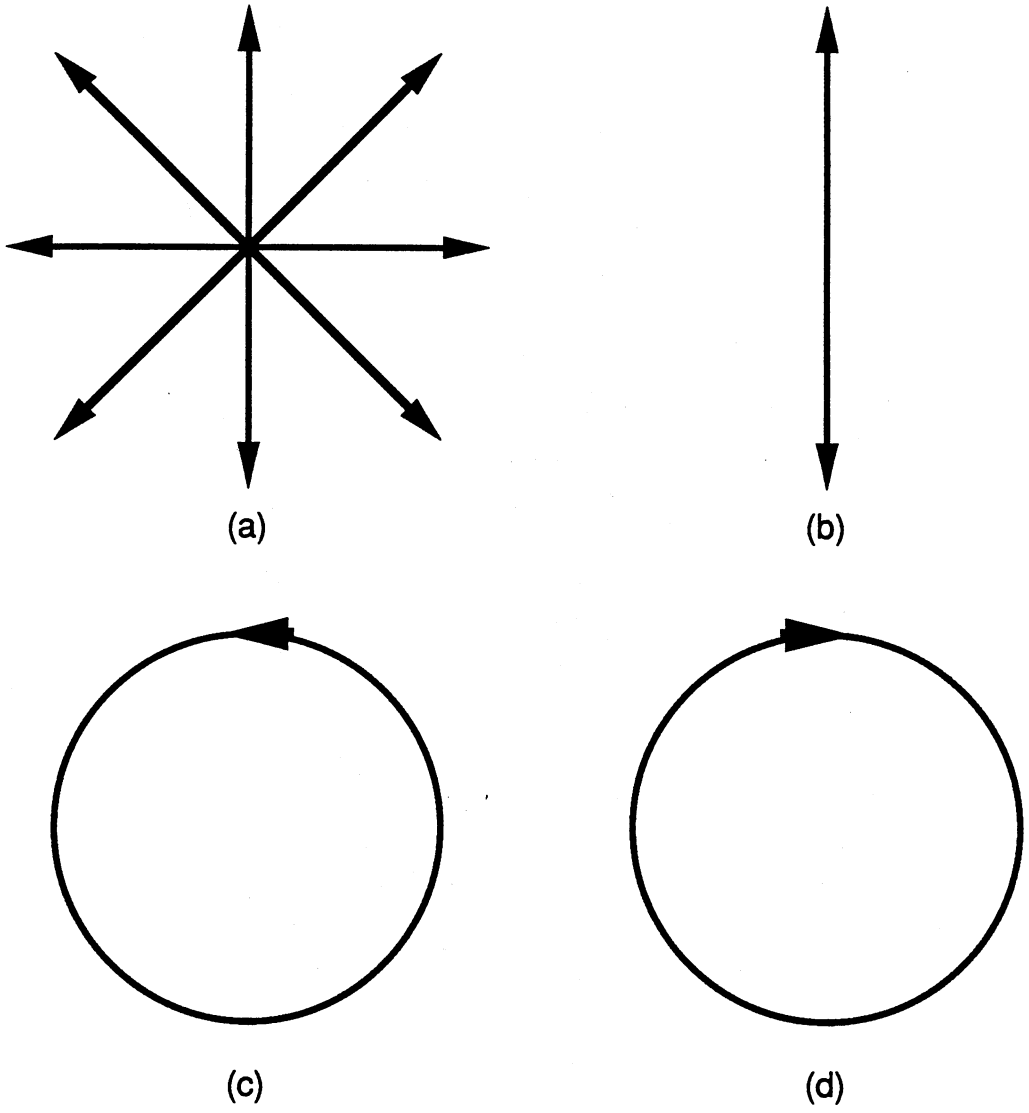


Figure 2. Polarization of monochromatic light beams: a) unpolarized light, b) linearly polarized light, c) left circularly polarized light, and d) right circularly polarized light. Direction of progagation is out of the page.

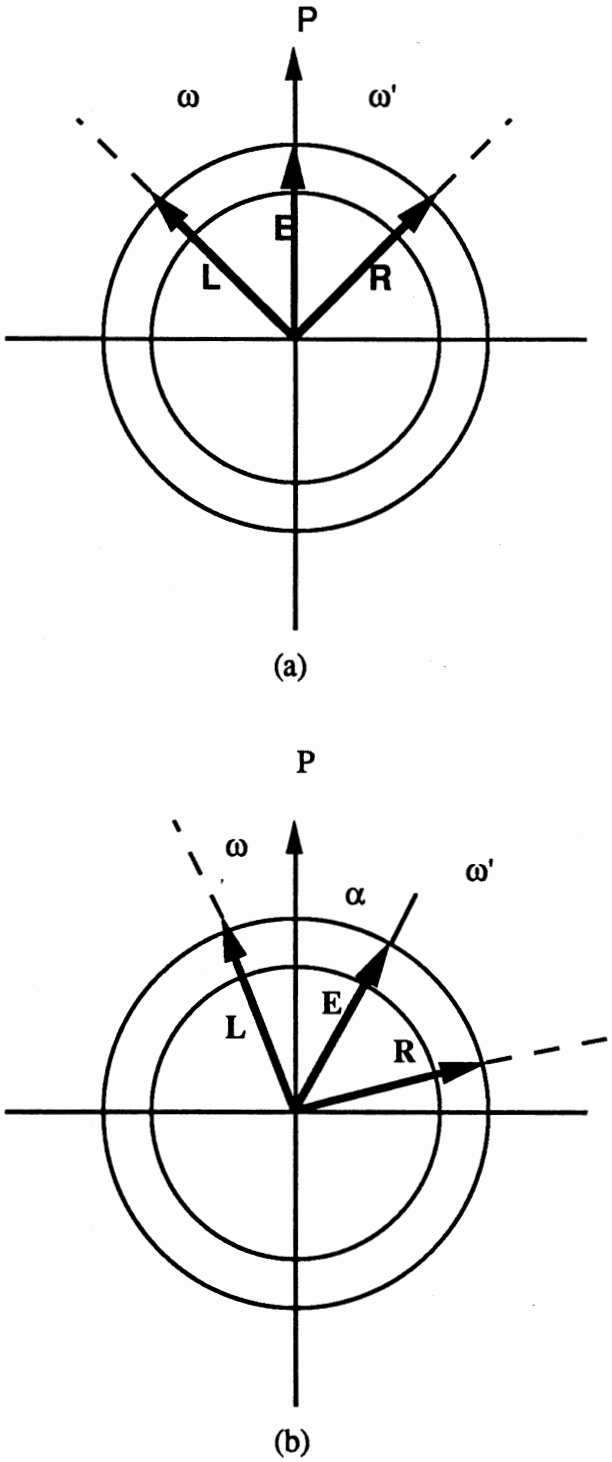


Figure 3. Direction of the electric field vectors emergingfrom a) an achiral medium and b) a chiral medium. P is the original plane of polarization, **L** and **R** are the left andright circularly polarized electric field vectors, **E** is the resultant electric field vector.

The circularly polarized electric field vectors **L** and **R** will exit an achiral media in phase with each other, i.e. with the same frequency. The angles between each vector and the original plane of polarization are always equal ($\omega = \omega'$), Figure 3a. The resultant linearly polarized field is in the same direction as the incident field. When circularly polarized beams interact with an optically active material, the emerging beams are out of phase with each other and their relative angles are different ($\omega \neq \omega'$). In this case the resultant field is still linearly polarized but is rotated from the original plane of polarization by an angle α, the optical rotation, as illustrated in Figure 3b.

In 1825, Fresnel correctly suggested that the optical rotation resulted from differences in the refractive indices, η, of the two circularly polarized beams (22).

$$\eta_L - \eta_R \neq 0 \qquad (1)$$

Changes in the refractive index correspond to changes in the propagation velocity of the two circularly polarized components while passing through the medium. Materials for which equation 1 is true are said to be circularly birefringent. If $\eta_L > \eta_R$, the optical rotation is counterclockwise, i.e. negative, and substance is levorotatory. Conversely if $\eta_L < \eta_R$, the rotation is clockwise, i.e. positive, and the substance is dextrorotatory (22).

The optical rotation, α, is directly proportional to the differences in the refractive indices:

$$\alpha = (\pi b/\lambda)(\eta_L - \eta_R)(1800/\pi) \qquad (2)$$

where α is measured in degrees, b is the cell path length, λ is the wavelength of the incident radiation, and η_L and η_R are the indices of refraction for the left and right circularly polarized beams respectively. $1800/\pi$ converts radians to degrees.

A new quantity $[\alpha]$, the specific rotation, is introduced to normalize the concentration, making it useful for comparison purposes:

$$[\alpha] = \alpha/c'b \qquad (3)$$

where α is the optical rotation from equation 1, c' is the concentration in g/cm^3 and b is the cell path length.

Equation 3 is useful for comparisons between similar molecules, but for drastically different molecules another quantity, the molar rotation [ϕ] is preferred. In equation 4 the optical rotation has been normalized to molecular weight, making it possible to compare results on a mole for mole basis:

$$[\phi] = [\alpha]M/100 \quad (4)$$

where [α] is the specific rotation from equation 3 and M is the molecular weight in g/mole. The division by 100 has no physical meaning but was introduced to keep the numbers small (31). The older literature often refers to [ϕ] as the molecular rotation, however, IUPAC has ruled this terminology to be incorrect (31).

Chiral media not only cause changes in the relative speed of the circularly polarized electric field vectors, but also cause these components to be absorbed to different extents as described by equation 5:

$$\Delta A = A_L - A_R \neq 0 \quad (5)$$

where A_L and A_R are the absorbances of the left and right circularly polarized components respectively. The absorbance of a substance in solution is related to the molar absorption coefficient, ε, by the Beer-Lambert law:

$$A = \varepsilon bc \quad (6)$$

where b is the cell path length, c is the analyte concentration in moles/L and ε has the units of liters per mole centimeter, A is the measured absorbance and is unitless. Equation 5 can be rewritten in terms of ε:

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R \neq 0 \quad (7)$$

where ε_L and ε_R are the molar absorption coefficients for the left and right circularly polarized beams respectively. The signed difference represented by equation 7 is defined by IUPAC (31) as the CD of a substance.

The differential absorbance of the two circularly polarized components changes the relative magnitude of their electric field vectors, **L** and **R** as pictured in Figure 4. The resultant electric field vector, **E**, no longer oscillates in a single plane, but traces out an

ellipse. Thus the emitted beam is both elliptically polarized and rotated by an angle α from the original plane of polarization because circular birefringence is also present. It is important to note that circular birefringence and CD always occur simultaneously in chiral media, however, the CD becomes measurable only in regions of absorption bands, i.e. equation 7 although accurate for all wavelengths is nonzero only in regions of an absorption band (30).

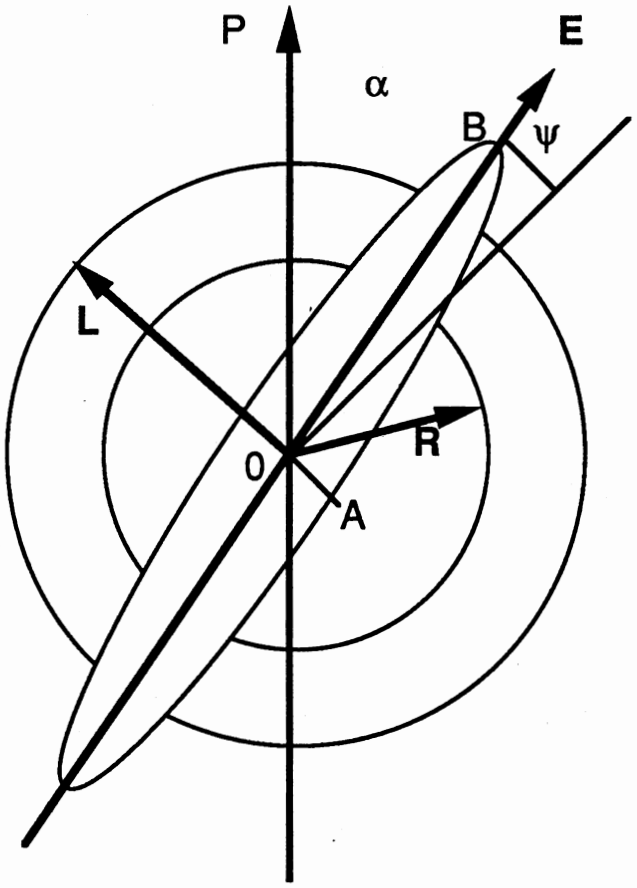


Figure 4. Production of elliptically polarized light in circular dichroism. P is the original direction of polarization, **L** and **R** are left and right circularly polarized light respectively, **E** is the resulting electric field which is now elliptically polarized.

The eccentricity of the elliptically polarized light is characterized by the ellipticity, ψ, which is defined as the arctangent of the ratio of the minor axis of the ellipse, OA, to the major axis of the ellipse, OB, as pictured in Figure 4 and described as follows:

$$\tan \psi = OA/OB \tag{8}$$

Since both the eccentricity and the difference between the absorption coefficients are small, equation 8 can be quantitatively approximated (22) as

$$\psi = \pi(\varepsilon_L - \varepsilon_R)/\lambda \tag{9}$$

where ε_L and ε_R are the molar absorption coefficients as defined in equation 7 and λ is the wavelength of the incident radiation. Note the similarity between this expression and equation 2 which uses Fresnel's definition of circular birefrigence ($\eta_L - \eta_R \neq 0$). Ellipticity is characteristic of CD just as optical rotation is characteristic of circular birefrigence and therefore it is not suprising that there are analogous expressions for their measurements.

The mathematical description of the specific ellipticity, $[\psi]$, is analogous to that for the specific rotation and is expressed as follows:

$$[\psi] = \psi/c'b \tag{10}$$

where ψ is the measured ellipticity in degrees, c' is the concentration expressed as g/ml and b is the cell path length expressed in cm. This quantity is useful for comparisons of molecules with similar molecular weights.

Normalizing the ellipticity to molecular weight allows for comparisons between molecules on a mole for mole basis. The resulting molar ellipticity, $[\theta]$, is analogous to the molar rotation used in defining circular birefrigence. The molar ellipticity is defined as follows:

$$[\theta] = [\psi]M/100 \tag{11}$$

where $[\psi]$ is the specific ellipticity from equation 10 and M is the molecular weight in g/mole. Since equations 4 and 11 are analogous, direct comparison between the magnitude of the rotation and the ellipticity for an individual molecule on a mole for mole basis is

possible. The molar ellipticity is also directly proportional to the difference between the absorption coefficients:

$$[\theta] = 3300(\varepsilon_L - \varepsilon_R) \quad (12)$$

where the numerical constant is the net result of all the conversion factors.

All of the commercial CD spectropolarimeters available today actually measure the absorbance difference rather than ellipticity, though the term ellipticity is still retained. Since CD is actually an absorbance measurement, the Beer-Lambert Law applies. In order to facilitate the quantitation of CD data, we have chosen a slightly different definition of the molar ellipticity, θ_M, that is more analogous to the the Beer-Lambert law.

$$\theta_M = \psi/cb \quad (13)$$

Here ψ is the measured ellipticity in degrees, c is the concentration in moles/L and b is the cell path length in cm. Because of this choice our values of molar ellipticity will be orders of magnitude different from those reported in the literature.

The Relationship of Absorption to Circular Dichroism

Circular dichroism as it has been described so far is a spectroscopic variation of normal absorption. Both phenomena are the result of the interaction of electrons with electromagnetic radiation. Absorption involves the interaction of electrons with unpolarized light and thus represents the more general case. This is discussed first.

Classical theory represents each valence electron as a small, harmonic oscillator. When an electromagnetic field of frequency υ is introduced, the electrons are displaced from their equilibrium positions. But like a harmonic oscillator, these electrons return to their equilibrium states by a series of damped oscillations of frequency υ_0, the natural frequency of the molecule. The displacement of electrons causes changes in the centers of the positive and negative charges resulting in an induced dipole moment. The resulting polarization is proportional to the amplitude of the electron movement and thus to the magnitude of the

electric field. The dielectric constant of the material characterizes the relative ease with which this polarization is induced.

If υ is remote from υ_0, then the electron displacement and the resulting polarization are not large and are in phase with the sinusoidally varying electric field. In this case the energy gained by the electrons in one phase is returned to the light beam in the next. The refractive index of the media can be related to both the velocity of the light beam (the exchange of energy slows down the beam) and the dielectric constant. The dielectric constant represents the molecular response of the substance while the refractive index represents the bulk response. Since the dielectric constant is propotional to the electric field, the refractive index is as well (23).

An electron, however, is not a true harmonic oscillator but behaves more like a quasi-elastically bound particle that exists in a discrete state characterized by a given energy. Transitions between levels can occur if symmetry considerations or changes in the electron spin do not prevent it, and if the incoming electromagnetic beam possesses the proper amount of energy to promote the electron to the next level. This energy requirement can be related to the frequency of the incident radiation by Planck's equation:

$$\Delta E = h\upsilon \qquad (14)$$

where ΔE is the energy between states, h is Planck's constant and υ is the frequency of the incident radiation which must correspond to υ_0 if a transition is to occur. The application of equation 14 quantizes the energy levels.

As υ approaches υ_0 there is a strong probability that an electron will be promoted to an excited state, one that is long lived enough to allow dissipative processes to occur. The net result is a reduction in the intensity of the beam and a permanent absorption of energy occurs. In this region of the spectrum there are large changes in the polarization of the molecule and thus in the refractive index. In addition, the coefficient of absorption is no longer zero but takes on some finite value.

So far only the induction of dipole transitions by the electric field has been discussed. The magnetic field associated with a light wave also induces dipole transitions, however these transitions in ordinary absorption produce negligable effects (26). In chiroptical phenomena the magnetic dipole transitions are important. Rotation has been shown to be a consequence of the combined effects of the electric and magnetic dipole moments which connect the ground and excited states. This conclusion results from quantum mechanical treatments that will not be covered here but are described in detail by Charny (26).

Since the basic physical phenomena of electronic transitions are common to both absorption and CD it is expected that many of the theoretical and experimental aspects of absorption apply to CD. For example, the most commonly encountered electronic transitions are $\sigma \longrightarrow \sigma^*$, $\sigma \longrightarrow \pi^*$, $\pi \longrightarrow \pi^*$, $n \longrightarrow \sigma^*$, and $n \longrightarrow \pi^*$ (32, 33). Only the $n \longrightarrow \pi^*$ and $\pi \longrightarrow \pi^*$ transitions occur at convenient wavelength for absorption and CD measurement. Such transitions are observed for carbonyl groups and conjugated systems, respectively (32). Since the transitions involved are common to both techniques, any parameters affecting the final excited state, such as temperature (33), pH, and solvent effects (32, 33) also affect CD spectra.

Analysis of CD spectra for analytical determinations is no different from the corresponding analysis of absorption data. A Beer-Lambert law plot is used to construct a linear calibration curve, according to:

$$A = \varepsilon bc \qquad (15)$$

where A is the measured absorbance, ε is the molar absorptitivty, b is the cell path length and c is the concentration in moles/L. All modern CD spectropolarimeters actually measure the difference in absorbance between the two circularly polarized components, as defined in equation 5. This is true even though the instrument is usually calibrated in terms of millidegrees in deference to historical precedent. Since CD is actually an absorbance measurement, equation 13 can be revised to an analogous form: $\psi = \theta_M bc$.

This modification of the Beer-Lambert law is subject to all the possible deviations from linearity that affect equation 15. Equation 13 holds for dilute solutions only, since high analyte concentrations cause a reduction in the distance between absorbing species to such a point that a molecule can affect the charge distribution of its neighbors (34). Other causes of nonlinearity in absorbance spectrophotometry such as nonzero intercepts, chemical equilibria (e.g. association, dissociation, and reaction of the absorbing species), polychromatic radiation, and stray light (35, 34) also affect the linearity of CD calibration plots.

Even though there are a large number of similarities between absorption and CD techniques, CD does have some real advantages. The presence of a chiral center is crucial to CD activity. This added structural requirement imparts added selectivity to CD measurements. Any chemical species which possesses a chromophore would be detectable in absorbance measurements and could possibly interfere with the analysis. The presence of just a chromophore or just a chiral center is not sufficient for CD activity and those molecules with either one or the other are eliminated as possible interferences. This added selectivity has been useful in determinations of pharmaceutical preparations where the presence of binders and dyes can make absorption data difficult to decipher. Another advantage CD possesses is the presence of not only positive Cotton bands, but negative ones as well. Often one undistinguished absorption band will result in a number of negative and positive Cotton bands that are easily identified and can be used in qualitative identifications.

Comparison of Chiroptical Methods

IUPAC has defined chiroptical techniques as those "optical (spectroscopic) methods which can differentiate between two enantiomers" (31). This definition is rather limiting, but is practical. Techniques normally classified as chiroptical include polarimetry, ORD,

and CD. Advances in instrument design has promoted studies on infrared CD, fluorescence CD, and Raman CD, but at the present time there are still a number of limiting factors that preclude widespread use of such techniques. Magneto CD and the Faraday effect (the inducement of circular birefringence by a magnetic field) are not classified as chiroptical phenomena by IUPAC (31). The following discussion will focus exclusively on polarimetry, ORD, and CD.

In polarimetric measurements the optical rotation is measured at a single wavelength. The wavelength and temperature of the measurement are recorded as a subscript and superscript to α, respectively, and the solvent employed is also specified. Polarimetry was developed by Biot in 1840 (24), making it not only the oldest chiroptical technique but one of the oldest instrumental techniques in general. Early developments in optical systems, especially in prisms and polarizers, were attempts to improve the sensitivity of polarimetric measurements.

The development of the bunsen burner in 1866 provided early chemists with a monochromatic light source, the sodium flame (24, 30). Consequently the sodium D line, actually a doublet at 589.0 nm and 589.6 nm, has become the standard wavelength for polarimetric measurements. Development of the mercury vapor lamp in the late 1920's provided chemists with four additional wavelengths, 579.1, 577.0, 546.1, and 435.8 nm, for polarimetric studies. The green mercury line at 546.1 nm is usually favored because of its brightness and spectral purity. Today sodium and mercury vapor lamps are the most common sources used in polarimeters. Filters are generally used to isolate the desired wavelength.

Prior to the development of modern instrumentation, polarimetry often provided the only structural information an organic chemist could obtain by instrumental methods. Even though the sodium D line lies in a region that is very insensitive to most colorless compounds, some structural information could be obtained because the sign and magnitude of the optical rotation is controlled by the same parameters that control the corresponding

ORD curve (30). Early chemists also used polarimetric measurements in purity determinations, where the combination of optical rotation, refractive index, and melting point measurements were used to characterize a substance.

Polarimetry also found favor among analytical chemists. It was the method of choice for determinations of sugar in raw and refined forms. A specially modified polarimeter, the saccharimeter, was developed especially for such determinations (24). However analytical applications of polarimetry are complicated by the fact that the relationship between the measured optical rotation and the concentration of an analyte is not strictly linear, thus the specific rotation is not always constant. Values of the specific rotation for very dilute solutions are often used, or one of the following relationships proposed by Biot can be employed (36):

$$[\alpha] = B + Cq \tag{16}$$

$$[\alpha] = B + Cq + Dq^2 \tag{17}$$

$$[\alpha] = B + Cq/D+q \tag{18}$$

where q is the percentage of solvent in the solution and B, C, and D are constants determined from several measurements taken at different concentrations.

Polarimetry and ORD measure the same phenomenon, optical rotation, but ORD is generally measured over a broad wavelength region while polarimetric measurements are made at a few discrete wavelengths. Consequently ORD curves contain more information. However, prior to the development of the photoelectric polarimeter in 1955 (23), ORD measurements were tedious and required a well trained staff. These difficulties coupled with the early development of the sodium flame made polarimetric data easier to obtain and it thus became the preferred technique.

Substances without a chromophore produce a decrease in the magnitude of the optical rotation as the wavelength increases:

$$\alpha = A/\lambda^2 \tag{19}$$

where A is a constant and λ is the wavelength of the incident radiation. The resulting ORD curves rise or fall monotonically with decreases in wavelength as shown in Figure 5a. These curves usually exhibit no inflection points or changes in sign and consequently are termed plain or normal ORD curves. If an optically active chromophore is present in the molecule, the optical rotation increases dramatically as the wavelength of the incident radiation approaches the absorption maximum of the chromophore. Just before the incident wavelength becomes equal to the absorption maximum, the magnitude of the optical rotation decreases rapidly, passing through zero rotation, until another inflection point is reached where the rotation again increases, but at a much slower rate. Figure 5b is an example of the resulting ORD spectrum, here a sigmoidal curve is superposed over the plain ORD curve. The plain curve in this type of spectra is referred to as the background rotation. Such behavior is classified as anomalous ORD. The positive and negative maxima are usually referred to as peak and trough extrema, respectively. The point where the anomalous curve changes sign is called the crossover point and usually corresponds to the absorption maximum of the chromophore.

In wavelength regions far from the optically active absorption band, the background rotation is described by the Drude equation:

$$\alpha = \Sigma^{n}_{i=1} A_i/(\lambda^2 - \lambda_i^2) \qquad (20)$$

where A_i represents the rotation constants, λ is the wavelength of measurement, and λ_i represents the wavelengths of the optically active absorption bands. Equation 19 is an early form of the Drude equation. Those molecules with no chromophores have λ_i below 200 nm. In this case λ is much larger than λ_i and equation 20 reduces to equation 19. For ORD curves with one absorption maxima the Drude equation can be simplified to a one term expression and can then be readily solved. For cases of two or more chromophores the equation becomes difficult to solve for the absorption maximum. Traditionally equation 20 was used to locate absorption maxima that could not be observed directly because of instrumental limitations. Djerassi (30) has pointed out that the advent of modern

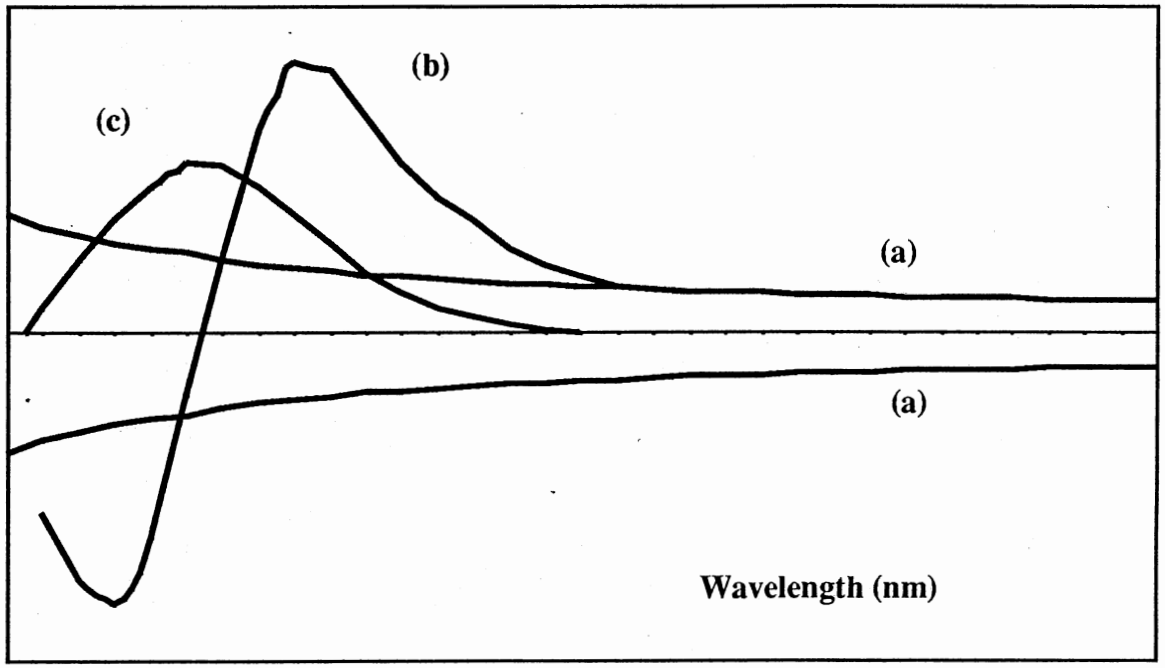


Figure 5. Typical chiroptical spectra: (a) plain ORD curves, (b) anomalous ORD curve with a single Cotton effect, and (c) CD curve with a single positive Cotton effect.

instrumentation allows direct observation of the absorption maximum via ORD or simple UV-visible spectrophotometry, and thus the Drude equation has become an interesting artifact.

Since ORD is a spectral scan, it provides more information than a single polarimetric measurement does. ORD can distinguish between molecules with similar structures much more readily than can polarimetry because of differences in the anomalous regions of their spectra. Thus ORD is more useful in the analysis of unseparated mixtures than is polarimetry, and since the optical rotation in the region of the optically active absorption band is usually much larger than at the sodium D line, ORD possesses greater sensitivity.

One of the major drawbacks to ORD is the presence of background rotation. Even for simple ORD spectra the background can make it difficult to establish the baseline since it is constantly changing. In some cases the continous background from chromophores which absorb in the far UV is so great that it can conceal the anomalous curve.

Background rotation is not a problem in CD measurements since $\Delta\varepsilon$ is significant only at wavelengths corresponding to an optically active absorption band. The CD curve often possesses a shape very similar to its corresponding absorption curve, Figure 5c. The wavelength of the CD maximum is usually very close to that of the absorption maximum. However, due to the definition of $\Delta\varepsilon$ (equation 7) the CD maximum is not always positive, but is actually a signed quantity. Therefore CD spectra can exhibit positive and negative maxima, positive and negative minima, and positive and negative inflection points. These spectral details arise from changes in ε_L with respect to ε_R as the absorption band is traversed and are very useful for qualitatively identifying an analyte.

Those wavelength regions which correspond to optically active absorption bands produce both CD curves and anomalous ORD curves. Such curves are called Cotton effects after the French physicist Aime Cotton who carried out the early investigations of both phenomena. In ORD if the peak occurs at a longer wavelength than the trough, the anomalous curve is termed a positive Cotton effect. If the trough precedes the peak, it is a

negative Cotton effect. A similar convention holds for CD curves, positive signals are termed positive Cotton effects, and vise versa. Multiple Cotton effects are possible for those analytes with more than one optically active absorption band (31).

Anomalous ORD and CD curves contain the same information since both phenomena result from electronic transitions. Because of their common source there is a mathematical relationship between the two referred to as the Kronig-Kramers transform. Knowledge of the CD curve over a given spectral region allows the corresponding ORD spectrum to be calculated and vice versa (27).

$$[\phi(\lambda)] = 2/\pi \int_0^\infty [\theta(\lambda')] (\lambda'/\lambda^2 - \lambda'^2)\, d\lambda' \quad (21)$$

$$[\theta(\lambda)] = -2/\pi \int_0^\infty [\phi(\lambda')] (\lambda'^2/\lambda^2 - \lambda'^2)\, d\lambda' \quad (22)$$

The resulting correlations are generally semi-quantitative and must be calculated for each individual transition.

CD has many analytical advantages over ORD. CD curves tend to be simpler in form than ORD curves, especially when multiple transitions are involved. This simplicity combined with the absence of background rotation makes CD data easier to analyze. Quantitation of mixtures is less complicated because signals occur only at wavelengths corresponding to the absorption bands of the individual molecules. The baseline outside these regions is well defined. Weak Cotton effects and overlapping absorption bands are best seen in CD spectra, again because background rotation is absent.

CD activity does require the presence of a chromophore in close proximity to the chiral center. ORD and polarimetry are less restrictive. They require only the presence of a chiral center. However, there are ways to induce CD activity in those analytes that either lack the chiral center or the chromophore. β-cyclodextrin (discussed in Chapter VII) is a chiral molecule known to complex with substrates that possess a phenyl or naphthyl ring, thus inducing the necessary chirality needed for CD activity. Chromophores can be introduced to chiral molecules by employing a variety of colorimetric reactions: a practice already commonly used in simple UV-visible absorption spectrophotometry.

Another basis for comparison of polarimetry and CD is the growing interest in chiroptical detectors in liquid chromatography (LC). Yeung and coworkers have developed laser based polarimetric and CD detectors for LC (21, 37). Stopped-flow CD detectors have been developed by Jasco, Inc. (19) and by Westwood, Games, and Sheen (38).

Polarimetric detectors are ideal if total separation of a mixture is possible. Polarimeters are relatively inexpensive to purchase and to operate and will respond to absorbing and nonabsorbing analytes equally well. This detector has two useful modes of operation: 1) the direct mode where the rotation caused by an analyte is measured (37), and 2) the indirect mode where the change in the rotation of the background for an optically active solvent is measured (39). The second mode can be employed in the analysis of achiral analytes.

The LC detectors mentioned above were used for stopped-flow analyses (19, 38) and measured short spectral scans in order to gain structural information as well as to identify the LC peaks. Spectral CD scans are far more useful than single wavelength data. Employing CD detectors with only single wavelength capability reduces this detector to no more than an expensive polarimeter with a more limited range of applications because nonabsorbing chiral analytes are invisible. However, for those mixtures which can not be separated, the capability of CD to differentiate compounds would be useful. But coupling CD to HPLC is not necessary in determinations of many complex mixtures. CD has been shown to be capable of identifying and quantitating a number of analytes in complex mixtures and matrices without a prior chromatographic separation step. Such applications have recently been reviewed by Purdie (40).

Development of on-the-fly fully scanning CD detectors is difficult due to source stability problems (39) and other instrumental parameters. These technical problems could be overcome if sufficient demand warranted further investigation and development.

CHAPTER III

INSTRUMENTATION

Introduction

All CD and UV spectra were measured by a model J-500A automatic recording spectropolarimeter produced by Japan Spectroscopic Co., Ltd. (JASCO). The light source is a 450 watt xenon arc lamp which requires water cooling. Boil off from a liquid nitrogen cylinder is used to purge the monochromator and sample chambers. Nitrogen purging is necessary because the ozone generated by the xenon lamp can damage the optical system. The UV spectra were obtained by switching to the HT setting on the instrument.

Initially data acquisition was handled by the JASCO DP-500 data processor. CD spectra were output to a chart recorder and the signal heights were measured manually. Recently, the data processor was replaced by a JASCO model IF-500-2 interface which allowed the J-500A to be coupled to an IBM-AT computer clone. CD signals were then measured digitally by the computer and CD spectra were printed on a Hewlett-Packard 7475A graphics plotter. The data presented in Chapters IV, V, VI, and VII were obtained using the DP-500, while data in Chapter VIII were acquired using the new computer system.

The instrument was calibrated daily with a 0.025% (W/V) solution of androsterone in dioxane as suggested by JASCO (41). Instrument parameters, such as sensitivity and number of repeats to be signal averaged, were optimized in order to obtain the best possible

spectra in a reasonable amount of time. Optimum conditions for each analysis are specified in the corresponding chapter.

A Sartorius 2703 analytical balance (Brinkman Instruments, Inc.) was used for sample weights of greater than 10 mg. Weights less than 10 mg were measured on a Cahn RG Electrobalance (Cahn Instrument, Co.).

Principles of Measurement

CD signals can be measured in two different ways. The first involves direct measurement of the eccentricity of the elliptically polarized transmitted light beam, the second measures the absorption difference between the left and right circularly polarized transmitted beams, ΔA. All commercially available CD spectropolarimeters, including the J-500A, use the latter type of measurement.

Direct measurement of ΔA is difficult. If Beer's Law is substituted into equation 7, the source of the difficulty becomes more obvious.

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R = (1/bc) \log (\phi_R/\phi_L) \quad (23)$$

Where b is the cell path length, c is the analyte concentration in moles/L, and ϕ_R and ϕ_L represent the radiant power (flux) of the right and left circularly polarized beams after passage through the sample. Measurement of ϕ_R and ϕ_L will result in a value for $\Delta\varepsilon$, however, the ratio of ϕ_R/ϕ_L is almost unity. The small difference between these two fluxes makes accurate measurement of $\Delta\varepsilon$ difficult ($\Delta\varepsilon$ is usually on the order of 10^{-2} to 10^{-3} while ε can be on the order of 10^3 to 10^5).

JASCO engineers have overcome this limitation by choosing to measure two other fluxes:

$$\phi_A = 1/2(\phi_R + \phi_L) \quad (24)$$

and

$$S = (\phi_R - \phi_L) \quad (25)$$

where ϕ_A is the average radiant power after passage through the sample and S is the difference in the radiant power of the left and right circularly polarized light. ϕ_A produces a direct signal at the photomultiplier tube, while S produces an alternating signal. Substitution of equations 24 and 25 into equation 23 yields the following approximation:

$$\Delta\varepsilon \propto (1/bc)(S/\phi_A) \tag{26}$$

If E_A and E_S represent the photomultiplier output voltages corresponding to ϕ_A and S respectively, equation 26 can be rewritten as:

$$\Delta\varepsilon \propto (1/bc)(E_S/E_A) \tag{27}$$

where $E_S << E_A$. Comparison of the relative magnitudes of these two voltages provides an accurate measurement of the CD signal.

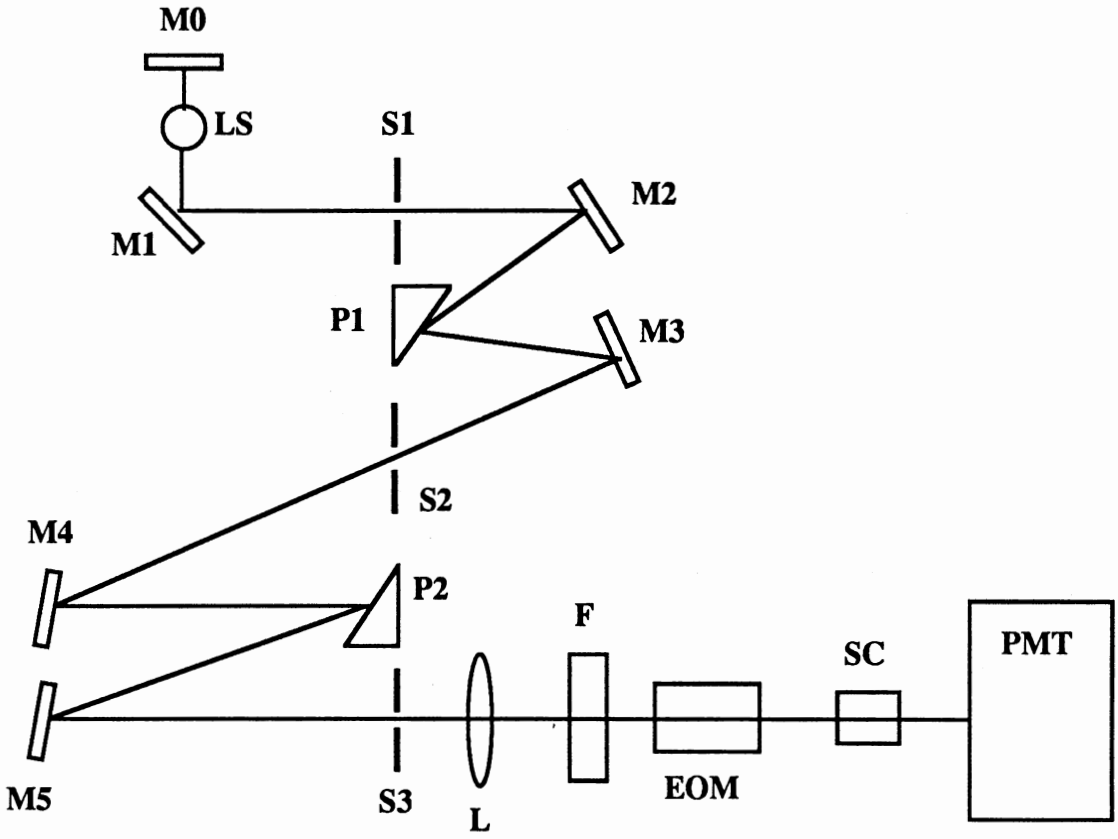
Description of the J-500A Optical System

The optical system of a CD spectropolarimeter is very similar to that of a conventional UV-visible spectrophotometer. However, the need for monochromatic circularly polarized light in CD measurements requires more complex and diverse optical components.

Figure 6 is a schematic representation of the J-500A optical system. A spherical mirror, M1, focuses the light beam on to the entrance slit S1. S1 marks the entrance of the first of two monochromators, the second is marked by slit S2. The need to keep stray radiation at a minimum makes the double monochromator a crucial part of the J-500A design.

Prisms P1 and P2 serve a dual purpose. Both are made from crystal quartz and have different axial directions with respect to each other. This allows them to function as birefringence polarizers in addition to acting as dispersion elements. The ordinary ray emerging from P2 is monochromatic and linearly polarized. Lens L is used to focus this beam onto the filter F which filters out any remaining unpolarized light. EOM is the electro-optic modulator which produces the circularly polarized light. In the J-500A a Pockels cell is employed. Application of an electric field to the Pockels cell crystal causes a

change in the refractive index and propagation velocity for the resulting ordinary and extraordinary beams. These beams are linearly polarized and are perpendicular to each other , but are out of phase. When the phase difference is a quarter wavelength ($\pi/2$) or any uneven number of quarter wavelengths, the resulting light beam is circularly polarized. The direction of polarization is changed by changing the direction of the electric field. The Pockels cell alternately produces left and right circularly polarized light at a frequency of 50 kHz. After interaction with the sample, the photomultiplier tube receives the transmitted beams and electronically recombines them as described above.



MO, M1, M2, M3, M4, M5: spherical mirrors
LS: light source
S1, S2, S3: slits
P1: first prism (horizontal axis)
P2: second prism (vertical axis)
L: lens
F: filter
EOM: electro-optical modulator (Pockels cell)
SC: sample cell
PMT: photomultiplier tube

Figure 6. Optical system for the J-500A spectropolarimeter (adapted from reference 41).

CHAPTER IV

DETERMINATION OF β-LACTAM ANTIBIOTICS

Introduction

The first β-lactam antibiotics, the penicillins, were discovered in 1928. Nearly thirty years was to pass before their analogs, the cephalosporins, were introduced. The ability of β-lactam antibiotics to interfere with cell wall production in bacteria has made them the second most widely prescribed class of drugs for treating bacterial infections (42). Because of the widespread use of β-lactams, analytical determinations of these compounds in pharmaceutical and biological samples is important. Early analysis methods included turbidimetric assay, iodometric titration, colorimetric reactions, and thin-layer chromatography (43, 44). A recent review (45) includes variations on these older techniques in addition to the more modern techniques such as polarography, reverse phase HPLC, flow injection analysis, and mass spectrometry.

Detection of β-lactam antibiotics by CD is not new. Most early CD work (46-49) concentrated on elucidating structural features of the β-lactam ring. However, Rasmussen and Higuchi (50) did determine the penicillin content by measuring changes in the specific rotation and in the CD signal after the addition of penicillinase. CD was reported to be the method of choice, but was limited by the UV absorption at 231 nm. We have characterized the CD spectra of nine penicillins and three cephalosporins dissolved in aqueous pH 5.4 buffer. No enzymatic reaction was necessary, nor was there any interference due to excessive UV absorption at 231 nm.

Experimental

The β-lactams amoxicillin, ampicillin, cloxacillin, dicloxacillin, methicillin, nafcillin, oxacillin (Bristol Myers), cephalexin, cephalothin, and cephapirin (Bristol Myers and Sigma Chemical Co.) or their sodium salts were obtained for study. The potassium salts of penicillin V (Pen-V) and penicillin G (Sigma Chemical Co.) were also obtained. All were used without further purification. Pen-V tablets (250 mg) (Parke Davis) were obtained from a local pharmacy. A sample of filtered and arbitrarily diluted Pen-V fermentation broth was furnished by Eli-Lilly, Inc. Lactose (Fisher), starch, (Mallinckrodt), and caffeine hydrobromide (Matheson Coleman and Bell) were used in the preparation of in-house mixtures. The pH 5.4, 7.6, and 9.2 buffers were obtained from Micro Essential Laboratory and prepared as specified on the label. The CD spectra of the β-lactams in pH 5.4 buffer exhibited the best signal quality. The spectra obtained from samples in pH 7.6 and 9.2 buffers showed considerably more noise.

The penicillin content in the laboratory samples was varied from approximately 7% to 70% by weight. Varying proportions of lactose, starch, or caffeine were added and the preparations mixed thoroughly. These additives are typical of those present in pharmaceutical preparations. Lactose and starch are chiral but are nonabsorbing, while caffeine is not chiral but does absorb UV radiation. None should affect the actual quantitation of the CD data, but their presence may affect the signal quality, thus affecting the precision of the determinations. Starch was also chosen because of its insolubility in the aqueous buffer chosen for sample extraction. Centrifugation was required to remove insoluble matter remaining after extraction of commercial preparations. Starch was added to laboratory mixtures to test for possible adsorption of the analyte on insoluble materials that are present in the commercial preparations.

Pen-V tablets were first powdered by shaking in a Wig-L-Bug for approximately two minutes. Three samples of approximately 4 mg were chosen at random from each tablet.

These samples were extracted with 25 mL of pH 5.4 buffer solution, centrifuged, and the CD spectrum was then measured. The fermentation broth was first defrosted, 50 μL aliquots were taken and diluted to 10 mL with buffer. The CD spectrum was run directly.

Results and Discussion

The basic structures of the penicillin and the cephalosporin homologues are shown in Figure 7. The identities of the R and R_1 substituents, as listed in Table I, distinguish the individual lactams.

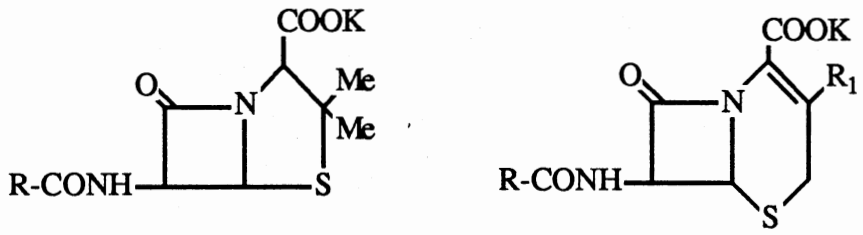


Figure 7. Molecular structures of the penicillins and the cephalosporins.

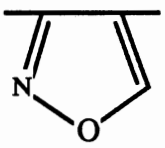
The β-lactam ring is the active chromophore in these compounds. It is responsible for the UV absorption occurring in the 320-220nm region, Figure 8. The CD spectra, Figure 9, were recorded over the same spectral region. A comparison of the UV and CD reveals that the CD is more useful in analytical determinations because there are no definite absorption bands with discrete maxima in the UV spectra. All nine penicillins produced a strong positive Cotton band maximizing at 230 ± 2 nm, Figure 9a. The three cephalosporins exhibited two Cotton bands, the first a positive maximum at 260 ± 2 nm

TABLE I

MOLECULAR STRUCTURES AND MOLAR ELLIPTICITIES FOR PENICILLINS AND CEPHALOSPORINS

Name	R[a]	R_1	θ_M (nm)
amoxicillin	2-amino(*p*-hydroxyphenyl)-		+398 (230)
ampicillin	2-amino-2-phenyl-		+431 (230)
cloxacillin	3-(*o*-chlorophenyl)-Z-		+333 (230)
dicloxacillin	3-(2,6-dichlorophenyl)-Z-		+323 (230)
methicillin	2,6-dimethoxyphenyl-		+265 (230)
nafcillin	2-ethoxynaphthalenyl-		+237 (230)
oxacillin	3-phenyl-Z-		+482 (230)
Pen-G	benzyl-		+394 (230)
Pen-V	benzoxy-		+363 (230)
cephalexin	2-amino-2-phenyl-	$-CH_3$	+395 (260) -632 (230)
cephalothin	2-(2-thienyl)-	$-CH_2OCOCH_3$	+452 (260) -600 (230)
cephapirin	2-(4-pyridylthio)-	$-CH_2OCOCH_3$	+501 (260) -674 (230)

[a] The symbol Z is used to represent:



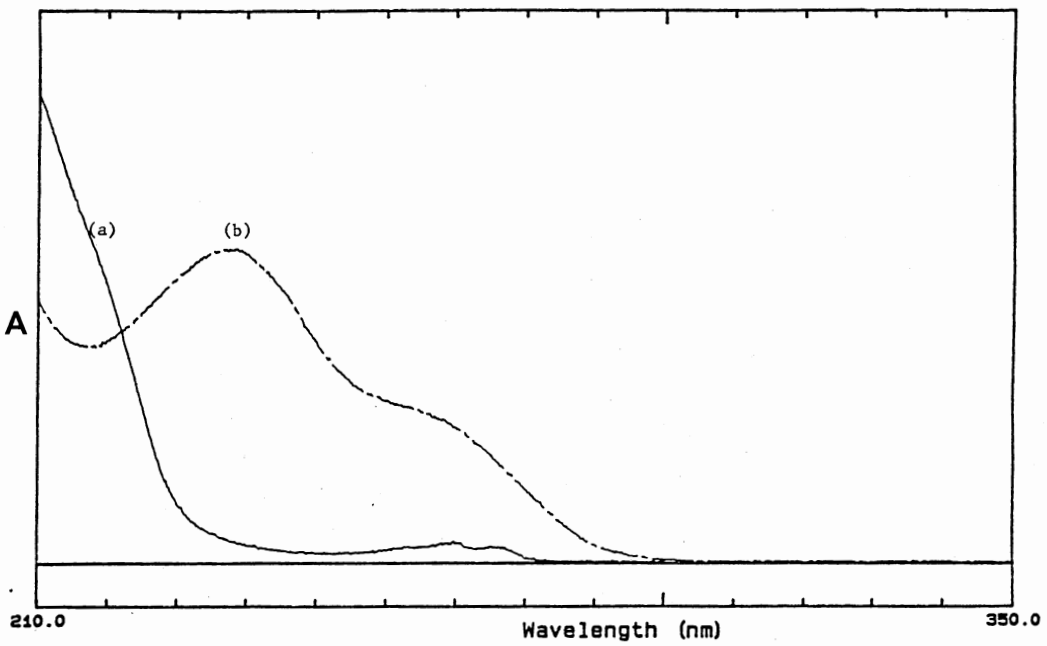


Figure 8. UV absorption curves for (a) Pen-V at 1.4×10^{-4} M and (b) cephalothin at 1.3×10^{-4} M in pH 5.4 buffer.

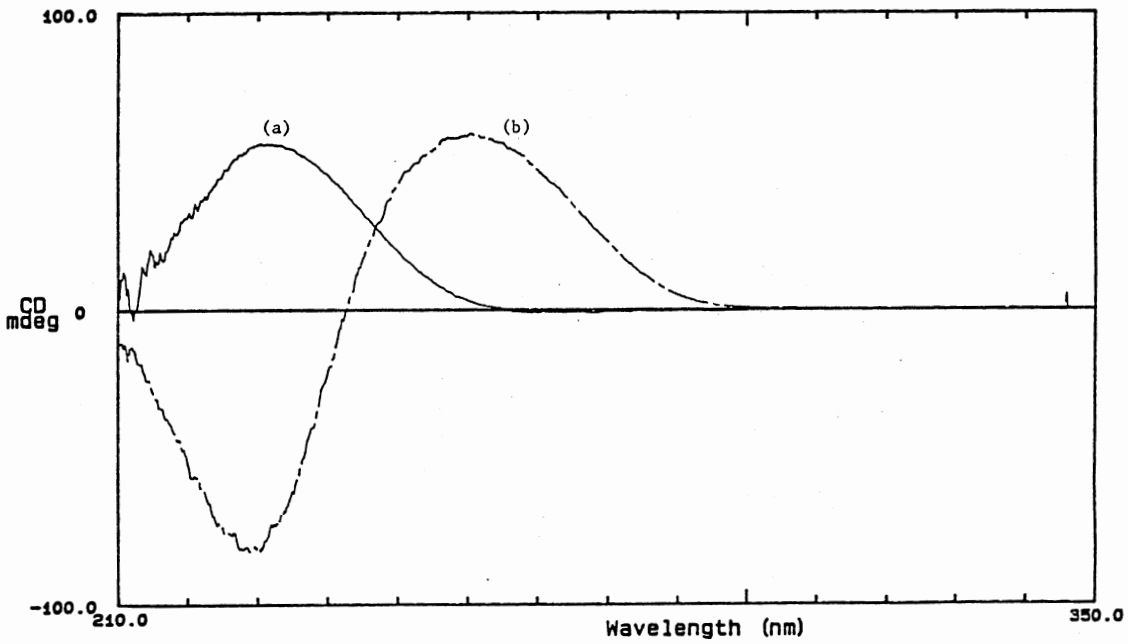


Figure 9. CD spectra for (a) Pen-V and (b) cephalothin in pH 5.4 buffer. Same concentrations as in Figure 8.

associated with the $\pi \rightarrow \pi^*$ transition. The second maximum at 230 ± 2 nm is negative and separated from the first by a cross over point of zero ellipticity at 243 nm, Figure 9b. Discrimination among analogs of a group (i.e., penicillin or cephalosporin) is not possible since the only spectral difference present is the magnitude of the molar ellipticities, θ_M, Table I. The molar ellipticities for ampicillin, amoxicillin, Pen-V, and cephalothin were obtained from calibration curves. The molar ellipticities reported for the other compounds were calculated from the maxima at only one concentration in order to obtain a rough idea of the relative intensities.

A summary of the in-house and commercial lactam mixtures studied is presented in Table II. Samples S1-S6 are in-house laboratory blinds. The addition of caffeine, a strong absorber, decreased the S/N ratio in the resulting spectra, but presented no other difficulties during the determinations. Both lactose and starch possess chiral centers, but only the lactose is soluble in the aqueous buffer system. Those mixtures containing the soluble additive exhibited excellent correspondence between the theoretical and measured values. However, there is a decrease in correspondence for those samples containing the insoluble additive, indicating the possibility of analyte adsorption on the undissolved starch. Even so, reproducibility was good, within ±0.5% for most of the samples. The mixture containing the caffeine exhibited a much larger standard deviation (±2%). This decrease in precision is probably linked to the decrease in the S/N ratio.

Determination of Pen-V in tablet form was a straightforward procedure, but the reproducibility was poorer than that observed with the in-house mixtures. This should not be surprising since the tablets are prepared from aliquots taken from a much larger and less uniform sample. The reported Pen V content, $63.3 \pm 0.9\%$ (Table II), was normalized to total sample weight, making it possible to directly compare the results from all analyzed tablets.

The determination of Pen-V in the fermentation broth presented no difficulties even in the presence of such a complex matrix. The composition of the broth as reported by Lilly

TABLE II

DETERMINATIONS OF IN-HOUSE AND COMMERCIAL LACTAM MIXTURES

Mixture	Lactam	Additive	Theoretical Percent	Measured Percent
S1	Pen-V	lactose	23.9	23.6
S2	Pen-V	lactose	40.5	39.5
S3	Pen-V	caffeine	72.3	72.2
S4	Pen-V	starch	7.1	6.3
S5	Pen-V	starch	29.6	28.2
S6	Pen-V	starch	63.5	63.8
PC1	Pen-V Cephalothin	lactose	1.28 0.63	1.53 0.65
PC2	Pen-V Cephalothin	lactose	2.22 4.79	2.53 4.74
PC3	Pen-V Cephalothin	caffeine	6.47 5.58	7.19 5.46
PC4	Pen-V Cephalothin	lactose	3.50 1.80	4.11 1.68
Tablet	Pen-V	?	(250 mg)	63.3 ±0.9 (249.0 ± 41.4 mg)
Fermentation Broth	Pen-V	see text	(7.94 +0.1 mg)	(8.07 ± 0.12 mg)

was 7.94 mg/mL Pen-V and less than 0.1 mg/mL *p*-hydroxy-Pen-V (as the potassium salts), 1.01 mg/mL phenoxyacetic acid and 0.12 mg/mL *p*-phenoxyacetic acid (as the sodium salts), inorganic salts of alkali and alkaline-earth metals plus ammonium and trace amounts of heavy metal cations, and various unknown organic compounds typically found in fermentation broths. The organism and several large proteins were removed in the filtration performed by Ely-Lilly. From this information the only CD active components present were the two penicillins. The presence of the other components caused little distortion in the resulting CD spectrum, however, there was a slight asymmetry in the short wavelength end of the band as compared to the standard. There was little loss in the S/N quality. The reported Pen-V content, 8.07 mg/mL, is actually representative of the total penicillin content calculated as if the signal were produced solely by Pen-V. We have assumed that the θ_M for *p*-hydroxy-Pen-V and for Pen-V are the same. Since the reported value is within 1% of the theoretical value, the assumption is acceptable. Another source of deviation resulted from the process of freezing and defrosting the broth. When the frozen broth is defrosted, some of the remaining cells burst, releasing their cellular fluid thus diluting the original sample. Initial samples were only slightly affected by this process, but later samples showed significant decreases in Pen V content. An independent analysis performed by Lilly on the same batch of frozen broth yielded a similar result.

Samples PC1-PC4, Table II, are in-house mixtures of Pen-V and cephalothin. Determinations of the relative concentrations were performed without a separation step. Quantitation of the cephalosporin was straightforward when it was present in large excess because it was essentially the only contributor to the positive band at 260 nm, Figure 9. For the other mixtures determination was accomplished by employing a simple curve fitting program in which weighted contributions from the standard curves were added in order to simulate the experimental curve (51). Agreement between theoretical and calculated values is good, Table II.

This work is a further endorsement of the analytical capabilities of CD in the direct determination of pharmaceuticals. Assuming that all lactams could be extracted by the same procedure, the relative time taken for detection by CD is very short. Further time savings could be achieved by automation. In addition, the developed procedure requires no derivatization or separation steps, nor does it require the addition of an internal standard.

CHAPTER V

DETERMINATION OF *RAUWOLFIA SERPENTINA* ALKALOIDS

Introduction

Rauwolfia serpentina is a plant native to subtropical regions of India. The ancient Indians used this plant to treat snake bite and dysentery (52). In 1933, Chopra, Guptia, and Mukherjee reported that *R. serpentina* extracts possessed hypotensive activity (52). However, it wasn't until 1952 that Bein and coworkers (52) isolated reserpine from the root of the plant. Reserpine is the alkaloid responsible for the antihypertensive and sedative activity of the extract.

In addition to reserpine, *R. serpentina* contains as many as 22 other alkaloids. Two of these, rescinnamine and deserpidine, also possess antihypertensive activity (52, 53). Reserpine, rescinnamine, and deserpidine are all used to treat mild to moderate hypertension (54). A pharmaceutical formulation of raw *R. serpentina* root is also available and is employed in cases of mild hypertension (55).

HPLC has been used to determine the relative concentrations of the alkaloids present in multi-component pharmaceutical formulations (56) and in *R. serpentina* root extracts (57). In contrast to the above HPLC procedures, most other reported assays determine only the reserpine and/or the rescinnamine concentration. Results are generally reported as total reserpine or total reserpine group alkaloids. Fluorometric (58, 59) and electrochemical (60) detection is often employed, both as stand alone techniques and as detectors for LC

methods (61, 62). GC (63) and GLC (64) have also been used. Many of the above methods require derivatization steps during sample preparation to eliminate interferences.

The CD spectra for four indole alkaloids present in *R. serpentina* have been characterized and calibration curves obtained for reserpine, rescinnamine, and ajmaline were used in the quantitative analysis of laboratory mixtures. Analytical determinations of reserpine in a few pharmaceutical preparations are also reported.

Experimental

The indole alkaloids, reserpine, rescinnamine, ajmaline, all as free base,(Sigma Chemical Co.), yohimbine hydrochloride (Aldrich Chemical Co.), and the diuretic substances furosemide and hydrochlorothiazide (Sigma Chemical Co.) were obtained and used without further purification. A standard of *R. serpentina* root was obtained from the United States Pharmacopeial Convention (USP) and was used without further purification. Reserpine tablets (0.25 mg) (Serpasil®, Ciba), and 50 mg *R. serpentina* tablets (Raudixin®, Squibb) were obtained from a local pharmacy. All solvents and other reagents used in the extraction procedures were of analytical grade quality.

In-house binary and tertiary mixtures of ajmaline, reserpine, and rescinnamine were prepared from chloroform stock solutions. The content of each alkaloid was varied from approximately 10 to 90% by weight for the binary mixtures and from 10 to 70% for the tertiary mixtures. Laboratory mixtures of hydrochlorothiazide and reserpine were prepared from solid standard material. The resulting chloroform solution was agitated for 45 minutes and centrifuged to remove any remaining hydrochlorothiazide which is only partially soluble in the chosen solvent. Weights were chosen to approximate a commercially available dosage form, Serpasil-Esdrix #1®, which contains both substances.

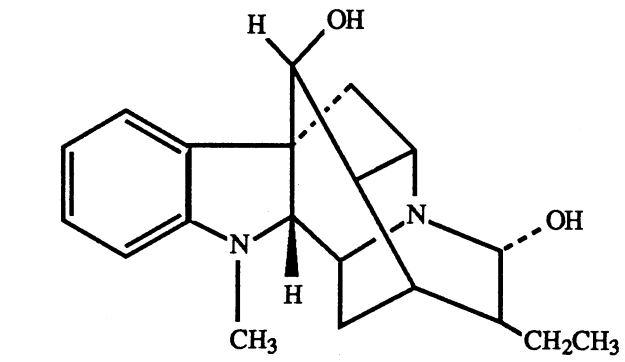
Individual reserpine tablets were reduced to a fine powder by shaking in a Wig-L-Bug. The powder was extracted with chloroform and the resulting solution agitated for 45 minutes. Centrifugation was required to reduce the amount of suspended particulate matter.

Entire *R. serpentina* tablets and 50 mg aliquots of USP root powder were extracted according to the procedure described by Cieri (57), but with the following modifications. The volume of H_2SO_4 was reduced from 60 to 25 mL and the volume of $CHCl_3$ used in each extraction step was reduced from 30 to 10 mL. Pooled $CHCl_3$ extracts from the Raudixin® tablets were washed with one 10 mL portion of 0.1 N NaOH to remove interferences due to dyes used in the tablet coating (58). The $CHCl_3$ fraction was evaporated under air to a volume of approximately 5 mL. Anhydrous $CaCl_2$ was added to remove any remaining moisture. The $CHCl_3$ was decanted and combined with $CHCl_3$ washings of the $CaCl_2$. The sample was diluted to a final volume of 10 mL. The extraction procedure was carried out under reduced lighting to avoid photooxidation (65).

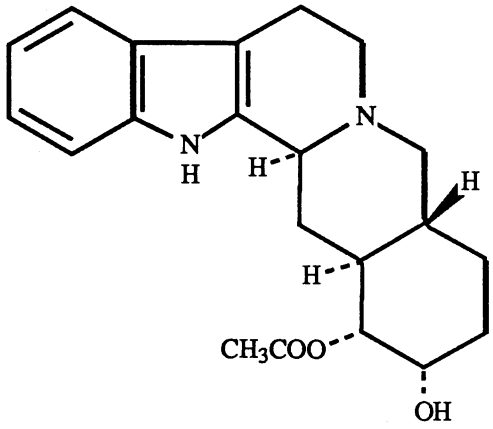
Results and Discussion

The molecular structures of the four CD active indole alkaloids investigated are given in Figure 10. All four exhibit absorption bands in the 300-210 nm range. Since CD activity occurs only in the vicinity of an absorption band, CD spectra were obtained for the 370-240 nm region. A distinctive CD spectrum was observed for each alkaloid, Figure 11. The spectrum of reserpine consists of a single negative Cotton band while the spectrum of yohimbine hydrochloride shows a single positive band. The spectra for ajmaline and rescinnamine are more complex having two and three Cotton bands, respectively. Table III details the molar ellipticities for each compound at the corresponding wavelength maxima.

The determination of reserpine in Serpasil® tablets was straightforward. The results from six tablets yielded an average reserpine content of 0.249 ± 0.034 mg/tablet, compared



(a)



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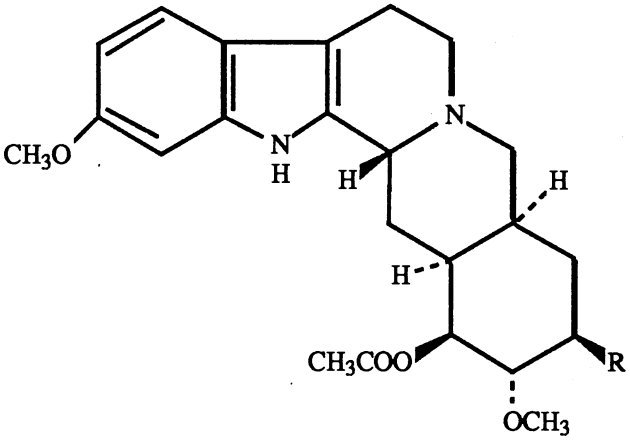
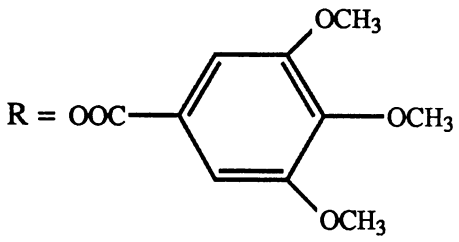
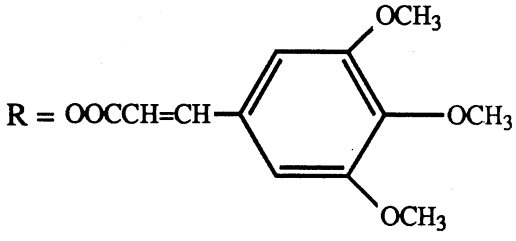




(c)



(d)

Figure 10. Molecular structures of the indole alkaloids (a) ajmaline, (b) yohimbine, (c) reserpine and (d) rescinnamine.

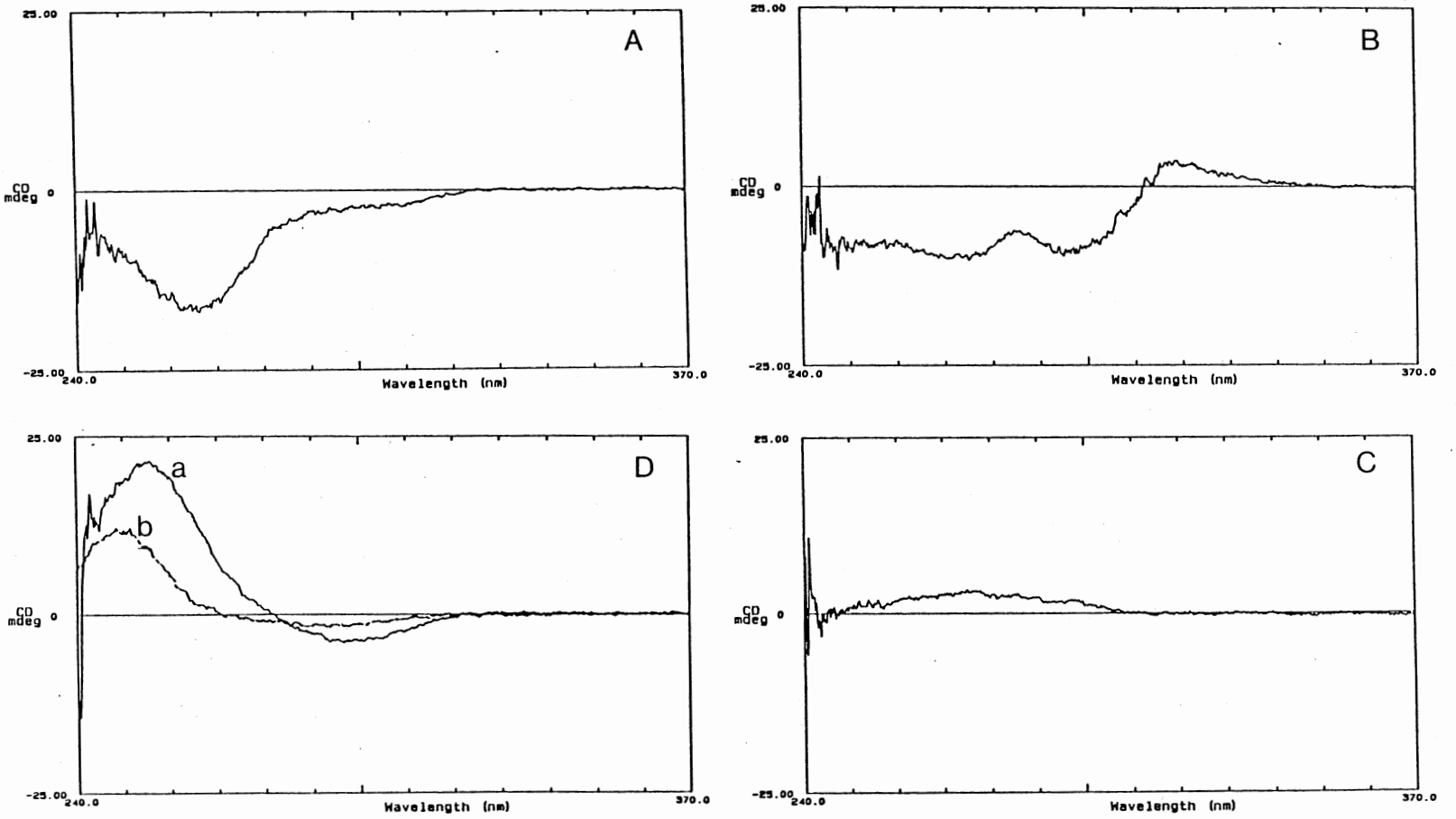


Figure 11. CD spectra of the indole alkaloids (A) reserpine, (B) rescinnamine, (C) yohimbine HCl in $CHCl_3$ and (D) ajmaline in (a) $CHCl_3$ and (b) methanol-sulfuric acid.

TABLE III

MOLAR ELLIPTICITIES FOR THE INDOLE ALKALOIDS OF *RAUWOLFIA SERPENTINA*

Indole Alkaloid	θ_M[a] (nm) (deg/M cm)
ajmaline	-70 (298) +394 (255)
rescinnamine	+57 (322) -131 (297) -153 (274)
reserpine	-262 (266)
yohimbine hydrochloride	+62 (269)

[a]All molar ellipticities for chloroform solutions.

with a labeled content of 0.250 mg/tablet, Table IV.

Diuretics are often prescribed in conjunction with antihypertensives, and often both medications are combined in a single formulation. The diuretics, hydrochlorothiazide and furosemide, absorb in the UV but are achiral, so are not CD active. The presence of either diuretic did not interfere with the determination of reserpine. The calculated reserpine content of a hydrochlorothiazide/reserpine laboratory mixture RH1 compared well with the theoretical content, Table IV.

A more complex extraction procedure was needed for the determination of reserpine present in the root material due to the large number of other alkaloids present. It had been demonstrated (6) that ajmaline, yohimbine, along with other alkaloids remain in the H_2SO_4 fraction, while reserpine and rescinnamine transfer to the $CHCl_3$ fraction. Analysis of USP

TABLE IV

DETERMINATION OF RESERPINE IN LABORATORY AND COMMERCIAL MIXTURES

Mixture	Content	Theoretical Quantity	Measured Quantity
Serpasil®	Reserpine	0.250 mg/tablet	0.249 ± 0.034 mg/tablet
USP R. serpentina (root)	Reserpine Rescinnamine	0.15-0.20% NA	0.165 ± 0.002% NA
Raudixin®	Reserpine Rescinnamine	0.15-20% NA	0.164 ± 0.017% NA
RH1	Reserpine Hydrochloro-thiazide	0.142 mg 24.6 mg	0.137 mg NA

root standard and Raudixin® tablets yielded a reserpine content well within the reported analytical range, Table IV.

The positive Cotton band at 322 nm typical for rescinnamine was not present in the spectra for the root extracts. Any contribution to the total signal from rescinnamine at 297 or 274 nm was concealed by the very large reserpine signal. Reserpine is present in such large excess and has such a large θ_M value, that the rescinnamine contribution to the total signal height is negligible. This made direct determination of rescinnamine in either the USP samples or the Raudixin® tablets impossible.

The limits on the detectability of the minor components in a mixture can be estimated from the study of laboratory mixtures. This was done for mixtures of

reserpine/rescinnamine, reserpine/ajmaline and reserpine/rescinnamine/ajmaline. A simple curve fitting program (51) was used to analyze the experimental data. The program simulates the experimental curve by adding weighted contributions from the standard curves. The results obtained from this procedure are recorded in Table V. In an effort to present the data for the many mixtures investigated both efficiently and succinctly, they have been collected under broad ranges of composition (column 3) and the cumlative average errors and median errors are listed as single figures in columns 4 and 5.

TABLE V

DETERMINATIONS OF INDOLE ALKALOIDS IN LABORATORY MIXTURES

Mixture	Indole Alkaloid	Theoretical Percentage	Average Error	Median Error
RC 6-10	reserpine	9.8 - 88.4%	15.7%	2.8%
	rescinnamine	11.6 - 90.2%	5.4%	4.7%
RA 1-5	reserpine	9.9 -90.0%	20.5 %	4.2%
	ajmaline	10.0 - 90.1%	7.8%	5.3%
RCA 1-9	reserpine	9.0 - 69.9%	62.1%	30.6%
	rescinnamine	10.0 - 70.0%	18.0 %	13.6%
	ajmaline	15.1 - 64.8%	21.7 %	20.5%

Relative errors are generally lowest for those components present in the largest amounts and are largest in the opposite situation. The average and median errors include both of these cases. The median errors are all lower than the corresponding average errors,

indicating that a few mixtures in each set have especially large errors. The best results were obtained from the 75%/25% reserpine/ajmaline or reserpine/rescinnamine binary mixtures. Calculated compositions for those mixtures had a relative error of approximately 0.8%. Poor results were obtained for those mixtures in which reserpine was the minor component. For example, a reserpine content of 10% yielded an experimental result of 2.5% (a 75% error) for both binary mixtures. The corresponding ajmaline or rescinnamine content was approximately 97.5% (a relative error of 9% when compared with the theoretical value of 90%). The 90%/10%, 50%/50%, and 25%/75% reserpine/ajmaline or reserpine/rescinnamine mixtures exhibited errors as large as 20% in the estimations of the minor component. While error in the estimation of the major component did not exceed 6%.

Correspondence between the theoretical percent and calculated percent for the tertiary mixtures was not as good as that for the binary mixtures. Again, the largest errors were associated with mixtures containing 10% reserpine. The best correspondence was obtained for those mixtures where one alkaloid was present in large excess (approximately 70%). In both the binary and tertiary mixtures, rescinnamine has the best correspondence between measured and theoretical percent. Examination of Figure 12 reveals that the rescinnamine peak at 322 nm is not greatly affected by the presence of the other two alkaloids. So rescinnamine can be more easily quantitated even at lower concentrations. Ajmaline has a large molar ellipticity, which promotes good correspondence even at low concentrations. Reserpine has neither of these advantages and consequently has the poorest correspondence in all three mixtures. At low concentrations the reserpine signal is dwarfed by the signals of the other two alkaloids making accurate quantitation difficult.

This work is another example of the utility of CD in the determination of pharmaceutically important substances. CD detection allowed the extraction procedure to be simplified, resulting in reduced analysis time. Determinations did not require derivatization of the analyte or the addition of internal standards.

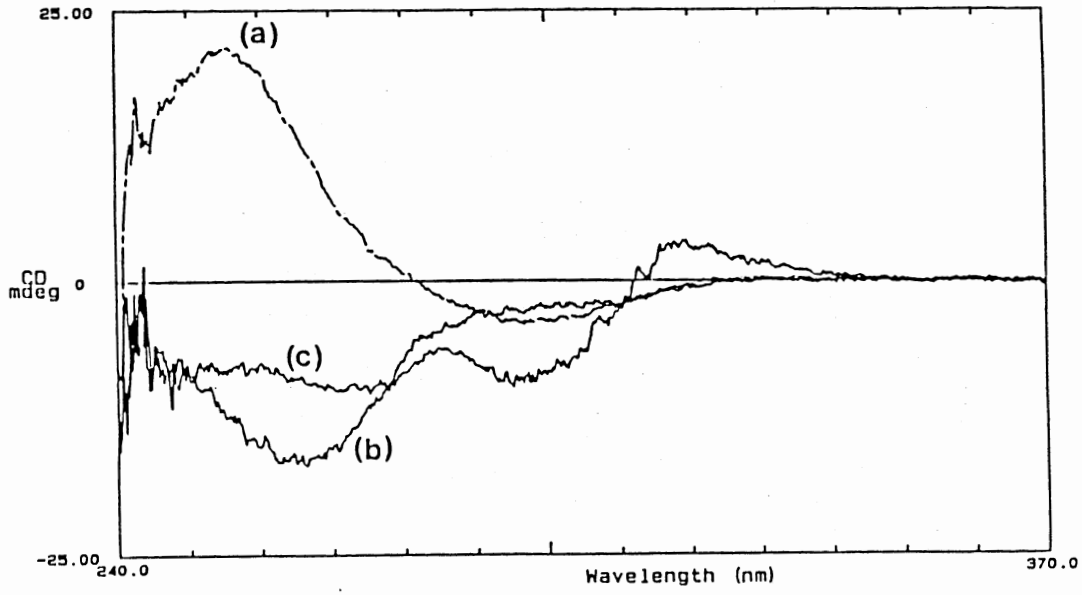


Figure 12. Comparison of (a) ajmaline, (b) rescinnamine, and (c) reserpine CD in $CHCl_3$.

CHAPTER VI

DETERMINATION OF WATER SOLUBLE VITAMINS

Introduction

Vitamin C (L-ascorbic acid), vitamin B_2 (riboflavin), and vitamin B_{12} (cyanocobalamin) are classified as water soluble vitamins. These molecules function as coenzymes or oxidation inhibiting agents in metabolic pathways. Their role in biochemical processes makes them essential nutrients, best obtained from foodstuffs. However, a large number of commercially prepared vitamin supplements are available. The proliferation of such preparations and the vitamin fortification of deficient foodstuffs has prompted the search for an accurate and efficient quality control procedure. Present USP procedures (66) rely on titrimetric (for ascorbic acid), spectrophotometric (for cyanocobalamin), and fluorimetric (for riboflavin) techniques. These procedures often require extensive sample preparation to eliminate the interferences commonly found in the complex matrices of pharmaceutical formulations and foodstuffs.

Other spectrophotometric and polarographic methods developed for these determinations have been reviewed by Hashmi (67). The preparation and determination of L-ascorbic acid samples have been summarized in a variety of sources (68-70) and determinations of B_{12}.by colorimetric, electrochemical, radioassay, microbiological assay, and enzymatic assay techniques have been reviewed by Kirschbaum (71).

Potentiometric (72), flow injection analysis (73), and a number of spectrophotometric (74-77) techniques were recently described for the determination of ascorbic acid.

Fluorescence detection (78) is still preferred in the determination of riboflavin, though Perez-Ruiz (79) does describe a spectrophotometrically monitored photoreduction of B_2 that can be used in analytical determinations. Radioassay and microbiological assays for B_{12} are rapidly being replaced with HPLC techniques (80). HPLC has become increasingly more important in determinations of vitamins, especially in the simultaneous determinations of individual components of multivitamin mixtures (81, 82). Fluorimetric detection is most commonly used for determinations of riboflavin (83, 84), while electrochemical and spectrophotometric methods are widely employed for determinations of ascorbic acid (85). Further applications of HPLC in determinations of vitamins have been reviewed elsewhere (86, 87).

A number of problems are associated with HPLC methodology. Typical problems include incompatibility between columns and stabilizing compounds, co-elution of compounds, and the need for dual detector systems to measure the widely disparate concentrations of vitamins in multicomponent preparations. CD detection circumvents some of these problems. It is more selective than UV, and the common stabilizers are not CD active so they are not a problem. CD detection of vitamins has been explored in the past. Firth et. al. (88) characterized the CD spectra of vitamin B_{12} and its derivatives. The primary objective of the study was the correlation of spectral changes with the different conformations of such compounds. Yeung and coworkers (89) explored the use of fluorescence-detected CD to analytically determine riboflavin, but no real samples were analyzed.

In the following work the CD spectra of ascorbic acid, riboflavin, and cyanocobalamin are characterized and the data used for the analytical determinations of these vitamins in commercial vitamin preparations. Seven common components of fruits and fruit juices are also characterized and are used to evaluate the composition and the vitamin C content of liquid and solid food samples.

Experimental

The three water soluble vitamins C (Aldrich Chemical Co.), B_2 and B_{12} (Sigma Chemical Co.) were used without further purification, as were the following compounds: L-dehydroascorbic acid, L-ascorbic acid-6-palmitate (Aldrich Chemical Co.), D-isoascorbic acid (D-ascorbic acid), L-malic acid, (-)-quinic acid, and (-)-shikimic acid (Sigma Chemical Co.) commonly associated with vitamin C food products. Marquee™ Vitamin C (distributed by Fleming Co.), Nature Made Vitamin B_2, Nature Made Vitamin B_{12} (Nature Made Nutritional Products), One-A-Day® Maximum Formula multivitamin (Miles Laboratories, Inc.) Regal-Natal 1 mg + Iron multivitamin (Regal Labs, Inc.), Thera-vites M multivitamin (Natural Wealth), and Nature Made Stress B-complex multivitamin (Nature Made Nutritional Products) tablets were obtained from a local pharmacy. Minute Maid® orange juice concentrate, Welch's® cranberry juice concentrate, TV grapefruit juice concentrate, TV apple juice concentrate, Gerber® apple juice, Sunsweet® prune juice, unsweetened cherry Kool-Aid® drink mix, green peppers, and apples were obtained from a local grocery.

All vitamin C standard solutions, extracts of samples, and possible interferences were prepared using a solution of 5.5×10^{-5} M EDTA (Aldrich Chemical Co.) in a 5.4 pH buffer (Micro Essential Laboratories). Addition of EDTA (90) retards the oxidation of ascorbic acid to dehydroascorbic acid (91) long enough to allow linear calibration curves to be obtained. A pH of 5.4 produced the best S/N ratio and the largest CD signal.

0.5-2 mL volumes of the cranberry, grapefruit, prune, apple, and orange juice samples were diluted to 10 mL with EDTA-5.4 buffer solution. Pulp was removed from the orange juice by centrifugation at low rpm. For analysis of the apples and green peppers, weighed slices were liquefied in a blender, extracted with 25 mL of EDTA-5.4 buffer, and centrifuged to reduce the amount of suspended particulate matter. 0.05 g portions of Kool

Aid® were dissolved in 25 mL of the EDTA solution. The food extracts and Kool Aid® solutions were diluted by a factor of ten before CD spectra were measured.

All tablets analyzed for vitamin C content were first powdered by grinding in a mortar. Powdered forms of the Marquee vitamin C tablet, the One-A-Day multivitamin tablet, and the Thera-vites M multivitamin tablet were extracted with 50 mL of EDTA-5.4 buffer solution. Volumes of 1 to 3 mL were further diluted to a final volume of 10 mL. The red outer coating of the Thera-Vites M tablet was removed in the crushing process because the dyes in the coating decreased the signal to noise ratio making quantitation difficult. A second set of One-A-Day tablets was crushed and three tablets were mixed together. The resulting solid mixture was divided into 2 to 5 mg portions and extracted into 10 mL of EDTA solution. The same procedure was followed for the Regal-Natal multivitamins, except that only one tablet was sampled at a time. All solutions were agitated for 10 to 15 minutes and centrifuged before final dilution and measurement.

Solutions of the B_2 and B_{12} standards and samples were prepared using a 4.8 buffer (Micro Essential Laboratories). A pH of 4.8 was chosen because B_{12} is most stable in the 4.5 to 5.0 pH range (92). The B_2 and B_{12} vitamin tablets were crushed in a mortar. Powdered B_{12} tablets were extracted with 10 mL of 4.8 buffer solution and agitated for 15 minutes under reduced lighting conditions to avoid decomposition (67). Samples were centrifuged before CD spectra were obtained. The powdered B_2 tablets were extracted with 100 mL of 4.8 buffer, agitated for approximately 15 minutes, and centrifuged. Volumes of 1 to 3 mL were diluted to a final volume of 10 mL and the CD spectra measured.

Nature Made Stress tablets were powdered as before and divided into 0.2 g portions. Each portion was extracted with 25 mL of 4.8 buffer for thirty minutes followed by centrifugation to remove any insoluble material. Volumes of 2 to 3 mL were then diluted to a final volume of 10 mL with 4.8 buffer and the CD spectrum obtained in order to determine the B_2 content. Determination of vitamin C content on the other hand required

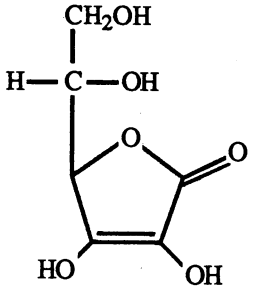
only 0.01 mL of the initial solution which was diluted to a final volume of 10 mL with EDTA buffer solution.

Results and Discussion

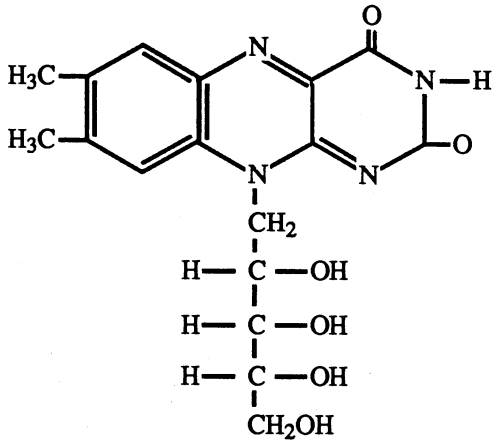
The molecular structures of the three water soluble vitamins are shown in Figure 13. The α–β unsaturated ketone in ascorbic acid limits its absorption to the shorter wavelength regions, 310 nm to below 210 nm, and thus is transparent at longer wavelengths. Vitamin B_2 and B_{12} are both highly conjugated systems and the absorption bands lie in the visible region of the spectrum, 500 to 310 nm and 580 to 310 nm respectively. At concentrations commonly found in commercial preparations, neither of the B vitamins exhibited a measurable CD signal below 300 nm. The CD spectra were measured for the wavelength regions as given above, Figure 14. Each vitamin has a unique spectrum allowing for easy qualitative identification. The maxima of the Cotton bands and the corresponding molar ellipticities are listed in Table VI.

The CD spectrum for vitamin B_2 is especially interesting. CD activity is usually strongest whenever the chiral center and the chromophore are adjacent to each other. If there is one or more atoms between the chiral center and the chromophore, CD activity becomes significantly less likely. In vitamin B_2 the five carbon sugar moiety which possesses the chiral center is separated from the conjugated ring system by two atoms, a carbon and a nitrogen. Normally such systems are not CD active, however, the acidic pH of the solvent might protonate the nitrogen connected to the sugar and the associated rigidity of the ring system could prevent inversion at the nitrogen atom. Under these conditions, the nitrogen would be chiral. Its location in the tertiary ring system would thus fulfill the requirements for CD activity.

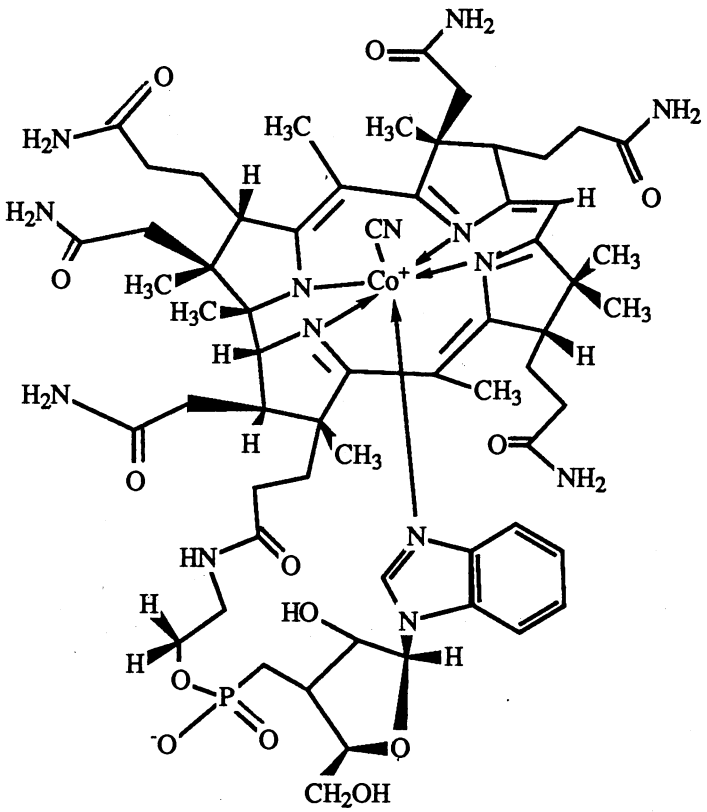
The determination of vitamin B_2 in tablet form was straightforward. The average vitamin content of six Nature Made B_2 tablets was 23.7 ± 1.7 mg/tablet. Agreement



VITAMIN C
(L-ASCORBIC ACID)



VITAMIN B_2
(RIBOFLAVIN)



VITAMIN B_{12}
(CYANOCOBALAMIN)

Figure 13. Molecular structures of the water soluble vitamins.

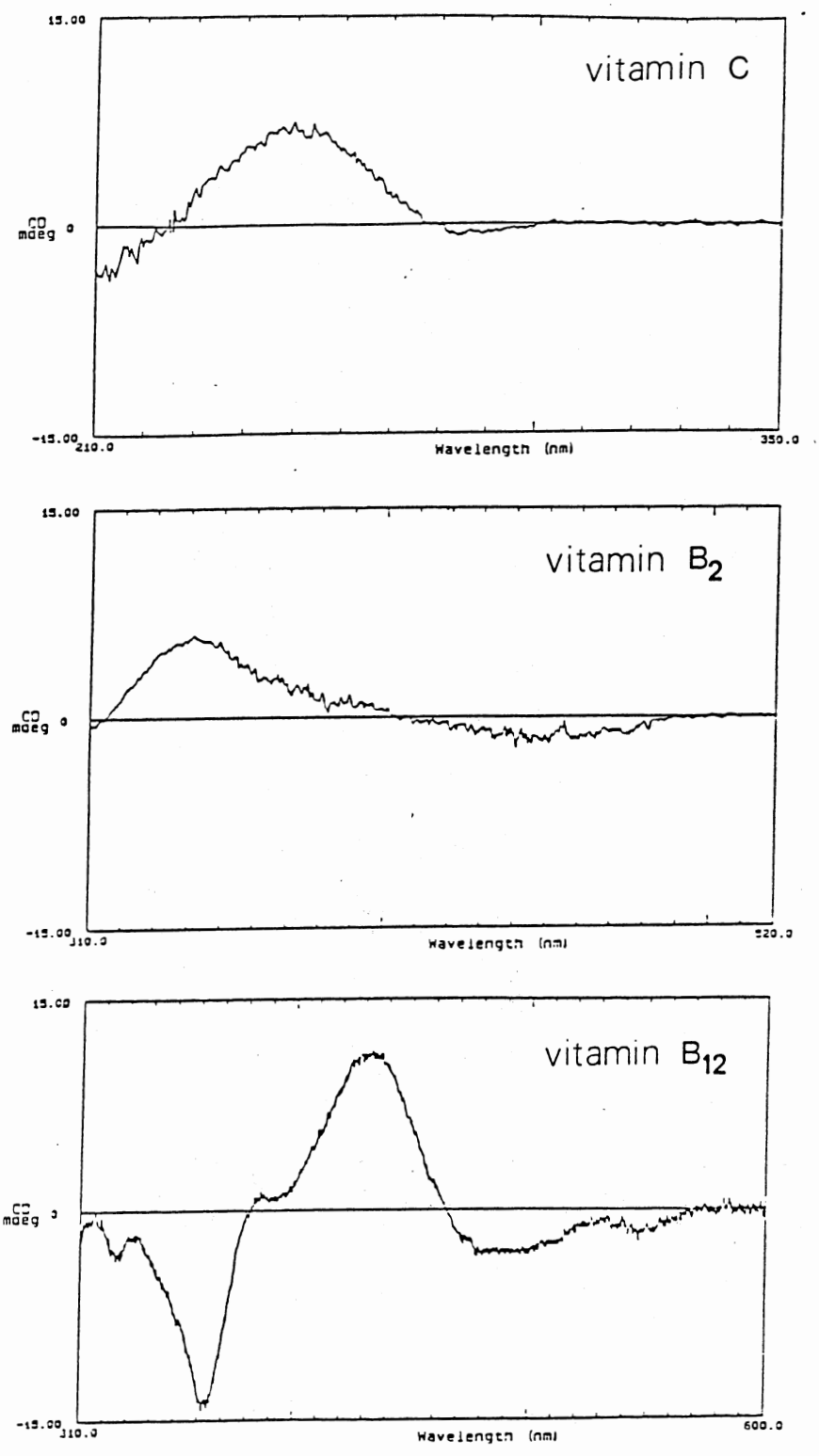


Figure 14. CD spectra of vitamin C (1.98×10^{-4} M),vitamin B_2 (1.76×10^{-4} M), and vitamin B_{12} (2.74×10^{-5} M).

TABLE VI

MOLAR ELLIPTICITIES OF THE WATER SOLUBLE VITAMINS

Vitamin	Solvent	θ_M (nm) (deg/M cm)
B_{12}	4.8 buffer	-117 (500) +437 (430) -587 (363)
B_2	4.8 buffer	-11 (440) +32 (342)
C	EDTA in 5.4 buffer	-3 (285) +105 (251)

between calculated and labeled content, 25 mg/tablet, is good, Table VII. Normalizing the B_2 content to tablet weight allowed direct comparison of the results all of the analyzed tablets. The same normalization procedure was used for all of the commercial preparations discussed below and in Table VII.

Analysis of four Nature Made B_{12} tablets yielded an average B_{12} content of 0.36 ± 0.02 mg/tablet, which does not correlate well with the labeled content of 0.25 mg/tablet. Analysis of four additional tablets taken six months later yielded a similar result, Table VII. The second set of data were obtained and analyzed using the new computer system in order to eliminate any human error or bias. Comparison of the CD spectra for the standard and samples revealed no anomalous features; the curves matched exactly, Figure 15. In addition, the CD spectrum pictured in Figure 15 matches the CD spectrum of

TABLE VII

VITAMIN CONTENT OF PHARMACEUTICAL PREPARATIONS

Sample	Labeled Content (mg/tablet)	Calculated Content (mg/tablet)	Calculated Content (% of tablet)
Nature Made Vitamin B_2	25	23.7± 1.7	4.8 ± 0.32
Nature Made Vitamin B_{12}	0.25	0.36 ± 0.02[a] 0.36 ± 0.02[b]	0.07 ± 0.003 0.07 ± 0.002
Marquee™ Vitamin C	250	243 ± 8	72.2 ± 3
One-A-Day® Multivitamin Vitamin C	60	63.9 ± 9.5	5.5 ± 0.8
Thera-vites M Multivitamins Vitamin C	120	108.4 ± 12	13.7 ± 1.3
Regal-Natal Multivitamins Vitamin C	90	79.7 ± 18	4.9 ± 1.2
Nature Made Stress Tablets			
Vitamin B_2	15	16.8 ± 0.9	1.5 ± 0.8
Vitamin B_{12}	0.12	NA	NA
Vitamin C	600	591 ± 20	52.7 ± 1.7

[a]Average content of samples from June 23, 1988.
[b]Average content of samples from January 2, 1989.

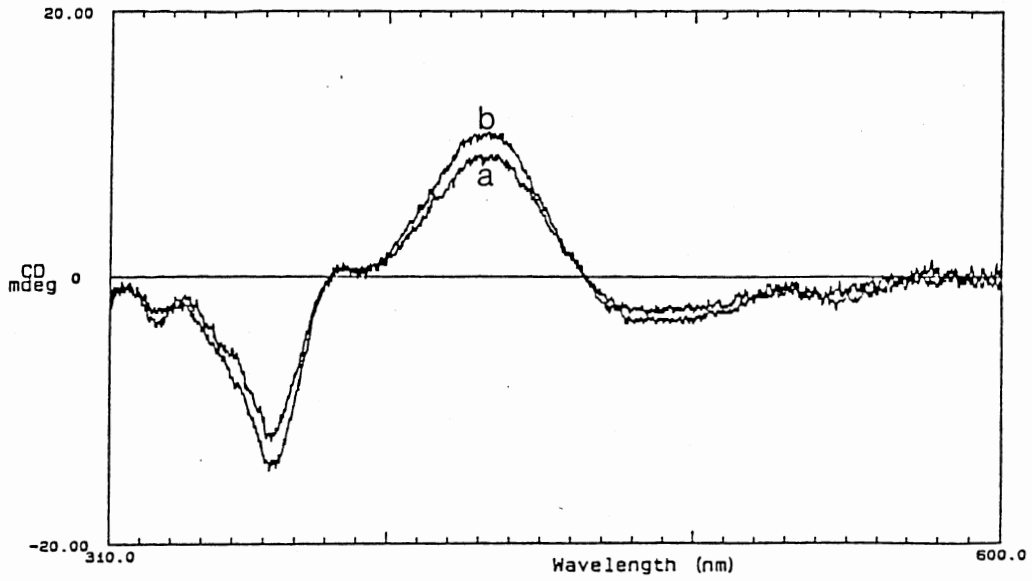


Figure 15. CD spectra of (a) B_{12} standard and (b) a Nature Made B_{12} tablet in 4.8 buffer.

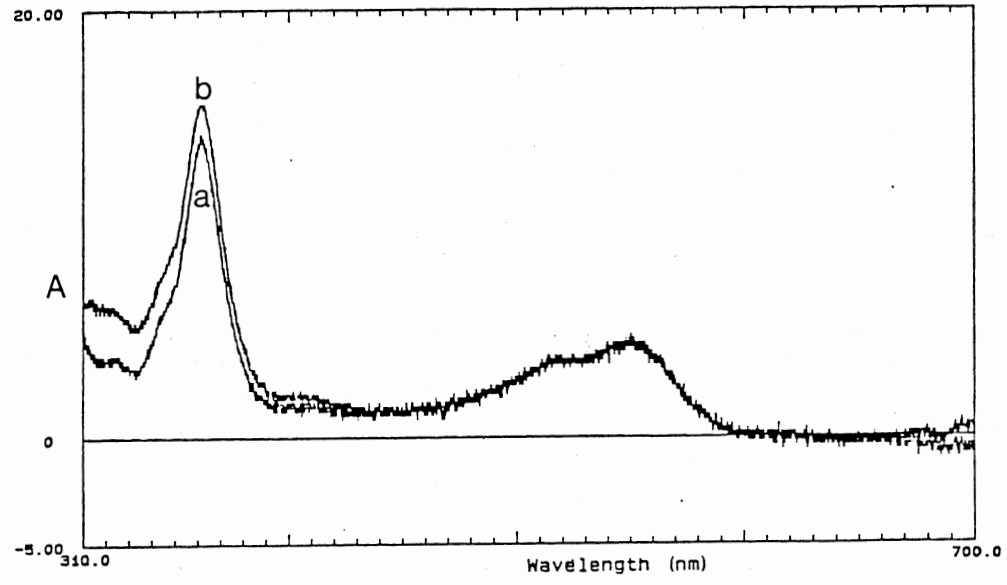


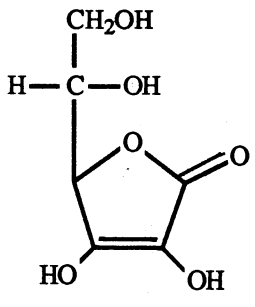
Figure 16. Absorption spectra of (a) B_{12} standard and (b) a Nature Made B_{12} tablet in 4.8 buffer. (Same solutions as in Figure 15).

cyanocobalamin recorded by Firth and coworkers (88). The presence of other CD active B_{12} derivatives can be eliminated. The CD spectra of dicyanocobinamide, vinylcyanocobinamide, aquocobalamin, hydroxocobalamin, selenocyanatocobalamin, and ethylcobalamin have also been characterized by Firth. All of these compounds possess CD spectra that are markedly different from that of cyanocobalamin. The positioning and intensity of the Cotton bands observed for these analogs is such that if one were present in the sample it would visibly distort the B_{12} spectrum.

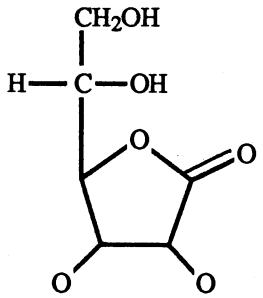
Tentative confirmation of the CD determination was obtained from an independent UV-visible absorption measurement of the sample against the standard. A result of 0.40 mg/tablet was obtained. However, the presence of other absorbers can not be ruled out, especially since the absorption curves for the standard and sample do not overlap at the 365 nm maximum but do at the 550 nm maximum, Figure 16.

One source of error that could account for anywhere from 7 to 36% of the difference between the measured and labeled values is the extent of water of crystallization. When B_{12} is recrystallized from water, the resulting crystal may contain as many as 22 moles of water per mole of B_{12} (71). The actual amount present depends on the recrystallization solvent and drying procedures used. These conditions are not known for the either the sample or the standard. Thermal analysis would be necessary in order to determine if there was a difference in the water content. Remaining differences may be due to lot variations or to deliberate overloading of the B_{12} tablets in order to prolong shelf life.

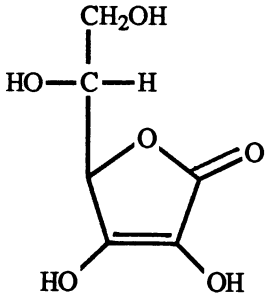
Determination of the vitamin C content in a sample can be a difficult process. In solution, L-ascorbic acid is oxidized to L-dehydroascorbic acid which is then converted to 2,3-diketogulonic acid (68), Figure 17. The rate of oxidation is proportional to the oxygen concentration and is catalyzed by metal ions (91). The oxidation process limits the accurate measurement of the ascorbic acid signal because the signal decreases too rapidly to allow time for the number of repeat scans needed to optimize the S/N. Dehydroascorbic acid



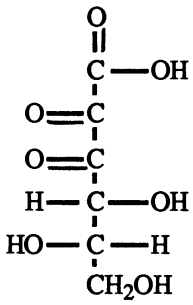
L-ascorbic acid



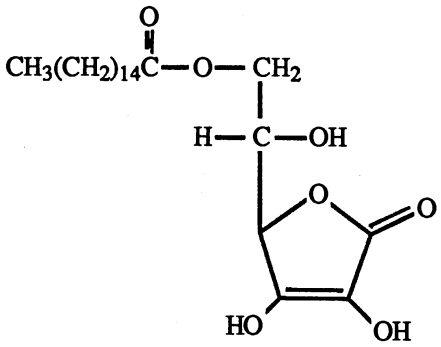
L-dehydroascorbic acid



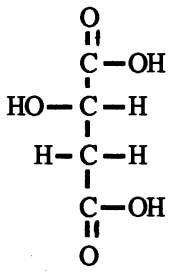
D-isoascorbic acid



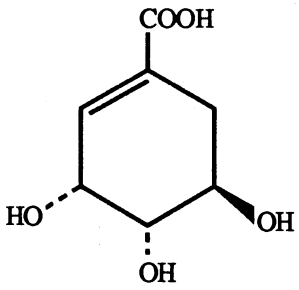
2,3-diketogulonic acid



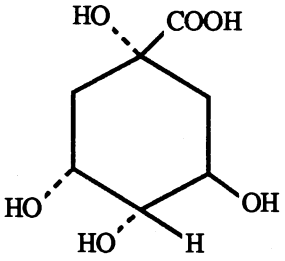
L-ascorbic acid-6-palmitate



L-malic acid



(-)-shikimic acid



(-)-quinic acid

Figure 17. CD active constituents of fruits and fruit juices.

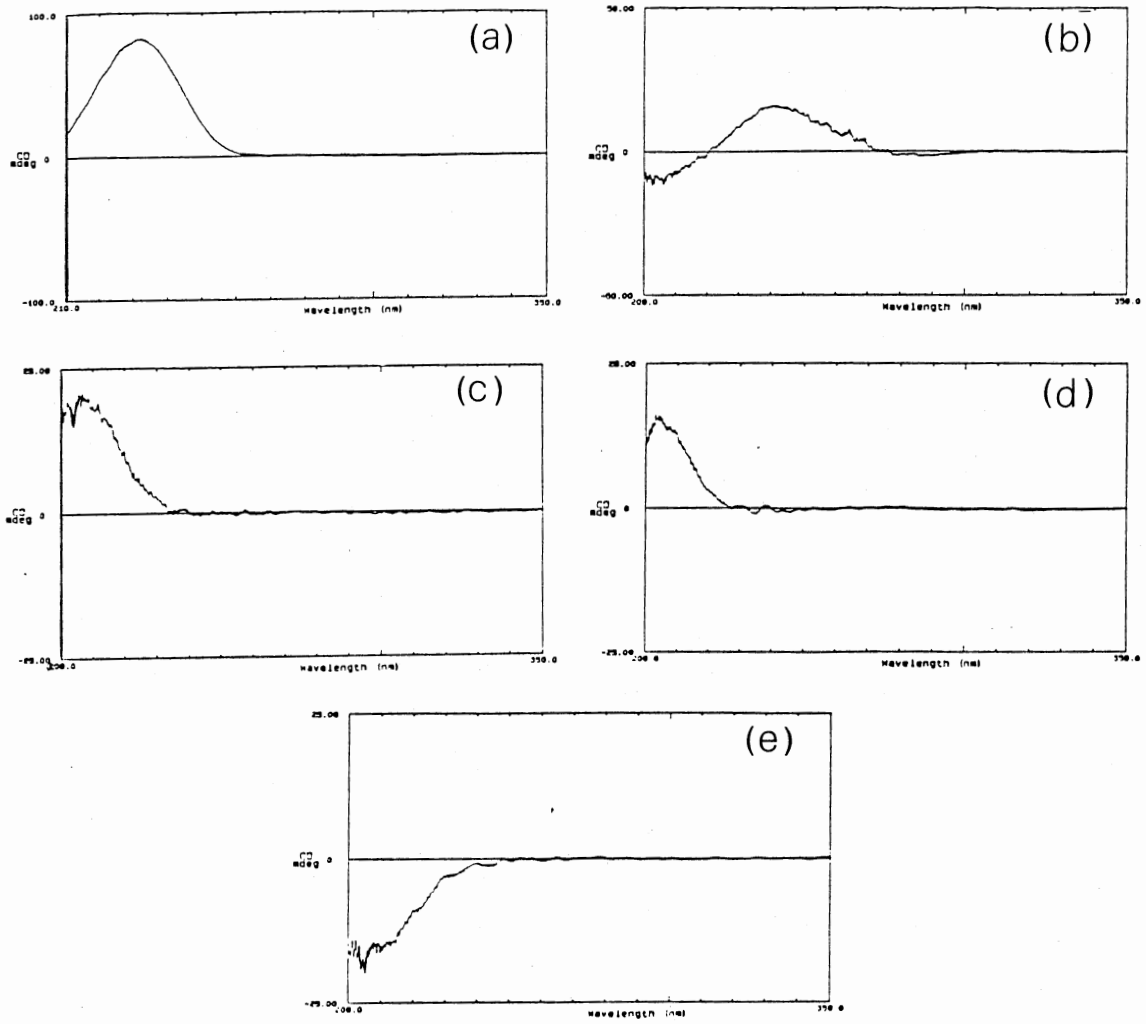


Figure 18. CD spectra of (a) L-dehydroascorbic acid (1.18×10^{-3} M), (b) D-isoascorbic acid (1.18×10^{-4} M), (c) L-malic acid (2.91×10^{-4} M), (d) (-)-quinic acid (1.06×10^{-5} M), and (e) (-)-shikimic acid (5.47×10^{-5} M).

exhibits a much cleaner signal than ascorbic acid, Figure 18a, and also possesses a larger linear calibration range. Initial attempts to determine L-ascorbic acid content by quantitative conversion to dehydroascorbic acid by reaction with norit (93) or quinone (94) resulted in incomplete conversion and such noisy signals that measurement was impossible.

Addition of EDTA to ascorbic acid solutions halts the oxidation process for over three hours. Once the ascorbic acid was stabilized, quantitation of vitamin C in tablet form was straightforward. Fourteen samples taken from four different Marquee™ Vitamin C tablets yielded an L-ascorbic acid content of 243 ± 8 mg/tablet. Agreement between labeled and calculated content was good, Table VII.

Quantitation of vitamin C in commercial multivitamin tablets was complicated by the presence of iron which catalyzes the oxidation process (82). The calculated and labeled ascorbic acid contents of three different multivitamin tablets are listed in Table VII. Correlations between measured and theoretical values are poor. The percent difference between measured and labeled content ranges from 7% for the One-A-Day® tablets to 11% for the Regal-Natal tablets. The increase in percent difference parallels the increase in iron content. One-A-Day® tablets contain 18 mg of iron, Thera-vites M contain 27 mg and the Regal-Natal contain 65 mg of iron. Increasing the EDTA concentration by a factor of twenty did not affect the results. Other stabilizers, such as metaphosphoric acid and oxalic acid (87) might yield better results. Another possibility is to physically remove the iron by forming an insoluble complex. Lam and coworkers (82) used cupferron to complex the iron, but reported that their method did not work on all multivitamin preparations.

If iron is not present in the multivitamin preparation, determination of the vitamin C content is straightforward. The analysis of Nature Made Stress tablets yielded an ascorbic acid content of 591 ± 20 mg/tablet compared to a labeled content of 600 mg/tablets, Table VII. The Stress tablets contain a number of other water soluble and fat soluble vitamins, but no iron. The percent difference between calculated and theoretical values is 1.4%, much less than the error reported for any of the multivitamin tablets with iron.

The Nature Made Stress tablets were also analyzed for vitamin B_2 and B_{12} content. Determination of both should be possible by measuring the net CD signal of the mixture and using a curve fitting program (51) to determine the relative concentrations of each. Analysis of samples taken from three tablets yielded a B_2 content of 16.8 ± 0.9 mg. Correlation between measured and labeled content is fair, Table VII. The determination of the B_{12} content was not possible. The typical B_{12} Cotton bands were not present in the spectrum of the extract. The labeled content is 0.12 mg/tablet, half the amount present in the Nature Made Vitamin B_{12} tablets. The volume used in the extraction of the Stress tablets is two and a half times that used in the Vitamin B_{12} tablets. It is possible that at this level the B_2 Cotton band at 342 nm hides the B_{12} band at 363 nm. However, due to the sign and magnitude of the B_{12} Cotton band, it should have caused a reduction in the B_2 signal resulting in a lower calculated content. A more likely explanation is the possibility that B_{12} was decomposed due to the presence of ascorbic acid, thiamine, and niacinamide (71). The literature reports significant losses of B_{12} in tablets and capsules after storage of only one year at room temperature (71).

The possibility of spectral interference from the vitamin C can be ruled out. As stated earlier, vitamin C is transparent in the wavelength regions used to quantitate both B vitamins. The B vitamins did not interfere with the determination of vitamin C discussed above because of the small quantities present. Higher concentrations of the B vitamins might present some minor interference, but this could be accounted for by employing a curve fitting routine.

Vitamin C is found in abundance in nature, especially in fruits and vegetables. B_2 is more common in grains and B_{12} is found predominantly in animal tissues. The analysis of such samples is often difficult because of the complexity of the matrices involved. The small quantities of vitamin B_2 and B_{12} present in such samples makes analysis even more difficult. However, the vitamin C content of many fruits and vegetables is such that it may

TABLE VIII

VITAMIN C CONTENT OF FRUITS, FRUIT JUICES, AND VEGATABLES

Sample	Labeled Content[a]	Measured Content
Kool Aid®	48 mg/package	42.3 ± 3.0 mg/package
TV grapefruit juice	NA	273.9 ± 11.4 mg/package
Minute Maid® orange juice	288 mg/package	272.5 ± 3.5 mg/package
Welch's cranberry juice	NA	404.6 ± 43 mg/package
TV apple juice	120 mg/package	72 mg/package
Gerber apple juice babies	42 mg/package	100.4 ± 3.6 mg/package
pregnant women	96 mg/package	
Sunsweet prune juice	6 mg/package	NA
apple	NA	8.9 ± 0.5 mg/slice (0.02% by weight)
green pepper	NA	17.4 ± .5 mg/slice (0.04% by weight)

[a]Labeled content calculations based on a U.S. RDA of 60 mg (95), except for the Gerber apple juice.
NA: not available

be quantitated by using only a simple extraction step. Kool Aid® unsweetened drink mix was extracted with EDTA-5.4 buffer solution and yielded a content of 42.3 ± 3.0 mg/package. This is in fairly good agreement with the labeled value of 48 mg/package, Table VIII.

The determination of L-ascorbic acid in TV grapefruit juice frozen concentrate and in green peppers was equally straightforward. The CD spectrum for the green pepper extract is shown in Figure 19. The CD for the grapefruit juice is similar but much noisier. The vitamin C content of the green pepper was determined to be 0.04% by weight. The actual weight is reported in Table VIII. No standard value can be reported for comparison since vitamin content is known to vary in crops from different regions, climates, and seasons. No nutritional information was provided for the grapefruit juice to compare with the measured ascorbic acid content of 273.9 ± 11.4 mg/package.

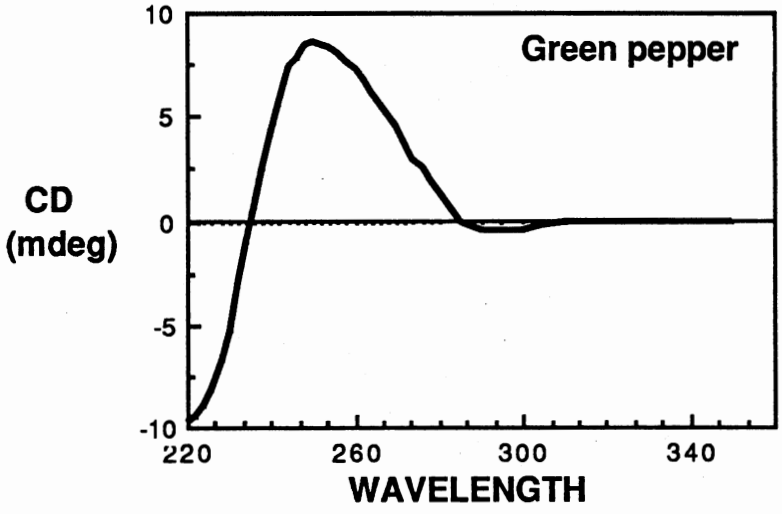


Figure 19. CD spectrum of green pepper extract (Extracted with EDTA-5.4 buffer solution).

The determination of vitamin C in other fruit juices was complicated by the presence of other CD active components. L-dehydroascorbic acid, D-isoascorbic acid, L-malic acid, (-)-quinic acid, and (-)-shikimic acid in variable amounts may be present in fruits and their juices. The molecular structures are shown in Figure 17. CD spectra for the standards in aqueous buffer are shown in Figure 18 and the molar ellipticities are given in Table IX.

TABLE IX

MOLAR ELLIPTICITIES OF OTHER COMPONENTS OF FRUIT JUICES

Compound	θ_M(nm) (deg/M cm)
L-dehydroascorbic acid	+69.1 (232)
D-isoascorbic acid	+134.5 (241) -99.8 (203)
L-malic acid	+71.4 (207)
(-)-quinic acid	+15.1 (203)
(-)-shikimic acid	-301.2 (206)

The dehydroascorbic and isoascorbic acids could present the greatest problem in the extracts because the overlap with the ascorbic acid band is large. Dehydroascorbic acid is formed during the oxidation of ascorbic acid. A reduction in the CD signal at 251 nm

and/or an increase in the signal height at 232 nm would be noticed if it was present. In the EDTA stabilized solutions no evidence of any decrease or increase in the signal intensity with time was found. D-isoascorbic acid is commonly used as a cheap antioxidant, as is ascorbic acid-6-palmitate (68). The palmitate possesses no CD activity and will not interfere with the analysis. D-isoascorbic acid could present a problem in the analysis of the cheaper commercial fruit juices. Its presence in the samples discussed below can not be ruled out, but is probably unlikely due to the quality associated with the chosen brands.

The Cotton bands at 340 and 235 nm in the CD spectrum of Minute Maid® orange juice frozen concentrate, Figure 20, do not correspond to any of the interferences characterized in Figure 18. However, they did not interfere with the quantitation of vitamin C from the 251 nm band. The resulting L-ascorbic acid content was found to be 272.5 ± 3.5 mg/package, in good agreement with the labeled content of 288 mg/package, Table VIII.

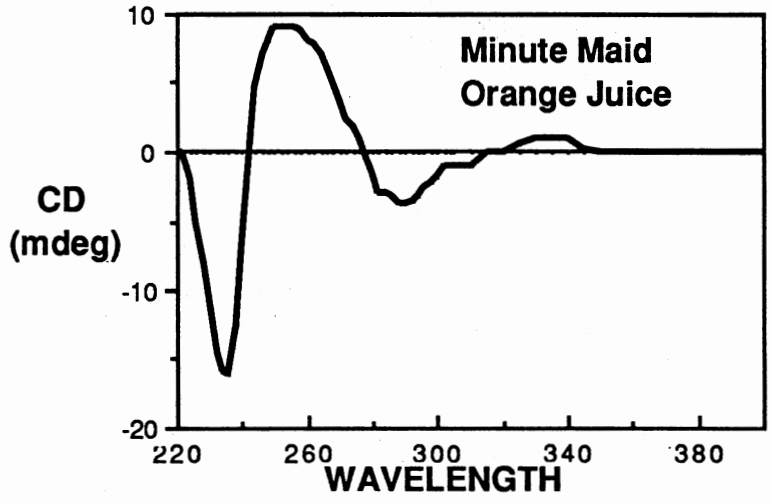


Figure 20. CD spectrum of Minute Maid® orange juice in EDTA-5.4 buffer.

CD spectra of the Gerber® and TV brand apple juices and the fresh apple extract all have a Cotton band at 210 nm, Figure 21, which can be assigned to L-malic acid (96). A simple curve fitting program (51) was used to quantitate the vitamin C and L-malic acid content of these samples. Of the total acid content measured from the spectrum, the ratio of L-malic acid to vitamin C varied from 98/2 (TV apple juice); to 91/9 for the Gerber product and fresh apple extracts. The correlation between the L-malic and L-ascorbic acid ratios for the Gerber apple juice and the fresh apple extract suggest that the reported values in Table VIII may have some validity. This is difficult to deduce otherwise because of the lack of standard values.

Welch's® cranberry juice concentrate also exhibited a band at 210 nm. Literature sources report the presence of malic, quinic, and shikimic acids in cranberry juice (96). All

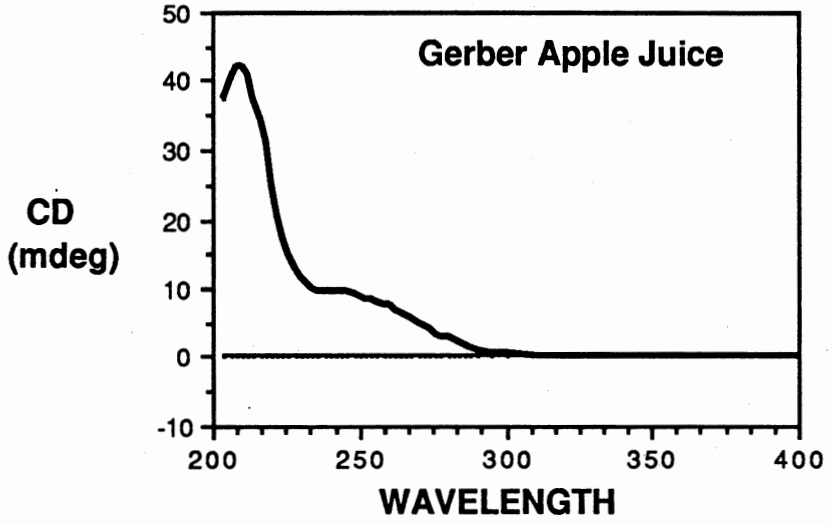


Figure 21. CD spectrum of Gerber® apple juice in EDTA-5.4 buffer.

of these components exhibit CD spectra in the 230 to 200 nm region, Figure 18. In addition, the presence of other natural pigments add considerably to the noise generated in the 240 to 220 nm region. Nevertheless a computer simulation suggested that the large noisy peak at 210 nm was due mainly to the presence of malic acid. This simulation suggested that 85% of the measured acid content was L-malic acid and 15% was L-ascorbic acid.

Analysis of prune juice extracts proved to be impossible. The presence of strong absorbers resulted in a complete loss of signal integrity due to noise. The use of bonded phase extraction procedures might eliminate some of these interferences and could prove useful in reducing or even eliminating the interferences encountered in the above samples.

This work is another example of the utility of CD in the analysis of complex samples. Sample preparation consisted of only a simple extraction step and no preconcentration or derivatization steps were required.

CHAPTER VII

INDUCTION OF CIRCULAR DICHROISM: DETERMINATION OF BENZODIAZEPIN-2-0NES

Introduction

CD activity requires the presence of both a chiral center and a chromophore. These dual requirements impart a degree of selectivity to CD which greatly reduces the number of interferences. But this inherent selectivity also reduces the number of detectable analytes. Nevertheless it is possible to induce CD activity by complexing an absorbing, but achiral molecule with a chiral, but nonabsorbing host. The resulting complex possesses both of the necessary structural features for CD activity.

Structurally ordered media are the best hosts for inducing chirality into an otherwise achiral molecule. The ability of cholesteric liquid crystals (97) and the cyclodextrins (98) to induce chirality has been demonstrated, with the latter compounds producing the most promising results. β-cyclodextrin has been used to induce chirality in phencyclidine (PCP) (99), and its analogs (100), barbiturates (101), and numerous other drugs (100, 102). The following discussion focuses on the induction of CD activity in the benzodiazepin-2-ones via complexation with β-cyclodextrin.

β-Cyclodextrin

Cyclodextrins are cyclic oligosaccharides composed of from six to twelve D-(+)-

glucopyranose units connected by α-(1,4)- linkages. The number of monosaccharide units is denoted by a Greek letter: α- for six, β- for seven, γ- for eight, etc.. The three smallest analogs are commercially available and are the most commonly used.

The molecular structure of β-cyclodextrin is shown in Figure 22. Structures for the other analogs differ only in the number of sugar monomers present in the macrocyclic structure. Schematically the cyclodextrins can be represented as truncated cones as shown in Figure 23. The larger opening is rimmed with the secondary hydroxy groups of the

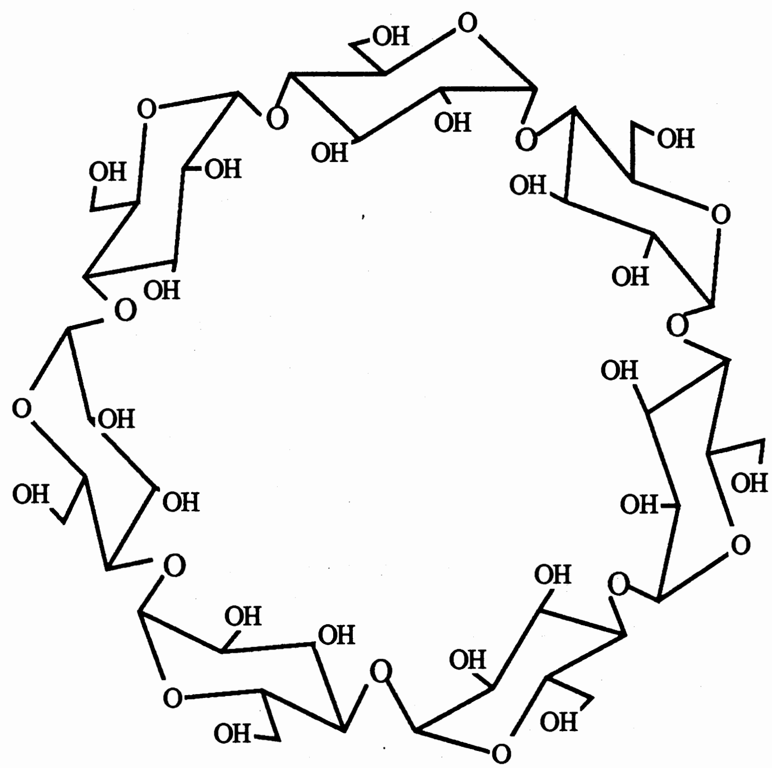


Figure 22. Molecular structure of β-cyclodextrin.

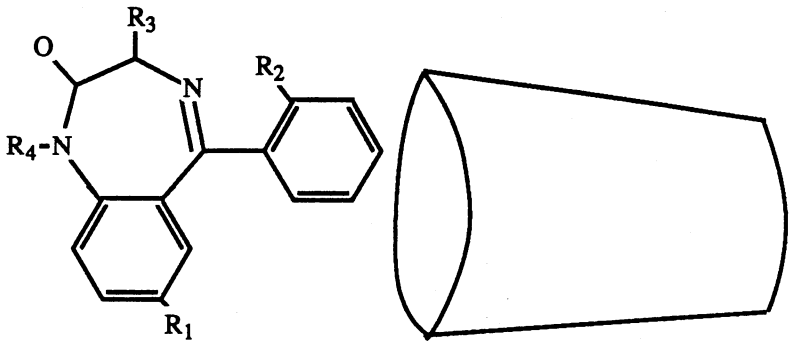


Figure 23. Truncated cone representation of β-cylcodextrin-benzodiazepin-2-one inclusion complex.

glucose units, all rotated in a clockwise direction. The interior of the cavity contains no hydroxy groups and is relatively hydrophobic. This hydrophilic exterior/hydrophobic interior combination allows for inclusion complex formation between cyclodextrins and such polar compounds as acids, amides, and some small ions. Highly apolar compounds such as aliphatic and aromatic hydrocarbons are complexed as well.

The forces responsible for complex formation include hydrogen bonding interactions, Van der Waals forces, London dispersion forces, release of high energy water (i. e. release of water molecules held in the relatively hydrophobic cyclodextrin cavity), and relief of macrocyclic ring strain (101). The relative contribution of each of these binding forces is still not clear and probably varies with the structural properties of the guest molecule. However, it is generally recognized that water plays a key role in complex formation. Inclusion complexes form much more readily in water. The resulting complexes consist of a 1:1 molar ratio of guest to host. In aqueous solutions, apolar substrates are favored during complex formation.

The cavity sizes for the first three analogs are given in Table X. The smaller the cyclodextrin analog the better it complexes smaller molecules, and vice versa. β-cyclodextrin forms inclusion complexes with moderate sized organic molecules such as

phenyls, biphenyls, or naphthyls (103). These or closely related structures are common to many pharmaceuticals. Consequently, β-cyclodextrin has proven to be the most efficient agent in inducing CD activity and has found some utility as a HPLC stationary phase and mobile phase additive.

β-cyclodextrin is transparent to UV-visible radiation over the wavelength range normally of interest in CD work so it does not adversely affect the S/N ratio. The smaller cyclodextrin analogs are quite water soluble and are stable at alkaline pHs. Acidic pH does promote acid catalyzed hydrolysis, but there are a number of ways to retard it and use the acidic pH range to its full advantage. β-cyclodextrin is easier to use for quantitative work

TABLE X

CAVITY DIMENSIONS OF COMMERCIALLY AVAILABLE CYCLODEXTRINS

Cyclodextrin Analog	Internal Diameter (Å)	Depth (Å)
α-cyclodextrin	4.5	6.7
β-cyclodextrin	~7.0	~7.0
γ-cyclodextrin	~8.5	~7.0

Adapted from reference 98

than the anisotropic cholesteric liquid crystalline phase material used in earlier studies (97). In addition to these advantages, β-cyclodextrin is readily available and is relatively inexpensive.

The Benzodiazepin-2-ones

The benzodiazepines are commonly prescribed anti-anxiety agents. Other indications include some cases of insomnia and treatment of alcohol withdrawal. Determinations of benzodiazepins have been accomplished by UV spectrophotometry (104), fluorimetry (105), polarography (106), voltammetry (107), flow injection analysis (108, 109), HPLC (110, 111), and GC (112). GLC and HPLC separations and determinations have been reviewed by de Silva (113). These methods have associated advantages and disadvantages. In most cases derivatization and/or chromatographic separations are necessary to remove interferences common to pharmaceutical and biological matrices.

All of the benzodiazepin-2-ones possess a chromophore. Three of the analogs possess chiral centers, but are actually racemic mixtures in solution. The rest are achiral. The presence of a chromophore makes these compounds good candidates for CD induction by introducing optical activity via complexation with β-cyclodextrin.

The following study is a continuation of the characterization and thermodynamic studies completed earlier (114). CD spectra for ten benzodiazepin-2-ones/β-cyclodextrin complexes have been characterized and the formation constants calculated. The effect that ring substitution has on complex formation and stability is discussed. In addition, the determination of the drugs in two different pharmaceutical preparations is discussed.

Experimental

The benzodiazepin-2-ones, clonazepam, delorazepam, diazepam, flurazepam,

lorazepam, nitrazepam, nordiazepam, oxazepam, temazepam, and the dehydroderivative medazepam (all from Hoffmann-LaRoche or Sigma Chemical Co.) were used without further purification. β-cyclodextrin was obtained from Eastman Kodak.

For the equilibrium studies, solutions were prepared by dissolving the drugs in 0.02 M sodium hydroxide and weighed increments of β-cyclodextrin were added in such a way that the host (β-cyclodextrin) was always in excess. Drug concentrations were typically less than 10^{-4} M because of the strong absorption by the aromatic chromophores. Saturation for β-cyclodextrin at room temperature is approximately 3×10^{-2} M. The sugar is stable in strong alkali for several hours, which is very long compared to the few minutes needed for data aquisition.

The same solution conditions, namely 10^{-2} M β-cyclodextrin in 0.02 M sodium hydroxide, were selected for the extraction from the pharmaceutical products which were obtained from a local dispensary. The alkali was added to the sugar stock solution just prior to obtaining the spectrum. The products used were tablets containing 5 mg of diazepam (Valium, Rugby) and capsules containing 30 mg of flurazepam (Dalmane, Mylan). Tablets were crushed and thoroughly mixed by shaking on a Wig-L-Bug for 2 minutes; 20 mg portions were withdrawn for extraction into 10 or 25 mL aliquots of the alkaline β-cyclodextrin stock solution. Undissolved solids were removed by centrifugation. Several extracts from several tablets were used in the determinations.

Results and Discussion

The general structural formula for the series of benzodiazepin-2-ones is shown in Figure 24. The R groups for each analog, the values obtained for the complex formation constants K_{DS}, and the resultant molar ellipticities, θ_{DS}, at the major maxima in the 250-300 nm wavelength range are listed in Table XI.

The CD spectra for the complexes formed with each of the analytes are shown in Figure 25. These are markedly different from those obtained for the barbiturates (99), the tetracyclines (100), the penicillins or the cephalosporins (Chapter IV), and some differences are observed between members of the group. Strong spectral similarities exist for lorazepam and oxazepam, each of which has a hydroxyl group at position R_3, and between diazepam and flurazepam each of which has an alkyl substituent at position R_4. The nitro substituents at R_1 in clonazepam and nitrazepam contribute towards a totally new Cotton band of positive sign at longer wavelengths. The spectrum for the β-cyclodextrin/diazepam complex in strong acid (98) bears a stong resemblance to the spectrum in strong alkali, with the latter being more intense.

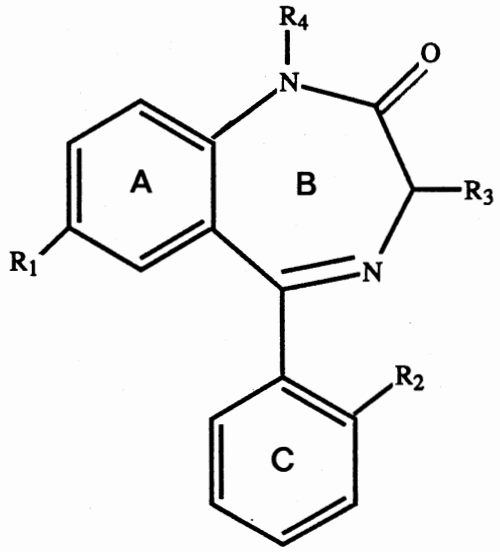


Figure 24. General structure for the benzodiazepin-2-ones. The R groups are identified in Table XI.

TABLE XI

GENERAL STRUCTURE FOR THE BENZODIAZEPIN-2-ONES AND FORMATIONCONSTANTS FOR β-CYLCODEXTRIN-BENZODIAZEPIN-2-ONE COMPLEXES

Compound	R_1	R_2	R_3	R_4	K_{DS}	θ_{DS}
Clonazepam	NO_2	Cl	H	H	812	100
Delorazepam	Cl	Cl	H	H	615	587
Diazepam	Cl	H	H	CH_3	208	218
Flurazepam	Cl	F	H	$CH_2CH_2NEt_2$	106	310
Lorazepam	Cl	Cl	OH	H	928	639
Nitrazepam	NO_2	H	H	H	479	72
Nordiazepam	Cl	H	H	H	133	1005
Oxazepam	Cl	H	OH	H	569	751
Temazepam	Cl	H	OH	CH_3	147	206
Lorazepam[a]	Cl	Cl	OH	H	96	246

[a]Result is for the reaction with γ-cyclodextrin.

Complex formation between guest (drug) and host (β-cyclodextrin) generally results in a 1:1 complex, according to the equilibrium reaction

$$\text{Drug (D)} + \text{Sugar (S)} \rightleftharpoons \text{Drug/Sugar Complex (DS)} \quad (28)$$

The formation constant, K_{DS}, is defined as

$$K_{DS} = [DS] / [D]\,[S] \quad (29)$$

Analytical concentrations of the drug, C_D, and sugar, C_S, under equilibrium conditions can be expressed as the sums of the molar concentrations of free drug, free sugar, and complex

$$C_D = [D] + [DS] \quad (30)$$

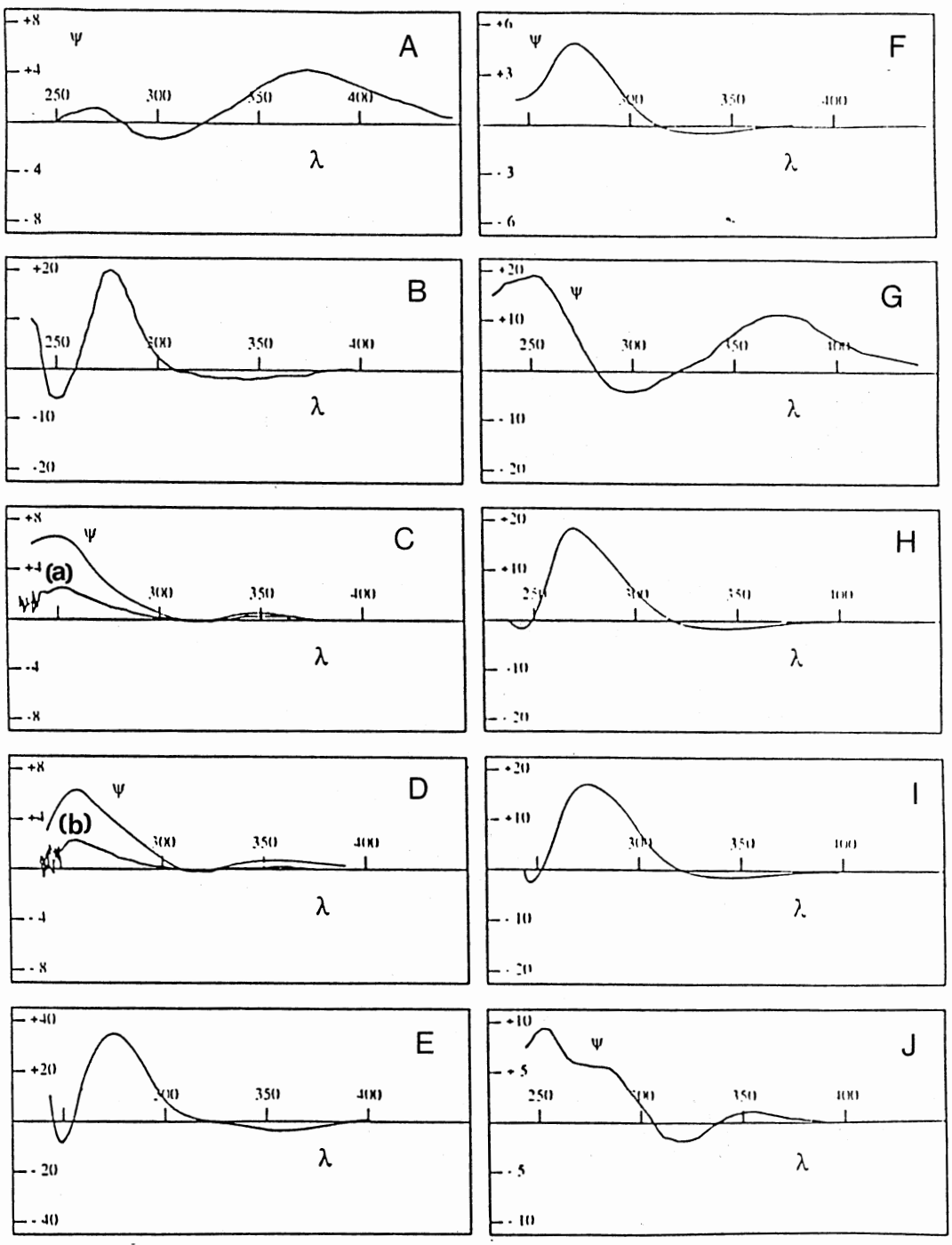


Figure 25. Induced circular dichroism, spectrum [induced ellipticity (millidegrees) vs. wavlength (nm)] for the 1:1 complex formed between β-cyclodextrin and drug: A. clonazepam, B. delorazepam, C. diazepam, plus (a) spectrum for extract, D. flurazepam, plus (b) spectrum for extract, E. lorazepam,F. medazepam, G. nitrazepam, H. nordiazepam, I. oxazepam, and J. temazepam.

$$C_S = [S] + [DS] \quad (31)$$

and the experimental ellipticity, ψ, is equal to the sum of the terms for each CD active component:

$$\psi = \theta_D\,[D] + \theta_{DS}\,[DS] \quad (32)$$

where θ_D and θ_{DS} are the molar ellipticity coefficients for free and complexed drug respectively for a one centimeter pathlength. For molecules which possess no CD activity, θ_D is zero and equation 32 reduces to $\psi = \theta_{DS}\,[DS]$. Rearrangement and substitution of equations 30, 31, and 32 into equation 29 yields an expression for K_{DS} in terms of ellipticity parameters.

$$K_{DS} = (\psi/\theta_{DS}) / \{(C_D - \psi/\theta_{DS})\,(C_S - \psi/\theta_{DS}) \quad (33)$$

Equation 33 does not hold for cases where the drug is CD active and a more complex form results because equation 32 can not be simplified. However, another derived equation attributed to Hildebrand (115) and used for UV-visible absorption data can be modified for use with CD data, equation 34.

$$(b\,[D]\,[S] / \psi - \psi_D) = \left\{([D] + [S] - [DS]) / (\theta_{DS} - \theta_D)\right\} + \left\{1 / K_{DS}\,(\theta_{DS} - \theta_D)\right\} \quad (34)$$

where b is the cell path length in centimeters, and ψ_D is the experimental ellipticity of the free drug. Solution requires a converging iterative procedure to calculate K_{DS} and θ_{DS}. Iteration is initiated by assuming [DS] = 0 and the values for K_{DS} and θ_{DS} are refined by substituting new values for [DS] upon completion of each cycle in the iteration. The calculation is terminated when successive K_{DS} and θ_{DS} values are invarient. The iterative calculations were performed by a microprocessor.

Determinations of the benzodiazepine components of the Valium and Dalmane tablets were straightforward. The observed ellipticities for the samples are directly proportional to the concentration of the complexed form [DS]. Data analyses can be handled in two different ways. The first would require construction of a calibration curve, the slope of which is an conditional molar ellipticity which can be used to obtain the concentration of the

pure benzodiazepine. If the equilibrium constants for the diazepam and flurazepam are known, a second approach can be employed. This involves substituting ψ/θ_{DS} for [DS] and solving equations 30, 31, and 34 by microcomputer. The latter method was chosen for convenience since the equilibrium constants had already been calculated.

Analysis of the tablets yielded a content of 4.98 ± 0.08 mg of diazepam per Valium tablet and 29.3 ± 0.6 mg of flurazepam per Dalmane tablet. Both values are within ±2% of the prescription amounts. The reproducibility was better than ±0.2% for samples taken from an individual tablet. There was no evidence for spectral interferences from other co-extracted components which were either inherently CD active or had had CD activity induced by complexation with β-cyclodextrin. This is apparent from the similarities between the CD spectra for the standards and the extracts shown in Figure 25. None of the other soluble or insoluble ingredients was identified. Because heats of reaction are relatively small, close temperature control is not necessary in the performance of the determination and the K_{DS} values at 25°C can be used for the ambient conditions (114). One obvious advantage of the present method over others is that a sophisticated separation procedure is not necessary, only a single centrifugation step was needed. While a single determination may take 20 minutes to complete, simultaneous multiple determinations can subsequently be done at a rate of ten per hour if a whole spectral scan is performed, and more frequently if data are taken at a single wavelength.

The nine benzodiazepin-2-one analogs form an interesting set wherein substitutions for the four R groups and the accompanying changes in K_{DS} and θ_{DS} might be compared and used to indicate which part of the benzodiazepin-2-one structure is encompassed in the sugar molecule on the formation of the complex. All of the structural variations needed to completely interpret the stability dependence are not satisfied by choosing only nine representatives of the series. However, some patterns of complex formation can be recognized.

The θ_{DS} values found in the benzodiazepin-2-one/β-cyclodextrin interaction are considerably larger than the values already reported for the barbiturate/β-cyclodextrin associations (100). The lowest value of 72 observed for nitrazepam is a factor of three greater than the highest barbiturate value, namely 27 for hexobarbital. However, the wider range in θ_{DS} for the benzodiazepin-2-ones suggests a stronger structural dependence. The range of values for K_{DS} is not as broad but the stabilities are on the whole greater than those observed for the barbiturates. This is also true for the comparison of the results for diazepam in 0.1 M hydrochloric acid (K_{DS} = 83; θ_{DS} = 151) (98) versus the present data. One other stability comparison which can be made and which might have a bearing on the later discussion is that for lorazepam in the analogous complexation reaction with γ-cyclodextrin. When offered the larger central cavity of the gamma analog, the stability was observed to decrease by a factor of ten, K_{DS} = 96. However, a decrease in stability of the complex with increasing cavity size is to be expected.

A general trend of increasing stability and decreasing induced ellipticity is observed relative to the values for the designated parent, nordiazepam. The differences in K_{DS} and θ_{DS} for individual and paired substitutions relative to nordiazepam are listed in Table XII. When the substituent changes are considered individually, the effects on K_{DS} are greatest for R_1, R_2,and R_3, while changes in R_4 exert little influence over the value of K_{DS}. The effects of the paired substituent changes parallel those observed for single substituent changes. Substitutions at the R_1/R_2 and R_2/R_3 sites dramatically increase the value of K_{DS}. A substitution at the R_3/R_4 site has little effect on the formation constant. These results suggest that R_1, R_2 and R_3 are all involved in complex formation, while R_4 is far removed from the interaction site.

These observations can best be explained by examining the possible complexation sites on the benzodiazepin-2-one ring system. Numerous studies have indicated that a naphthyl or biphenyl ring systems best fit the β-cyclodextrin cavity (116, 117). Phenyl groups are moderately complexed (117, 118), and the degree of complexation decreases with

increasing substitution (100, 102, 118). Thus the size and triple substitution of the seven membered diazo ring, B, should limit its inclusion in the sugar cavity. However, this still leaves two rings, A and C, as possible inclusion sites. Inclusion of either site is possible, though it does not occur with equal probability.

Inclusion of the C ring in the sugar cavity accounts for many of the trends observed in Table XII. The common R_2 substitutuent in this series is a hydrogen or chlorine atom (except for flurazepam). In studies of substituent effect on the retention of bisubstituted benzenes on β-cyclodextrin stationary phases, Fujimura (118) and Shono (119) both report increased capacity factors, k', for those analytes possessing halogen substitution[1]. The increase in the degree of complexation is due to the increased hydrophobicity of the halogens. The addition of a nitro group to position R_1 or a hydroxyl group to positions R_3 increases the degree of hydrogen bonding possible, thus also increasing the extent of complexation. These trends are also observed by Fujimura and Shono.

The possibility of a second inclusion complex formed by inclusion of the A ring, rather than the C ring, is introduced in order to explain the effect of changing the R_4 substituent, especially in temazepam where a simultaneous change in R_3 also occurs. Introduction of a methyl group at R_4 would sterically hinder the inclusion of the A ring. Introduction of the triethyl amine group would also increase steric hindrance to a much larger degree. The changes in K_{DS} support this interpretation, especially the decrease in stability noted for flurazepam. Fujimura (118) and Shono (119) also reported a decrease in complex stability when methyl groups were present. The second complexation site also explains the small increase in stability observed for temazepam. The addition of the hydroxyl group should greatly increase the stability as is seen in delorazepam and lorazepam, however only a small

---

1. Capacity factors should reflect the magnitude of the equilibrium constant since
$k' = K V_S/V_M$
where V_S and V_M are the volume of the stationary and mobile phase respectively. K is the distribution coefficient which can be related to the formation constant of the complex since it governs how much analyte is present in the stationary phase.

TABLE XII

CHANGES IN THE FORMATION CONSTANT AND THE MOLAR ELLIPTICITY WITH CHANGES IN THE SUBSTITUENTS OF THE BENZODIAZEPIN-2-ONE RING SYSTEM

Compound	R_1	R_2	R_3	R_4	ΔK_{DS}	θ_{DS}
Nordiazepam	Cl	H	H	H		
Nitrazepam	NO_2	-	-	-	+346	-933
Delorazepam	-	Cl	-	-	+482	-418
Oxazepam	-	-	OH	-	+436	-254
Diazepam	-	-	-	CH_3	+75	-787
Clonazepam	NO_2	Cl	-	-	+679	-905
Lorazepam	-	Cl	OH	-	+795	-366
Temazepam	-	-	OH	CH_3	+14	-799
Flurazepam	-	F	-	$CH_2CH_2NEt_2$	-27	-787

change is observed. If this hydroxyl group was removed from the site of interaction, its effect would be minimal. This is the situation if the A ring is the site of inclusion.

The small formation constant observed for the parent complex can also be explained using the same type of comparisons. Fujimura (118) reports a decrease in stability when amine groups are present. The lack of hydrogen bonding and hydrophobic groups coupled with the presence of an amine function on the diazo ring would decrease the ability of either site to interact with the β-cyclodextrin cavity.

The structural subtleties of molecular association are never fully understood no matter how impartial is the attempt to choose model compounds. Apparent inconsistencies arise when comparisons are made between different guests, such as has occurred in this case

between the barbiturates and the benzodiazepin-2-ones, which only serve to point up the limitations of our understanding of such interactions. Fujimura (118) and Shono (119) both used 20/80 methanol/water mobile phases. Solvent effects can have a marked effect on capacity factors and equilibrium constants. Extrapolation from one system to another should be made with caution. The above discussion should be viewed as a preliminary attempt to describe the general mechanism of complexation and compares the results for solution and column equilibria.

Because of the growing utility of β-cyclodextrin phases, an effort has been made to understand the mechanism of complexation. The final objective is to be able to predict which analytes will be separated using a given mobile phase. Because of the direct relationship between the equilibrium constants and the retention of an analyte on a column, CD determinations of equilibrium values in different solvents might help predict elution order and optimum separation conditions without the need for trial and error runs on the HPLC.

CHAPTER VIII

DETERMINATIONS OF ENANTIOMERIC EXCESS

Introduction

Pasteur's discovery of optically active molecules was mentioned in Chapter II. His separation of enantiomeric tartrate crystals not only played a key role in the development of chiroptical theory and methods, but was also the first enantiomeric "determination". For over a century, Pasteur's method of crystallization and manual separation was the only technique available for enantiomeric excess determinations. The procedure is tedious and does not work for all stereoisomers. Consequently, determinations of enantiomeric excess in pharmaceutical or clinical samples were usually not performed, even though the significance of stereoisomerism in biological systems was generally recognized.

Early attempts to determine the optical purity by using inclusion complex formation and GC techniques have been reviewed by Feibush and Grinberg (120). Melting range can be used to indicate whether a sample consists of just one enantiomer or a racemic mixture, however, discrimination between or quantitation of the enantiomers is impossible. Recently Raman optical activity spectroscopy (121), and NMR with chiral lanthanide shift reagents (122) have been utilized in optical purity determinations. These methods are ideal for detection of the differing stereochemical isomers, but quantitation can be difficult. Separation of stereoisomers on chiral HPLC columns has been the most successful determination method to date. An extensive review of chiral separation techniques has

appeared recently (123). HPLC data are readily quantitated, but often suffer from incomplete separation of the enantiomers.

The recent development of techniques capable of detecting the different enantiomers has sparked a renewed interest in enantiomeric excess determinations, especially in the pharmaceutical industry. The differing physiological and toxicological effects of racemates has been well documented. The development of analytical procedures for determination of enantiomeric excess has prompted the FDA to enact more rigid criteria for identifying and quantitating the enantiomeric concentrations and the pharmacological actions of the individual enantiomers (17).

CD is an ideal detector for optical purity determinations because of its inherent ability to detect chiral molecules. The sign of the CD signal, or lack of a signal in a sample known to possess stereoisomers, can indicate which isomer is present in excess or the existence of a racemic mixture, respectively. Polarimetry and ORD possess the same capability, but lack the added selectivity of CD and are more likely to suffer interference problems (Chapter II). The inherent advantages of CD detection prompted a series of investigations into its utility in determining the enantiomeric excesses of mixtures. Three methods were explored: standard addition, UV-absorption/CD, and CD/CD.

Enantiomeric Excess Determination by Standard Addition

Introduction

Standard addition is a common procedure in many analytical laboratories. Most introductory and some advanced texts covering quantitative chemical analysis include a description of this technique. Standard addition is generally employed when the sample matrix has some effect on the analytical signal and the matrix is too complex to duplicate in standard solutions.

The goal of optical purity determinations is to measure the analytical concentration of both stereoisomers. Initially standard addition procedures looked like a promising solution to this problem. If one of the enantiomers is treated as a matrix interference, standard addition of one of the isomers, preferably the one present in the lesser proportion, should produce a linear curve with a x-intercept corresponding to the concentration of that component. The concentration of the major component is the sum of the concentration at the x-intercept and the concentration calculated from the observed ellipticity, if any, of the CD maximum. Mixtures of D- and L-phenylethylamine were used to test this presumption.

Experimental

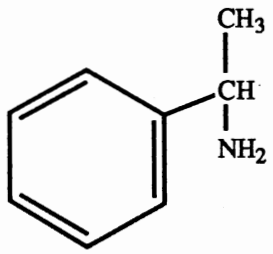
D-(-)- and L-(+)-phenylethylamine as freebase were obtained from Sigma Chemical Co. and were used without further purification. Stock solutions of approximately 1.5×10^{-1} M D- or L-phenylethylamine were used to prepare the enantiomeric mixtures. The relative content of both isomers was varied from 0% to 100% . 0.1 to 1.0 mL volumes of the D or L stock solution were added to the 10 mL volumetric flasks containing 1 mL of the enantiomeric mixture before the final dilution with 1 M HCl. CD spectra were obtained and standard addition curves were constructed from the measured ellipticity at 275 nm.

Results and Discussion

The structure of the phenylethylamine enantiomers is given in Figure 26. CD spectra of both stereoisomers have been characterized previously (124).

Standard additions of both the D and L forms were made for each prepared sample. Plots of measured ellipticity versus concentration of added enantiomer were constructed and the x-intercept (in concentration units) was compared with the total phenylethylamine concentration, the concentration of each enantiomer in a mixture, and the concentration of

the major isomer minus the concentration of the minor component (i. e. C_{EI} - C_{MI}). The best correspondence was found between the x-intercept concentration and the last value (average percent difference of 11.0%, median percent difference of 6.7%). No correlation was found between the x-intercept concentration and the other concentrations. In this case, standard addition techniques failed to yield the desired result, namely the concentrations of both enantiomers.



PHENYLETHYLAMINE

Figure 26. Molecular structure of Phenyl-ethylamine.

The failure of standard addition to solve this problem can be explained by reviewing the basic theory for both the analytical technique and CD measurement. The CD signal observed for an enantiomeric mixture is the sum of the signals from both isomers,

$$\psi = \theta_D[D] + \theta_L[L] \quad (35)$$

where ψ is the observed ellipticity, θ_D and θ_L are the molar ellipticities for the D and L isomers respectively, and [D] and [L] are the respective analytical concentrations of the isomers. For enantiomeric pairs,

$$\theta_L = -\theta_D \quad (36)$$

Substituting equation 36 into equation 35 gives,

$$\psi = \theta_L([L] - [D]) \quad (37)$$

The observed CD signal is therefore the difference in the analytical concentrations of the enantiomers. In order for standard addition to be successful, none of the analytical signals employed for a determination can be linear combinations (125, 126). Equation 37 shows that the observed ellipticity is a linear combination and thus standard addition procedures are ineffective in determining enantiomeric excess.

Enantiomeric Excess Determination by UV Absorption and CD

Introduction

A second approach to enantiomeric excess determinations requires the performance of two independent experimental measurements. One of the measurements must focus on the chiroptical properties of the analytes. Boehm et al (127) were the first to apply this concept to enantiomeric excess determinations. An ultraviolet detector in series with an optical activity detector, a polarimeter, was employed for the determination of enantiomeric ratios in conventional HPLC separations. This combination eliminates the need for a chiral separation, but because a polarimeter is employed, all other chiral analytes must be separated in order to ensure accurate measurement. CD could replace the polarimeter, but if it is limited to single wavelength detection no improvement over polarimetry can be expected. The small elution volumes and low analyte concentrations typical of HPLC require that either the analyte possess a very large optical rotation (or ellipticity in the case of CD) or a laser based light source must be used to produce the required sensitivity.

Nicotine is not a pharmaceutical compound, but it is of interest to the tobacco industry and to the medical community because of its relationship to cancer. The enantiomeric ratio of nicotine in the leaves used in cigarettes and smokeless tobacco may have pronounced

effects upon the type and extent of the physiological action and is an important parameter to know about commercial products.

Armstrong and coworkers (128) have reported the enantiomeric resolution of nicotine on a reverse phase HPLC column by employing β-cyclodextrin as a mobile phase additive. Baseline separation of the enantiomers is possible, but the retention time is extremely long, in excess four hours. The procedure is suitable but impractical for analysis of multiple samples.

In this work the determination of the enantiomeric excess of spiked tobacco leaf extracts, the UV measurement has been retained. In order to avoid the problems of small elution volume and low analyte concentration, CD spectra were measured for samples of the unseparated mixtures which were on the order of a few milliliters.

Experimental

Samples of D- and L-nicotine standards (as free base) and of tobacco leaf extracts spiked with the unnatural D-isomer were provided by the R. J. Reynolds Co. 2-propanol was used to extract the alkaloid from the leaves and for dilution of the standards . The only sample preparation required was dilution of the leaf extracts. Approximately 0.2 to 0.4 mL of extract was diluted to a final volume of 10 mL with 2-propanol. CD spectra were obtained directly. Total nicotine concentrations of the leaf extracts were determined from the absorption of the eluting bands after HPLC separation and were were provided by Reynolds subsequent to the completion of the CD experiment.

Results and Discussion

CD spectra of the isomeric standards and of one leaf extract are shown in Figure 27. The spectrum for the L-isomer bears a strong resemblance to that for the isomer dissolved

in methanolic-KOH solutions and used in the determination of nicotine in commercial forms of tobacco (129).

Molar ellipticities (θ_M) were determined from data taken at a number of wavelengths around the 272 nm maximum. These are compared in Table XIII for the D- and L-forms. If the isomeric purities were precisely the same, these values would be equal in magnitude and opposite in sign at every wavelength. The observed discrepancies in the present figures are due to instrumental limitations and should not be construed to be an accurate measure of the purity difference. Such a conclusion might have some meaning if the discrepancy is significantly greater and consistently the same throughout the entire spectrum. Theoretically the θ_M value for only one of the enantiomers is needed to calculate

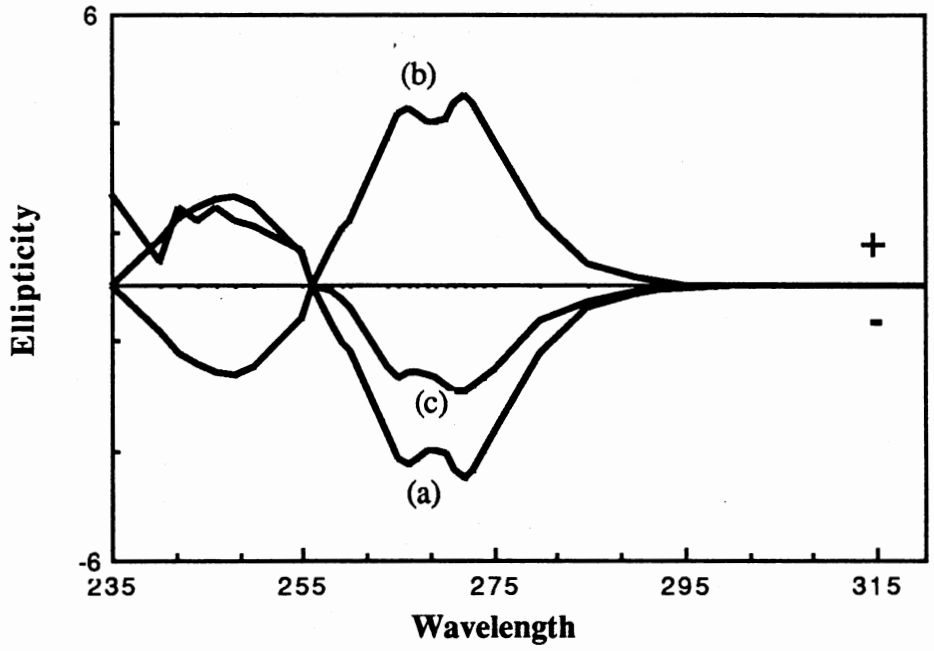


Figure 27. CD spectra of (a) L-nicotine standard, (b) D-nicotine standard, and (c) tobacco leaf extract (Table XIV, EXT3) in 2-propanol.

TABLE XIII

MOLAR ELLIPTICITIES AS A FUNCTION OF WAVELENGTH FOR D- AND L-NICOTINE ENANTIOMERS

Wavelength (λ nm)	θ_L (deg./M cm)	θ_D (deg./M cm)
269	-73.4	74.6
270	-78.7	78.1
271	-84.1	84.9
272	-91.4	91.0
273	-90.3	92.0
274	-85.9	86.0
275	-77.4	75.6
276	-66.6	64.5
277	-58.0	56.4
278	-48.6	48.0

the enantiomeric excess, but it must be assumed that the figure is for a standard which is 100% pure. The determination is only as good as the standard. This is true no matter what type of analytical procedure is employed. In the present case the enantiomeric excess has been calculated using both θ_D and θ_L values on the one hand, and only the larger value on the other, which assumes that the larger value is more representative of 100% purity. Because the standards appear to be very similar in purity there is essentially no difference in the calculated results. The results of the enantiomeric excess determination obtained from only θ_L are given in Table XIV.

The enantiomeric concentrations are calculated by expressing both the measured absorbance and ellipticity in terms of enantiomer concentration and solving the resulting set

of equations. The measured absorbance, A, is proportional to the sum of the enantiomer concentrations, i.e. the total nicotine concentration, C_T,

$$A = \varepsilon[D] + \varepsilon[L] = \varepsilon([D] + [L]) = \varepsilon C_T \quad (38)$$

where ε is the molar absorptivity, and [D] and [L] are the concentrations of the respective enantiomers. The experimental ellipticity, ψ, on the other hand, is proportional to the difference in enantiomer concentrations, equation 37. Solving equations 37 and 38 simultaneously yields the concentrations of both enantiomers present in the mixture.

TABLE XIV

ENANTIOMERIC EXCESS DATA FOR SPIKED TOBACCO MIXTURES

Sample	Concentration (mg/mL)	% L (theoretical)	% D (theoretical)
EXT1	1.069	37.5 (36)	62.5 (64)
EXT2	1.385	9.9 (10)	90.1 (90)
EXT3	1.451	50.0 (50)	50.0 (50)
EXT4	1.887	62.3 (65)	37.2 (37)
EXT5	1.374	13.1 (10)	86.9 (90)
EXT6	1.261	9.9 (10)	90.1 (90)

The correspondence between calculated enantiomer concentration and theoretical concentration is excellent, Table XIV. The largest deviations are observed for the

concentrations of the stereoisomer present in lesser amounts (average percent difference of 7.2%). Deviations between the calculated and theoretical values are much smaller for the component in large excess (average percent difference of 1.7%). Similar trends were observed for the two- and three-component *Rauwolfia* alkaloid mixtures (Chapter V).

The presence of other absorbers (129) required that the tobacco extracts be first separated by conventional HPLC before the total amount of nicotine in the samples could be determined. (The enantiomers co-elute from a conventional HPLC column.) Where other absorbing species are absent, total enantiomeric concentration would be obtained by a direct absorbance measurement. The S/N quality of the CD signal would be adversely affected by other absorbers and if extreme some type of sample clean up would be necessary. The presence of other CD active analytes are also cause for concern, though chiral analytes which do not absorb are not a problem. In the above analysis, no other chiral analytes were present and visual inspection of the spectra was sufficient indication of whether one isomer was in excess or whether the sample was a racemic mixture. Interference from other CD active compounds would be seen as a distortion of the spectrum of the principle analyte. The integrity of the original spectrum of either isomer is maintained even in enantiomeric mixtures, Figure 27c, thus the presence of another CD active compound would be recognized. In the worst possible case, sample clean up would be required. Conventional HPLC is one option, however, solid phase extraction procedures are showing great promise as a sample clean up technique (130). Even in the worst cases of interference, enantiomeric separation is unnecessary.

The combination of CD with absorption spectrophotometry, or other analytical techniques, provides a viable alternative to the often difficult problem of attaining complete enantiomeric separation. A word of caution is pertinent here in that the observed CD spectrum may be that for either a single analyte in low relative concentration, or for a mixture at a higher analytical concentration but with a small enantiomeric excess value. The

dichotomy is easily resolved by calculating the total enantiomeric concentration from an appropriate alternative method.

Enantiomeric Excess Determination By CD

Introduction

The lack of selectivity in UV absorption spectrophotometry might require additional steps in the sample preparation, steps which are not always necessary when CD detection is used. Analytical determinations of pen-V (Chapter IV), reserpine in *R. serpentina* root (Chapter V), L-cocaine in illicit drug samples (131), and nicotine in tobacco products (129) were accomplished using CD detection and required only a dilution step or a simple extraction procedure before determination. Employing only CD detection for the determination of enantiomeric excess would in many instances simplify sample preparations and reduce analysis times.

The ability of β-cyclodextrin to induce CD activity into achiral analytes was demonstrated in Chapter VII. β-cyclodextrin can also induce CD activity into total or partial racemic mixtures (124) and permits enantiomeric separation when used as either a chiral stationary phase or mobile phase additive (132). These may seem to be unrelated capabilities, but in fact both phenomena result from the preferrential complexation between β-cyclodextrin and one of the enantiomers of a pair.

The third technique for the determination of enantiomeric excess that has been used takes advantage of the difference in induced CD activities between the enantiomers and uses the resulting conditional ellipticity in place of absorbance as the second independent measurement. The other independent measurement is taken from the CD spectrum of the analyte in an achiral solvent. Optical purities for a number of D- and L-cocaine

enantiomeric mixtures are determined using this technique and some preliminary results for enantiomeric mixtures of pseudoephedrine are also reported.

Experimental

D-cocaine as free base, L-cocaine hydrochloride (National Institute on Drug Abuse, Research Technology Branch), L-pseudoephedrine hydrochloride (Sigma Chemical Co.) and D-pseudoephedrine hydrochloride (Burroughs Wellcome Co.) were obtained and used without further purification. Street samples of cocaine hydrochloride and samples of "crack" were obtained from the Forensic Laboratory of the Oklahoma City Police Department. β-cyclodextrin was purchased from Eastman Kodak Co.. All solvents employed were of analytical grade quality.

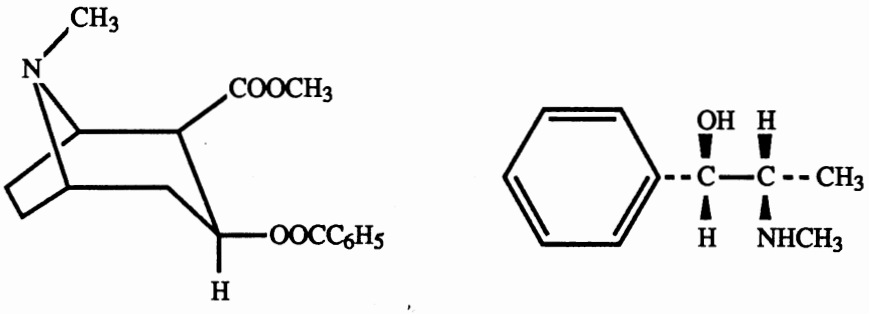
CD spectra were obtained for D- and L-cocaine standards, for prepared laboratory mixtures, and for extracts of street samples of cocaine hydrochloride and "crack", first in 0.1 M HCl and then in a solution of 10^{-2} M β-cyclodextrin (in 0.1 M HCl). Solutions were prepared such that both solvents contained exactly equal quantities of standard or sample. The content of each enantiomer in the laboratory mixtures was varied from 0 to 100%.

D- and L-pseudoephedrine standards and laboratory mixtures were dissolved in 0.005 M HCl or 10^{-2} M β-cyclodextrin (in 0.005 M HCl). Each solvent contained identical quantities of the enantiomers. The D- and L-pseudoephedrine content of the laboratory mixtures was varied from 10 to 90%.

Results and Discussion

Molecular structures of the naturally occurring cocaine and pseudoephedrine stereoisomers are shown in Figure 28. The CD spectra for the uncomplexed enantiomers

are mirror images of each other, Figures 29(a), 30(a) and 31(a and b). Complexation with β-cyclodextrin causes a blue shift in the short wavelength band of the D-cocaine spectrum, Figure 30. By comparison the equivalent band in the CD spectrum of L-cocaine exhibits a smaller blue shift, but the sign of the CD signal is inverted as the L-cocaine concentration is increased, Figure 29. The corresponding changes in the wavelength maxima are recorded in Table XV.



L-COCAINE D-PSEUDOEPHEDRINE

Figure 28. The molecular structures of the naturally occuring stereoisomers: L-cocaine and D-pseudoephedrine.

The CD spectra of the complexed pseudoephedrine enantiomers undergo a slight red shift and decrease in intensity when compared to the spectra for the uncomplexed forms, Figure 31. The difference between the molar ellipticities for the complexed D- and L-isomers is small, but readily measurable using the computer accessory, Table XV.

Initial attempts at developing the second independent CD measurement were focused on calculating the formation constant for the cocaine/β-cyclodextrin complex. The formation constant obtained for the L-cocaine complex, 491.5 ± 15, agrees with earlier results (133). Determination of the corresponding formation constant with equivalent accuracy for the D-

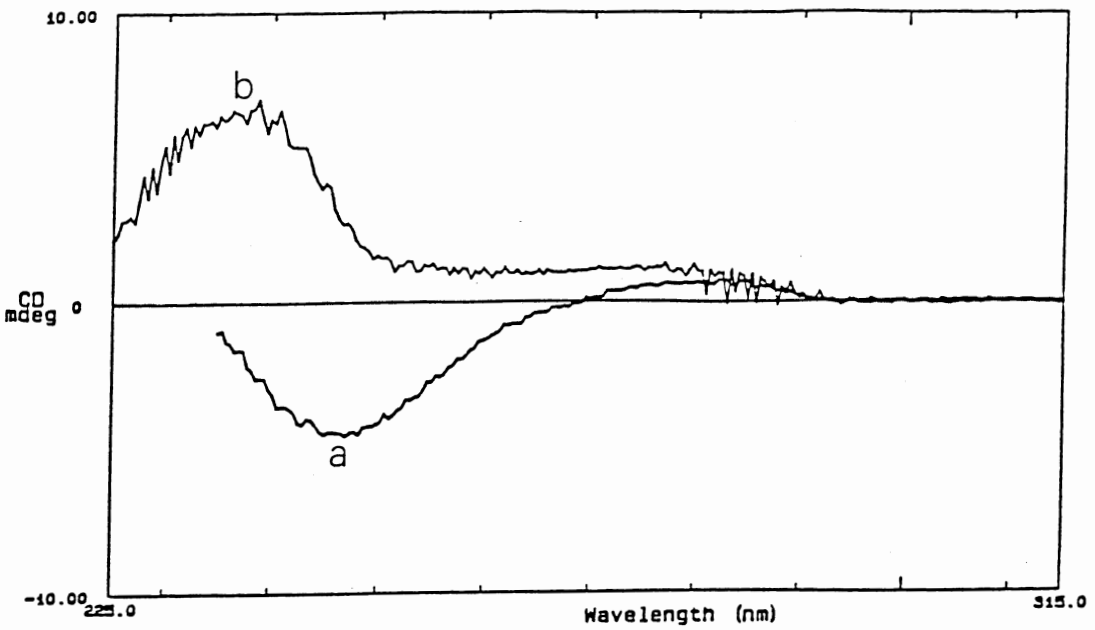


Figure 29. CD spectra for L-cocaine in (a) 0.1 M HCl and (b) 10^{-2} M β-cyclodextrin.

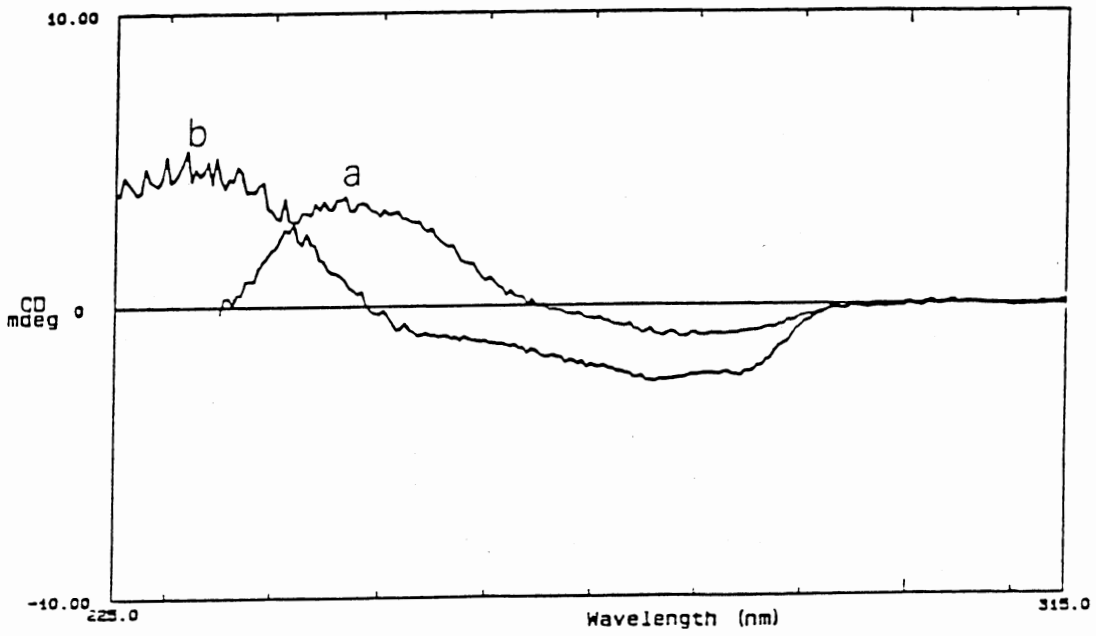


Figure 30. CD spectra for D-cocaine in (a) 0.1 M HCl and (b) 10^{-2} M β-cyclodextrin.

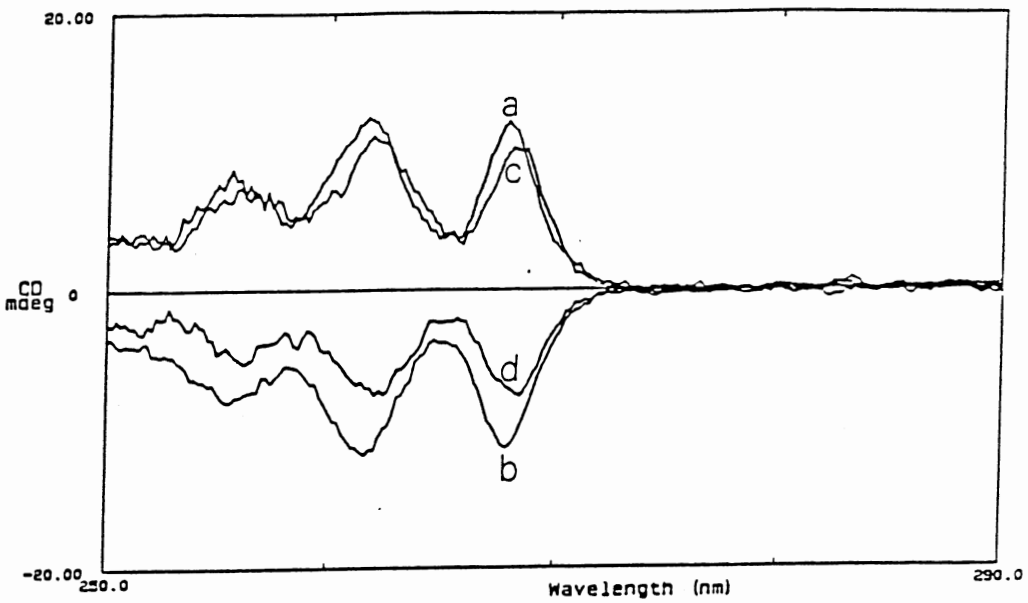


Figure 31. CD spectra for (a) L-pseudoephedrine in 0.005 M HCL, (b) D-pseudoephedrine in 0.005 M HCl, (c) L-pseudoephedrine in 1.5 x 10^{-2} Mβ-cyclodextrin and (d) D-pseudoephedrine in 1.5 x 10^{-2} M β-cyclodextrin.

TABLE XV

MOLAR ELLIPTICITIES FOR THE UNCOMPLEXED AND COMPLEXED FORMS OF THE D- AND L-STEREOISOMERS OF COCAINE AND PSEUDOEPHEDRINE

Enantiomer	Uncomplexed θ_M(nm) (deg/M cm)	Complexed θ_C(nm) (deg/M cm)	
L-cocaine	-55.7 (246.0)	+72.8 (236.0)	+54.7 (230.0)
D-cocaine	+49.3 (246.0)	+52.9 (236.0)	+57.1 (230.0)
L-pseudoephedrine	+2.3 (268.0)	+2.2 (268.2)	
D-pseudoephedrine	-2.4 (268.0)	-1.6 (268.2)	

cocaine complex was not possible. The small changes in the induced CD signal and the poor S/N quality of the signal precluded accurate measurement of the actual changes caused by complexation with β-cyclodextrin.

A second alternative was to measure the "conditional" molar ellipticity values (θ_C in Table XV) of the enantiomers in β-cyclodextrin by preparing calibration curves of the experimental ellipticity versus the total drug concentration. The measured ellipticity, ψ_C, is equal to the sum of the induced CD signals of both enantiomers,

$$\psi_C = \theta_{CL}[L] + \theta_{CD}[D] \qquad (39)$$

where θ_{CL} and θ_{CD} are the conditional molar ellipticities for the L- and D-isomers respectively. Equation 39 is used in place of the absorbance measurement represented by equation 38. The concentration of each isomer in the enantiomeric mixture is calculated by solving equations 37 and 39 simultaneously.

Correspondence between prepared and measured enantiomeric concentrations for the laboratory mixtures was excellent, Table XVI. The largest errors were associated with the enantiomer present in the lesser amount (a percent error between prepared and measured of 5.7%). Similar trends have been observed for multi-component alkaloid mixtures (Chapter V) and enantiomeric mixtures of nicotine (Table XIV). Accurate determination of the D-isomer when it is the minor enantiomer are more difficult than the corresponding determination of the L-form. This is expected since the induced signal for the D-isomer is smaller than that for the L-isomer and the associated maximum has a smaller S/N ratio because of the extra absorbance at the shorter wavelengths.

The data presented in Table XVI was measured from full spectral scans. Scanning just the maximum and a small portion of the baseline for each sample reduces analysis time dramatically, from one hour per sample to approximately 10 minutes. Correlation between prepared and measured percentages of the major enantiomer are still excellent (an average percent error of 2.8%), however the error associated with the minor enantiomer increases (an average percent error of 8.8%).

For the street cocaine samples the total cocaine content as a percentage of the total weight as well as the enantiomeric ratio is obtained. There are no reference materials with which to compare the results for the street samples, however, total cocaine content was determined from absorbance measurements. Excellent agreement between the absorbance (84.6 ± 4.3%) and CD (86.4 ± 7.8%) measurements was obtained. There was no evidence to indicate the presence of D-cocaine in these samples.

TABLE XVI

DETERMINATION OF ENANTIOMERIC EXCESS IN D- AND L-COCAINE MIXTURES

Sample	%L (Theoretical)	%D (Theoretical)
LAB1	- (0)	101.1 (100)
LAB2	15.5 (15.2)	84.5 (84.8)
LAB3	28.7 (29.7)	71.3 (70.3)
LAB4	51.1 (52.2)	48.9 (47.8)
LAB5	60.3 (60.0)	39.7 (40.0)
LAB6	79.6 (78.6)	20.4 (21.4)
LAB7	96.8 (100)	3.2 (0)
SPK1	19.4 (17.4)	80.7 (82.6)
SPK2	40.5 (36.0)	59.6 (64.0)
SPK3	56.7 (55.8)	43.4 (44.2)
SPK4	78.5 (77.1)	21.6 (22.8)
SPK5	98.9 (100)	1.1 (0)

Only one crack sample was investigated. CD measurement yielded a L-cocaine content of 91.5% by weight. Corresponding absorbance data was not obtained. The presence of D-cocaine was not evident in these samples.

Street cocaine samples spiked with the unnatural D-isomer were also examined. A L-cocaine content of 86.4% was assumed for the street samples based on the above CD data. D-cocaine content was determined directly from the weight added to each sample. Correlation between prepared and measured percent was excellent, Table XVI.

A problem encountered during analysis of the street samples, but not in the analysis of laboratory mixtures which were prepared from the standard materials, is the lack of equivalent purity in the two cocaine standards indicated by the large differences in θ_D, Table XV. The chemical data sheet supplied with the D-isomer indicated good correlation between melting point and polarimetric measurements obtained by the supplier and those values reported in the literature. Independent polarimetric and absorption measurements of both enantiomers support the conclusion from the CD data that the D-cocaine standard is of lesser purity. While equation 36 applies for uncomplexed enantiomers, a similar assumption for the molar ellipticities of the complexed forms would not be valid. The additional uncertainity in θ_{DDS} is expected to contribute more towards the errors in the calculated enantiomeric concentration, especially in cases of low D-cocaine content. Such observations have been reported for the laboratory mixtures.

Laboratory mixtures of D- and L-pseudoephedrine were also analyzed for enantiomeric excess. Measured percentages are very close to the prepared values, Table XVII. Recent determinations have yielded correlations of better than ±2% for all enantiomeric ratios. Anaylsis of real samples is still in the preliminary stages, but initial results are promising (134).

This work is another example of the versatility of CD as an analytical detector. CD detection eliminated the need to perform a chiral separation via HPLC thus reducing analysis time. Neither derivatization steps nor addition of internal standards was required.

TABLE XVII

ENANTIOMERIC EXCESS DETERMINATION OF LABORATORY MIXTURES OF D- AND L-PSEUDOEPHEDRINE

Sample	%L (Theoretical)	%D (Theoretical)
EPH1	6 (10)	94 (90)
EPH2	39 (40)	61 (60)
EPH3	50 (50)	50 (50)
EPH4	74 (75)	26 (25)

CHAPTER IX

CONCLUSION

The primary goal of the investigations described in this work was to develop selective analytical procedures for the determination of pharmaceuticals and related substances, without the need for chromatographic separation or extensive sample work up often required in a number of other analytical techniques. The successful determination of multi-component samples such as the β-lactam antibiotics, the indole alkaloids of *R. serpentina*, and the water soluble vitamins C, B_2, and B_{12} demonstrates how CD detection can simplify an analysis, thus reducing sample turn around time, a parameter extremely important in a pharmaceutical or clinical quality control laboratory where large numbers of samples are processed on a routine basis. Recent work indicates that the use of a microcomputer to record and manipulate the analytical signal might help to further reduce the analysis time, as well as improve the accuracy and reproducibility attainable during analysis of multi-component mixtures, especially when determining the concentration of the minor constituents. Automation would also reduce analysis time.

The successful application of CD detection to enantiomeric excess determinations another demonstration of the utility of this detector. Inherent selectivity for chiral molecules allows determinations to be accomplished after only a simple extraction step. Though present work has been limited to complexations using aqueous solutions of β-cyclodextrin; chiral crown ethers, NMR shift reagents, and metal ion complexes, and as well as other solvents could be used to extend the applicability of this technique. Other

variables such as pH and temperature might also be manipulated in order to maximize the differences between the resulting CD spectra.

Determination of enantiomeric excess via CD detection has definite advantages over the often preferred enantiomeric separation performed by chiral HPLC techniques. HPLC procedures often suffer from incomplete separations, band spreading, co-elution of other analytes, and long retention times. In addition, selecting the proper chiral stationary phase can be difficult and is often more art than science. CD studies of the changes in formation constants may prove to be useful in narrowing the possibilities and in choosing the proper solvent conditions. For example, the formation constants calculated for the cocaine enantiomers indicate that the D-cocaine/β-cyclodextrin complex is of lower stability. D-cocaine is also least retained by a β-cyclodextrin stationary phase (135). This implies that it might be feasible to employ CD studies of complex formation under varying conditions to predetermine the proper conditions for an HPLC separation. This would reduce the number of trials needed on the HPLC, thus extending column life and conserving expensive HPLC grade solvents. In depth studies are required before any significant correlations can be made.

CD detectors can also be interfaced directly with HPLC systems. Such a combination is useful for studying substances of natural orgin because of the associated chirality of these molecules. Generally CD detectors for HPLC applications are limited to one wavelength, which greatly reduces the potential of the detector. Full spectrum CD has much more utility, especially when baseline separation of chiral analytes is not possible. For situations were complete separation is not a problem, polarimeters are excellent detectors. They are less expensive both to purchase and maintain than CD instrumentation and they can detect nonabsorbing chiral analytes that are invisible to the CD. In view of these considerations and the evidence that CD can be used as a stand alone detector without the need for HPLC separations, further efforts are probably better focused on developing procedures which would increase the number of analytes detectable by CD.

Induction of CD activity in achiral molecules by complexation with β-cyclodextrin is one way to increase the number of detectable analytes and has been discussed in Chapter VI. However, the possibility of inducing CD activity in chiral molecules with no chromophore has been generally overlooked. Color induction is a standard tool in clinical chemistry when absorption is the method of choice and can be successfully applied to CD studies. Sterols, sterones, and the fat soluble vitamins D_2, D_3, and E have all exhibited induced CD activity upon color induction (136). Interferences are common in colorimetric techniques as applied to absorption measurements, however, in CD the produced colored species must meet the criteria for CD activity in order to be detected. Therefore, interferences typical in absorption measurements may only reduce the S/N in CD measurements.

These proposed applications of CD detection would be useful in forensic, clinical, and pharmaceutical laboratories where chiral molecules are common subjects of interest. HPLC and absorption spectrometry both in combination and individually have become the standard procedures in these areas in spite of the limitations associated with each. CD detection can circumvent many of these problems and provide information not presently easily attainable. For example, in the study of the pharmacokinetics of enantiomeric drugs. Presently such kinetics methods are handled by absorbance measurements which are insensitive to chiral subtleties present in most living systems. Enantiomers are metabolized differently or at different rates if they do follow the same metabolic pathway. Absorbance measurements provide an "average" kinetic profile that is in some cases markedly different from the profile of individual enantiomers. Using the techniques discussed in Chapter VIII, it would be possible to follow the kinetics of each enantiomer.

The development of the future applications of CD detection depends upon the outcome of more complete studies. The desirability of developing fast-scan capabilities or laser sources for CD work is contingent upon the acceptability and applicability of the proposed method. CD detection is by no means ideal for all situations, in some cases its very

selectivity excludes its use. In addition, CD methods are subject to one of the primary limitations of almost every analytical technique, the relative purity of the calibrating standard. However, CD detection has some unique characteristics that would be invaluable in quality assurance testing in the pharmaceutical laboratory and for screening tests in clinical chemistry.

LITERATURE CITED

1. Schuster, C. R. *Clin. Chem.* **1987**, *33*, 7B.

2. Finkle, B. S. *Clin. Chem.* **1987**, *33*, 13B.

3. Wendlandt, W. W. *Thermal Analysis* ; Elving, P. J.; Winefordner, J. D., Eds., Chemical Analysis; John Wiley & Sons: New York, 1986; Vol. 19, Chapters 4 and 7.

4. Smith, B. A.; Joseph, J. C. In *Analytical Aspects of Drug Testing*; Deutsh, D. G., Ed.; Chemical Analysis; John Wiley & Sons: New York, 1989; Vol. 100, Chapter 2.

5. Calatayud, J. M. *J. Pharmazie* **1986**, *41*, 92.

6. Smeyers-Verbeke, J.; Detaevernier, M. R.; Massart, D. L. *Anal. Chim. Acta* **1986**, *191*, 181.

7. Woolfson, A. P. *Anal. Proc.* (London), **1986**, *23*, 89.

8. Patriarche, G. J.; Vire, J. C. *Anal. Chim. Acta* **1987**, *196*, 193.

9. Gough, T. A.; Baker, P. B. *J. Chromatogr. Sci.* **1982**, *20*, 289.

10. *GLC and HPLC Determination of Therapeutic Agents*; Tsuji, K.; Morozowich, W., Eds.; Chromatographic Science Series; Marcel Dekker: New York, 1978; Vol. 9, Parts 1-3.

11. *Therapeutic Drug Monitoring and Toxicology by Liquid Chromatography*; Wong, S. H. Y., Ed.; Chromatographic Science Series; Marcel Dekker: New York, 1895; Vol. 32.

12. Watson, I. D. In *Advances in Chromatography*; Giddings, J. C.; Grushka, E.; Brown, P. R., Ed.; Marcel Dekker: New York, 1987; Vol 26, Chapter 4.

13. Hill, D. W.; Langner, K. J. In *Analytical Aspects of Drug Testing*; Deutsh, D. G., Ed.; Chemical Analysis; John Wiley & Sons: New York, 1989; Vol. 100, Chapter 5.

14. Wong, S. H. Y. In Analytical Aspects of Drug Testing; Deutsh, D. G., Ed.; Chemical Analysis; John Wiley & Sons: New York, 1989: Vol. 100, Chapter 6.

15. Voykner, R. D. In Anlytical Aspects of Drug Testing; Deutsh, D. G., Ed.; Chemical Analysis; John Wiley & Sons: New York, 1989, Vol. 100, Chapter 7.

16. Berry, A. J.; Games, D. E.; Perkins, J. R. *J. Chromatogr.* **1986**, *363*, 147.

17. De Camp, W. H. *Chirality* **1989**, *1*, 2.

18. Reitsma, B. H.; Yeung, E. S. *J. Chromatogr.* **1987**, *405*, 295.

19. Takakuwa, T.; Kurosu, Y.; Sakayanagi, N.; Kaneuchi, F.; Takeuchi, N.; Wada, A.; Senda, M. *J. Liq. Chromatogr.* **1987**, *10*, 2759.

20. Meinard, C.; Bruneau, P.; Perronnet, J. *J. Chromatogr.* **1985**, *349*, 109.

21. Synovec, R. E.; Yeung, E. S. *J. Chromatogr.* **1986**, *368*, 85.

22. Crabbe, P. *Optical Rotatory Dispersion and Circular Dichroism in Organic Chemistry*; Holden-Day: San Francisco, 1965.

23. Velluz, L.; Legrand, M.; Grosjean, M. *Optical Circular Dichroism: Principles, Measurements, and Applications*; Academic Press: New York, 1965.

24. Lowry, T. M. *Optical Rotatory Power*, Dover Publications: New York, 1935.

25. Crabbe, P. *ORD and CD in Chemistry and Biochemistry: An Introduction*, Academic Press: New York, 1972.

26. Charney, E. *The Molecular Basis of Optical Activity: Optical Rotatory Dispersion and Circular Dichroism*; John Wiley & Sons; New York, 1965.

27. Abu-Shumays, A.; Duffield, J. J. *Anal. Chem.* **1966**, *38*, 29A-58A.

28. Armstrong, D. W. *Anal. Chem.* **1987**, *59*, 84A-91A.

29. *Optical Rotatory Dispersion and Circular Dichroism in Organic Chemistry*, Snatzke, G., Ed.; Heyden and Son: London, 1967.

30. Djerassi, C. *Optical Rotatory Dispersion: Applications to Organic Chemistry*; McGraw-Hill: New York, 1960.

31. *Chiroptical Techniques; Nomenclature, Symbols, Units.* Spectroscopic Nomenclature Committee, International Union of Pure and Applied Chemistry.

32. Pavia, D. L.; Lampman, G. M.; Kriz, G.S. *Introduction to Spectroscopy: A Guide for Students of Organic Chemistry*; Saunders College: Philadelphia, 1979; Chapter 5.

33. Rao, C. N. R. *Ultra-Violet and Visible Spectroscopy: Chemical Applications*; Butterworths: Boston, 1975.

34. Skoog, D. A.; West, D. M. *Principles of Instrumental Analysis*, 2nd ed.; Saunders College: Philadelphia, 1980; Chapter 6.

35. Ingle, J. D.; Crouch, S. R. *Spectrochemical Analysis*; Prentice Hall: New Jersey, 1988.

36. Willare, H. H.; Merrit, L. L.; Dean, J. A. *Instrumental Methods of Analysis* ; 4th ed.; D. Van Nostrand: New Jersy, 1965.

37. Synovec, R. E.; Yeung, E. S. *Anal. Chem.* **1986**, *58*, 1237A.

38. Westwood, S. A.; Games, D. E.; Sheen, L. *J. Chromatogr.* **1981**, *204*, 103.

39. Yeung, E. S. *J. Pharm. Biomed. Anal.* **1984**, *2*, 255.

40. Purdie, N.; Swallows, K. A. *Anal. Chem.* **1989**, *61*, 77A.

41. *Model J-500 Automatic Recording Spectropolarimeter Instruction Manual*; Japan Spectroscopic: Tokyo, 1979.

42. Gerald, M. C. *Pharmacology: An Introduction to Drugs*; Prentice Hall: New Jersey, 1981; p.560.

43. Marrelli, L. P. In *Cephalosporins and Pencillins: Chemistry and Biochemistry*; Flynn, E H.; Academic: New York, 1972, Chapter 14.

44. Hamilton-Miller, J. M. T.; Smith, B. A.; Knox, R. *J. Pharm. Pharmacol.* **1963**, *15*, 81-91.

45. Gilpin, R. K.; Pachla, L. A. *Anal. Chem.* **1987**, *59*, 174R.

46. Mitscher, L. A.; Howison, P. W.; Sokoloski, T. D. *J. Antibiotics* **1974**, *27*, 215.

47. Richardson, F. S.; Yek, C. Y.; Troxell, T. C.; Boyd, D. B. *Tetrahedron* **1977**, *33*, 711.

48. Nagarajan, R. In *Cephalosporins and Penicillins: Chemistry and Biology;* Flynn, E. H., Ed.; Academic: New York, 1972; pp 651-657.

49. Boyd, D. B. In *Chemistry and Biology of β-Lactam Antibiotics;* Morin, R.B.; Gorman, M., Eds.; Academic: New York, 1982; pp 499-500.

50. Rasmussen, C. E.; Higuchi, T. *J. Pharm. Sci.* **1971**, *60* , 1608.

51. Han, S. M.; Purdie, N. *Anal. Chem.* **1986**, *58*, 113.

52. Phillips, D. D.; Chada, M. S. *J. Am. Pharm. Assoc. Sci. Ed.* **1955**, *44*, 553.

53. Bein, H. J. *Pharm. Reveiw* **1956**, *8*, 435.

54. *AMA Drug Evaluations*, 4th ed.; American Medical Association: Chicago, 1980, pp 568-570.

55. *Physicians Desk Reference*, 40th ed.; Medical Economics: Oradell, NJ, 1986, p 1766.

56. Butterfield, A. G.; Lovering, E. G.; Sears, R. W. *J. Pharm. Sci.* **1973**, *62*, 650.

57. Cieri, U. R. *J. Assoc. Off. Anal. Chem.* **1983**, *66*, 867.

58. Smith, W. M.; Clark, C. C. *J. Assoc. Off. Anal. Chem.* **1976**, *59*, 811.

59. Smith, W. M. *J. Assoc Off. Anal. Chem.* **1977**, *60*, 1018.

60. Wang, J.; Tapia, T.; Bonakdar, M. *Analyst* **1986**, *111*, 1245.

61. Cieri, U. R. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 542.

62. Wang, J.; Bonakdar, M. *J. Chromatogr.* **1986**, *382*, 343.

63. Settimj, G.; Di simone, L.; Del Giudice, M. R. *J. Chromatogr.* **1976**, *116*, 263.

64. Khayyal, S. E.; Ayad, M. M.; Girgis, A. N. *J. Chromatogr.* **1984**, *285*, 495.

65. Wright, G. E.; Tang, T. Y. *J. Pharm. Sci.* **1972**, *61*, 299.

66. *United States Pharmacopeia*, 20th ed.; United States Pharmacopeial Convention: Rockville, MD, 1980.

67. Hashmi, M. *Assay of Vitamins in Pharmaceutical Preparations*; John Wiley & Sons: New York, 1973.

68. Cooke, J. R.; Moxon, R. E. D. *Vitamin C: Ascorbic Acid*; Counsell, J. N.; Hornig, D. H., Ed.; Applied Science Publishers: New Jersey, 1982.

69. Al-Meshal, I. A.; Hassan, M. M. A. in *Analytical Profiles of Drug Substances*; Florey, K. Ed.; Academic Press: New York, 1982; Vol. 11, p 45.

70. Pachla, L. A.; Reynolds, D. L. *J. Assoc. Off. Anal. Chem.* 1985, 68, 1.

71. Kirschbaum, J. in *Analytical Profiles of Drug Substances*; Florey, K. Ed.; Academic Press: New York; Vol 10, p 183.

72. Nasser, T. A. K.; Al-Rikabi, A. M.; Mansoor, T. T. *Anal. Lett.* **1987**, *20*, 627.

73. Hernandez-Mendez, J.; Mateos, A. A.; Parra, M. J. A.; De Maria, C. G. *Anal. Chim. Acta* **1986**, *184*, 243.

74. Skaltsa, H. D.; Tzakou, O. A.; Koupparis, M. A.; Philianos, S. M. *Anal. Lett.* **1987**, *20*, 1679.

75. Thompson, R. Q. *Anal. Chem.* **1987**, *59*, 1119.

76. Lau, O.; Luk, S. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 518.

77. Karayannis, M. I.; Farasglou, D. I. *Analyst* **1987**, *112*, 767.

78. Barrary, M.; Abdel-Hamid, M.; Hassan, E.; Elsayed, M. *Pharamzie* **1986**, *41*, 483.

79. Perez-Ruiz, T.; Lozano, C. M.; Tomas, V. *Anal. Chim. Acta* **1987**, *198*, 63.

80. Hudson, T. S.; Subramanian, S.; Allen, R. J. *J. Assoc. Off. Anal. Chem.* **1984**, *67*, 994.

81. Amin, M.; Reusch, J. *Analyst* **1987**, *112*, 989.

82. Lam, F.; Holcomb, I. J.; Fusari, S. A. *J. Assoc. Off. Anal. Chem.* **1984,** *67,* 1007.

83. Augustin, J. *J. Assoc. Off. Anal. Chem.* **1984,** *67,* 1012.

84. Woollard, D. C. *J. Chromatogr.* **1984,** *301,* 470.

85. Wilson, C. W.; Shaw, P. E. *J. Agric. Food Chem.* **1987,** *35,* 329.

86. Wittmer, D. P.; Haney, W. G. in *GLC and HPLC Determination of Therapeutic Agents,* part 3; Tsuji, K., Ed.; Marcel Dekker: New York, 1979; Vol. 9, Chapter 36.

87. *Modern Chromatographic Analysis of the Vitamins*; De Leenheer, A. P.; Lambert, W. E.; De Ruyter, M. G. M., Eds.; Marcel Dekker: New York, 1985; Vol. 30, Chapters 5, 9, and 12.

88. Firth, R. A.; Hill, H. A. O.; Pratt, J. M.; Williams, R. J. P.; Jackson, W. R. *Biochemistry* **1967,** *6,* 2178.

89. Christensen, P. L.; Yeung, E. S. *Anal. Chem.* **1989,** *61,* 1344.

90. Lau, O.; Luk, S.; Wong, K. *Analyst* **1986,** *111,* 665.

91. Weissberger, A.; LuValle, J. E. *J. Am. Chem Soc.* **1944,** *66,* 700.

92. *The Merck Index,* 9th ed.; Windholz, M. Ed.; Merck & Co.: Rathway, NJ, 1976; pp 9670-9671.

93. Roc, J. H.; Kuether, C. A. *J. Biol. Chem.* **1943,** *147,* 399.

94. Patterson, J. W. *J. Biol. Chem.* **1950,** *183,* 81.

95. Bender, A. E. The All New Pocket Encyclopedia of Calories and Nutrition; Simon and Schuster: New York, 1985; p 22-23.

96. Frost, B. A. *Am. Lab. Lab Guide* April 1987, 8.

97. Bowen, J. M.; Crone, T. A.; Hermann, A. O.; Purdie, N. *Anal. Chem.* **1980,** *53,* 2436.

98. Bender, M. L.; Komiyama, M. *Cyclodextrin Chemistry*; Hafna, K.; Lehn, J. M.; Rees, C. W.; Schleyer, P. R.; Trost, B. M.; Zahradnik, R., Ed.; Spinger-Verlag Berlin Heidelberg: New York, 1978; Chapter 1,2.

99. Bowen, J. M.; Purdie, N. *Anal. Chem.* **1981,** *53,* 2239.

100. Han, S. M.; Purdie, N. *Anal Chem.* **1984,** *56,* 2822.

101. Han, S. M.; Purdie, N. *Anal. Chem.* **1984,** *56,* 2825.

102. Atkinson, W. M.; Han, S. M.; Purdie, N. *Anal. Chem.* **1984,** *56,* 2827.

103. Ward, T. J.; Armstrong, D. W. J. *Liq. Chromatogr.* **1986,** *9,* 407.

104. El Yazbi, F. A.; Abdel-Hay, M. H.; Korany, M A. *Pharmazie* **1986**, *41*, 639.

105. Procopio, J. R.; Hernandez, P. H.; Hernandez, L. H. *Analyst* **1987**, *112*, 79.

106. Vire, J. C.; Gallo Hermosa, B.; Patriarche, G. J. *Anal. Lett.* **1986**, *19*, 1839.

107. Alonso, R. M.; Jimenez, R. M.; Fogg, A. G. *Analyst*, **1988**, *113*, 27.

108. Latorre, C.; Hernandez Blanco, M. Abad, E. L.; Vicente, J.; Hernandez, L. *Analyst* **1988**,*113*, 317.

109. Ruiz, E.; Hernadez Blance, M.; Abad, E. L.; Hernandez, L. *Analyst* **1987**, *112*, 697.

110. Gordon, S. M.; Freeston, L. K.; Collins, A. J. *J. Chromatogr.* **1986**, *368*, 180.

111. Fatmi, A. A.; Hickson, E. A. *J. Pharm. Sci.* **1988**, *77*, 87.

112. Raymond, G. G.; DeGennaro, M. D. *Anal. Lett.* **1986**, *19*, 239.

113. de Silva, J. A. F. in *GLC and HPLC Determinations of Therapeutic Agents*, Part 3; Tsuji, K., Ed.; Chromatic Science Series; Marcel Dekker: New York, 1978; Vol. 9, pp 612-636.

114. Han, S. M.; Purdie, N. Swallows, K. A. *Anal. Chim. Acta* **1987**, *197*, 57.

115. Monk, C. B. *Electrolytic Dissociation*; Academic Press: New York, 1961; p 186.

116. Wark, T. J.; Armstrong, D. W. in *Chromatographic Chiral Separations*; Zief, M. Crane, L. G.,Eds.; Chromagtographic Science Series; Marcel Dekker: New York, 1988; Vol. 40, Chapter 5.

117. Saenger, V. W. *Agnew Chem.* **1980**, *92*, 343.

118. Fujimura, K.; Ueda, F.; Ando, T. *Anal. Chem.* **1983**, *55*, 446.

119. Kawaguchi, Y.; Tanaka, M.; Nakae, M.; Funayo, D.; Shono, F. *Anal. Chem.* **1983**, *55*, 1852.

120. Feibush, B.; Grinberg, N. in *Chromatographic Chiral Separations*; Zief, M.; Crane, L. J., Eds.; Chromatographic Science Series; Marcel Dekker: New York, 1988; Vol. 40, Chapter 1.

121. Hattori, T.; Higuchi, S.; Tanaka, S. *J. Raman Spec.* **1987**, *18*, 153.

122. Kime, K. A.; Sievers, R. E. *Aldrichimica Acta* **1977**, *10*, 54.

123. *Chromatographic Chiral Separations*; Zief, M.; Crane, L. J., Eds.; Chromatographic Science Series; Marcel Dekker: New York, 1988; Vol. 40.

124. Han, S. M.; Atkinson, W. M.; Purdie, N. *Anal. Chem.* **1984**, *56*, 2827.

125. Saxberg, B. E. H.; Kowaiski, B. R. *Anal. Chem.* **1979**, *51*, 1031.

126. Weiz, B. *Fresenius Z. Anal. Chem.* **1986**, *325*, 95.

127. Reitsma, B. H.; Yeung, E. S. *J. Chromatogr.* **1986**, *362*, 353.

128. Armstrong, D. W.; Spino, L. A.; Han, S.M.; Seeman, J. I.; Secor, H. W. *J. Chromatogr.* **1987**, *411*, 490.

129. Atkinson, W. M.; Han, S. M.; Purdie, N. *Anal. Chem.* **1984**, *56*, 1947.

130. Harkey, M. R. in *Analytical Aspects of Drug Testing*; Deutsch, D. G., Ed.; Chemical Analysis Series; John Wiley & Sons: New York, 1989; Vol. 100, Chapter 3.

131. Bowen, J. M.; Purdie, N. *Anal. Chem.* **1981**, *53*, 2237.

132. Ward, T. J.; Armstrong, D. W. in Chromatographic Chiral Separations; Zief, M.; Crane, J., L., Eds.; Chromatographic Science Series; Marcel Dekker: New York, 1988; Vol. 40, Chapter 5.

133. Bowen, J. M.; Purdie, N. Anal. Chem. 1981, 53, 2239.

134. Engle, A.R.; Purdie, N. unpublished results.

135. Armstrong, D. W.; Han, S. M.; Han, Y. I. *Anal. Biochem.* **1987**, *167*, 261.

136. Purdie, N.; Murphy, L.H.; Purdie, R., unpublished results.

VITA

Kathy Ann Swallows

Candidate for the Degree of

Doctor of Philosophy

Thesis: ANALYTICAL APPLICATIONS OF CIRCULAR DICHROISM IN PHARMACEUTICAL DETERMINATIONS

Major Field: Chemistry

Biographical:

Personal Data: Born in Cape Giradeau, Missouri, July 4, 1962, the daughter of LeRoy and Carol Swallows.

Education: Graduated from Edmond Memorial High School, Edmond, Oklahoma, in May 1980; received Bachelor of Science Degree in Chemistry from Central State University in Edmond, Oklahoma in May 1985; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in December 1989.

Professional Experience: Teaching and Research Assisstant, Department of Chemistry, Oklahoma State University, June 1985 to July 1989. Staff Chemist at Phillips Petroleum, Bartlesville, Oklahoma, August 1989 to present.