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Analytical applications of circular dichroism*

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Abstract: The goal of the present research has been to develop a selective analytical detection procedure for the easy identification and assay of drugs and related substances present in complex mixtures, without a chromatographic separation step or other work up. The detector of choice is circular dichroism (CD) spectropolarimetry which has the appropriate balance between degree of analytical selectivity and breadth of application. Applications that are described are selected from work carried out in the context of forensic, clinical, and pharmaceutical chemistries, and specifically include discussions of the analyses of enantiomeric mixtures of nicotine and cocaine, the D and E vitamins, and cholesterol and other steroids.

Keywords: *Circular dichroism; enantiomeric excess determination; nicotine; cocaine; cholesterol screening; vitamins; spectropolarimetry.*

Introduction

Circular dichroism (CD) was introduced in the 1950s and was touted as having the potential to determine absolute configurations. Its use was quickly abandoned when it was realized that only relative, and not absolute, stereochemistries could be established. Since that time a few laboratories have been evaluating the method as an analytical technique. To date, it has been used successfully as a stand-alone detector [1, 2] and significant progress is being made in its development as an on-line detector for high-performance liquid chromatography (HPLC) [3, 4]. As a stand-alone detector it is essential that a full spectrum be measured. As an HPLC detector, single wavelength detection is sufficient.

CD can operate as a stand-alone detector because of the analytical selectivity inherent in the method. In order to be CD-active an analyte must absorb electromagnetic radiation and be optically active; in other words the molecule should possess a chiral chromophore. Both properties together are essential for analytical identification, but only the absorption function is needed for quantitative determinations. Compounds with either one of these properties or neither are transparent to the detector.

The property measured in the modern CD instrument is the difference in the absorbances of the left and right circularly polarized beams of incident radiation. These

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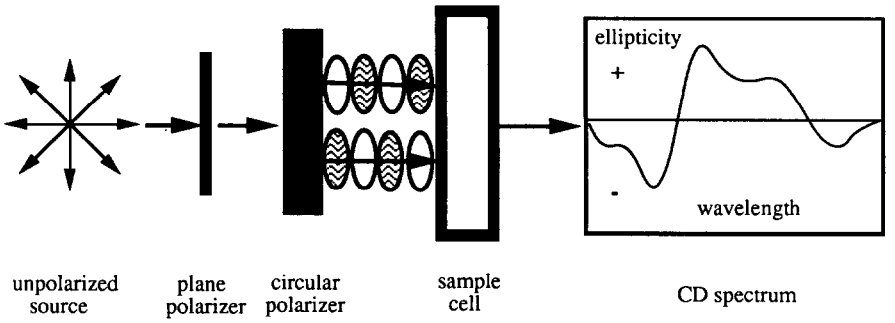

Figure 1
Block diagram of the CD instrument in which a difference in absorption is measured.

differences are proportional to a quantity referred to as the ellipticity; they are plotted on the ordinate of the spectrum as a function of wavelength. If a molecule is achiral the ellipticity is zero at all wavelengths. Chiral analytes absorb the two circularly polarized beams to differing degrees to produce positive and negative CD bands in a spectrum that serves as a fingerprint for that species (Fig. 1). Since absorption is necessary for CD activity and a function of absorbance is measured instrumentally, the spectral data obey the Beer–Lambert law, as might be expected. Simply put, CD is a modified form of absorption spectrophotometry. One disadvantage that may limit its usefulness is the relatively small magnitude of the CD signal compared with the absorbance signal, but other advantages far outweigh this deficiency.

The method is not only limited to those compounds which meet both structural requirements. The missing property can be introduced into a molecule which meets only one of these requirements, by way of chirality induction on the one hand, or colour induction on the other, both techniques broaden the scope of the method enormously. Compounds studied in this laboratory are listed in Tables 1–3. Although not all studies are complete, details of those that are can be found in two review articles [1, 2].

Two recent applications are discussed in the present paper; the determination of enantiomeric excess (EE) using CD, and the use of colour induction reactions for the analysis of water insoluble vitamins and steroids.

Enantiomeric excess determinations

Enantiomeric excess (EE) or optical purity determination is a problem that is becoming increasingly important as quality control regulations in the pharmaceutical industry become more and more stringent [5]. Although enantiomers have the same chemical composition, they do have different structural and biological properties, and therefore must be assayed independently. In instances where only one isomer of an enantiomeric pair is pharmacologically active (the "eutomer"), the other isomer, sometimes referred to as the "distomer", can be considered to be an impurity. Because of the chemical similarity of the distomer to the primary drug, it may be more difficult to remove than other impurities; indeed, even after a successful separation the distomer could be regenerated by racemization. Therefore, the tolerance level of the distomer must be firmly established and effectively controlled. This means that routine methods for the determination of EE are in need of development.

Table 1
Chiral analytes investigated by CD

(A)	*Forensic analyses* D-LSD; cocaine morphine; heroin THC; cannabidiol amphetamine; methamphetamine strychnine; curare; colchicine	(C)	*Clinical analyses* cholesterol; methandriol urine ketosteroids male and female sex hormones cortisone; hydrocortisone epitestosterone; androsterone mestanolone
(B)	*Pharmaceuticals* aureomycin penicillin V and cephapirin codeine and morphine vitamins B_2, B_{12}, and C vitamins D_2, D_3, and E quinine; cinchonine; reserpine digoxin; digitoxin	(D)	*Plant extracts* marihuana opium tobacco Rauwolfia serpentina Digitalis

Table 2
Achiral analytes investigated by CD

Barbiturates:	allo-; amo-; barbital; buta-; hexo-; pento-; pheno-; seco-
Benzodiazepines:	clonazepam; delorazepam; diazepam; lorazepam; medazepam; nitrazepam; nordiazepam; oxazepam; temazepam
Racemates*:	D-L-α-phenethylamine; D-L-methadone; D-L-penicillamine; D-L-nicotine; D-L-cocaine; D-L-pilocarpine; D-L-hyosycamine
Miscellaneous:	dilantin; β-phenethylamine; meperidine; PCP; PCP pyrrolidine; PCP morpholine

*The racemates are achiral because of the exact internal compensation of optical activity for enantiomers present in equal concentrations.

Table 3
Chiral analytes investigated by colour induction

Sterols:	cholesterol; methandriol; stanozolol; ergosterol; stigmasterol; estradiols; diosgenin; dihydrocholesterol
Sterones:	testosterone; 17-α-methyltestosterone; 17-ketosterones (urine test); 19-nortestosterone; dihydrotestosterone; epitestosterone; digoxin; digitoxin; androsterone; cortisone; mestanolone; 5-cholesten-3-one; 7-ketocholesterol
Vitamins:	D_2; D_3; E (tocopherol acetate)
Sugars and glycosides*	

*Potential candidates for future study.

Methods currently being used are not easily transformed into broad based routine procedures. Most involve the formation of diastereoisomeric pairs by reacting both enantiomers with a single chiral adduct. The diastereoisomers have different physical properties, such as non-equivalent chemical shifts in NMR, or different retention times on chromatographic columns, both of which allow for their quantitative distinction. The number of possible reactions is quite limited. Derivatization reactions for chromatog-

raphy can be pre-column, when conventional stationary phases are used, or on-column whenever a suitable chiral stationary phase is available [6]. Pre-column derivatization also includes the use of chiral mobile phases. These latter procedures are probably better focused for the purposes of enantiomeric enrichment than for EE determination.

A second approach to EE determination is based on two independent experimental measurements, without derivatization. One of these measurements, however, must focus on the chiroptical properties of the analytes. A useful combination of measurements is absorption coupled with optical rotation [7]. A necessary prerequisite for the accurate polarimetric measurement of optical rotation is that all other chiral compounds must have been separated and removed. Separation is achieved by conventional liquid chromatography (LC) column materials and achiral eluting solvents, and the eluate flows sequentially through an absorbance and a polarimetric detector. CD can be used in place of polarimetry, but if it is limited to a single wavelength of detection, one cannot improve the performance over polarimetry. Very small eluted volumes and relatively low analyte concentrations tax to the limit the level of detection of the chiroptical detectors currently available to such an extent that laser-based radiation sources are required.

Experimental

Nicotine

In the Authors' first introduction of CD to EE determination the HPLC-absorbance measurement was retained, but the CD spectrum was measured for the *unseparated* mixture. This avoids the problem due to small volumes and poor detection limits. Results for laboratory-prepared mixtures and for extracts from tobacco leaves which had been deliberately, but anonymously, spiked with known amounts of the unnatural *R*(+)-isomer, are given in Table 4. Reagent grade 2-propanol was used for the extraction. The chromatographic separation was carried out using a reversed-phase column and 100% methanol as eluent, as recommended in the literature [8]. Duplicate separations were carried out for the tobacco extracts both before and after adding the *R*(+)spike. CD spectra (over the 320–230 nm wavelength range) for the standards, the laboratory mixtures, and the spiked plant extracts were run in 2-propanol without any further preparation other than sample dilution (0.2–10 ml). Molar ellipticity values for the

Table 4
Enantiomeric data for nicotine mixtures*

Sample	Concentration (mg ml^{-1})	%*S*(−)(theor.)	%*R*(+)(theor.)
Lab 1		84.4 (85)	15.6 (15)
Lab 2		89.9 (90)	10.1 (10)
Lab 3		95.4 (95)	4.6 (5)
Lab 4		98.3 (98)	1.7 (2)
Ext 1	1.07	62.1 (64)	37.9 (36)
Ext 2	1.37	88.8 (90)	11.2 (10)
Ext 3	1.45	50.0 (50)	50.0 (50)
Ext 4	1.90	36.2 (35)	62.8 (65)
Ext 5	1.36	8.7 (10)	91.3 (90)
Ext 6	1.26	92.8 (90)	7.2 (10)

*For the tobacco extracts the "theoretical" figures for the *S*(−) isomer were calculated from the total absorbance measured before the addition of the *R*(+) spike.

standards were measured from calibration curves for both isomers at the wavelengths of the maximum signals. The equation for total absorbance, which gives the concentration sum, and the equation for total experimental ellipticity, which gives the concentration difference, can be solved simultaneously. Alternatively, knowing the total concentration from the absorbance, one can assume that it is equal to the concentration of the isomer in excess, and calculate the maximum possible CD signal, H_{max}. The signal reduction from the theoretical maximum is therefore assumed to be due to the presence of the other component of opposite polarity. The fraction of the lesser component, F, in the mixture is easily calculated from the relationship: $F = (H_{max} - H_{obs})/2H_{max}$. Agreement between the prepared and calculated values was better than ±2%, expressed as the precision (RSD) in the measurements for the major component in the mixture.

Cocaine

For cocaine the EE was measured without chromatography and using only CD. The work is still preliminary at this time. In order to provide for two independent measurements, two solvent systems are employed, one of which can be chiral. Spectra were run for the standards, for prepared laboratory mixtures, and for extracts of street samples of cocaine hydrochloride and the refined base, "crack", first in 0.01 M HCl, and then in a solution of 10^{-2} M β-cyclodextrin (BCD) in the same medium. Both solutions contain identical weights of drug. BCD is known to be an excellent complexing agent and a good host for cocaine [9]. It is also a popular chiral stationary phase material in HPLC [6]. The spectra for the uncomplexed enantiomers are images of each other. On complexation the short wavelength positive band in the spectrum for the D-isomer undergoes a blue shift with no change in sign. By comparison the equivalent band in the CD spectrum of the L-isomer changes sign on complexation with BCD, Fig. 2. The spectrum in Fig. 2 is actually that for a street sample which had been dissolved in both solvent systems and the spectra run without any chromatographic separation. The sample of course is predominantly L-cocaine.

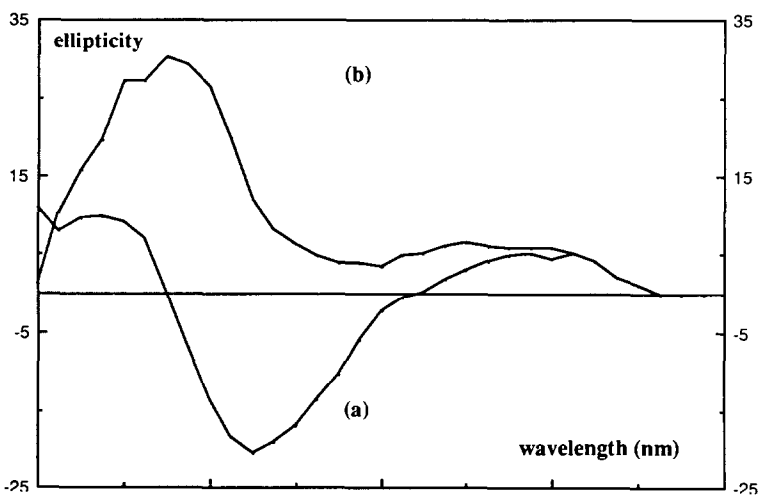

Figure 2
CD spectra of sample of street cocaine in: (a) dilute aqueous acid and (b) dilute aqueous acid plus 10^{-2} M β-cyclodextrin.

Rather than rely upon the calculation of the formation constants for the two enantiomeric complexes with BCD in order to obtain the total drug concentration [9], we elected instead to measure the "conditional" molar ellipticity values, represented by θ' and θ'', for the D- and L-forms of the drug in 10^{-2} M BCD, by preparing calibration curves of the experimental ellipticity against the total drug concentration. The concentrations of each isomer in the enantiomeric mixtures are calculated by solving the simultaneous equations that describe the total experimental ellipticities Ψ in both media:

$$\Psi_1 = \theta_D[D_1] + \theta_L[L_1] \text{ and } \Psi_2 = \theta_D[D_2] + \theta_L[L_2] + \theta'_D[D_2'] + \theta''_L[L_2''].$$

Correspondence between the prepared and measured EE values for the in-house laboratory mixtures are excellent. For street 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, but there is substantial evidence for the presence of the unnatural D-isomer.

A problem encountered in the analyses of the street specimens, but not in the study of the laboratory mixtures which were prepared from the initial reference materials, is the lack of equivalent purity in the two standards. Evidence from independent polarimetric and absorption measurements supports the conclusion from the CD data that the D-isomer standard sample is of lesser purity and perhaps has undergone partial racemization to the L-form. While θ_D might be taken to be equal to $-\theta_L$, a similar assumption for the molar ellipticities of the complexed forms is not valid. The additional uncertainty in the θ_D' value is expected to contribute more towards the errors in the EE values calculated. The problem of non-equivalent purities between the enantiomer standards is probably very general and accordingly the goal of being able to measure the absolute EE to better than $\pm 1\%$ might be unrealistic.

Colour-induction reactions

The Authors' interest in this area began specifically with the sterols, although it has been found to have utility in the study of sterones as well. A number of colour-induction reactions have been characterized for the analysis of steroids which use absorbance or fluorescence detection [10]. Of these, only a few can be used for CD detection. An advantage that was expected to be gained by introducing CD detection was enhanced selectivity. As an illustration of what in fact can be accomplished, the Authors can report from some unfinished findings that vitamins D_2 and D_3 are mutually distinguishable, as are the cardiac glycosides digoxin and digitoxin, because of the uniqueness of the CD spectra for the products of their reactions with colour-inducing reagents. The spectra of the D vitamins in turn are easily distinguishable from that for vitamin E, using the same chemical reaction, and it is conceivable therefore that the water-insoluble vitamins in multivitamin tablets could be assayed directly without resorting to extensive workup and chromatography.

Besides selectivity, another benefit from using CD detection is the fact that colour instability appears to be less of a problem than it is in absorption or fluorescence detection. This is believed to be due to the fact that an absorption *difference* is measured, a property that is less sensitive to change with time than the change in the total absorption.

Many of the anabolic steroids and their metabolites are analogues of testosterone, and have similar, if not identical, CD spectra in non-aqueous solvents. A particular reagent (CR) that the Authors have used, but are unable at the time of writing to disclose the

details on, is very effective as a way to differentiate among a large number of sterols and sterones. The CD spectra of the products of these reactions are uniquely different. Testosterone and epitestosterone can be used to illustrate the effectiveness of the reagent. These two epimers have identical CD spectra in chloroform solutions. The colours of the products of their reactions with (CR) are straw yellow and royal blue, respectively and therefore the absorption spectra and the CD spectra are totally different. This pair is of special interest because it is their concentration ratio which is taken as the standard evidence for steroid abuse [11]. The analytical work on this system is being continued in an attempt to establish the limits of detection and evaluate the problems of interferences from the 17-ketosteroids present in urine specimens.

Serum cholesterol

The reagent (CR) and the same reaction conditions have also been used for the determination of serum cholesterol. The spectrum of the orange-coloured product of the reaction is shown in Fig. 3, and as far as the Authors know it is unique to cholesterol. The long wavelength band, with a positive maximum at 525 nm, has been assigned to cholesterol associated with the combined very low density and low-density lipoprotein (VLDL + LDL) fractions; the signal at 390 nm has been assigned to the cholesterol associated with the high-density lipoprotein (HDL) fraction. The assignments were made after relating the changes in the CD spectrum with the selective precipitation of the low-density lipoprotein fractions with phosphomolybdate ion, i.e. the shaded curve in Fig. 3.

Experimental

The NBS cholesterol standard reference material (SRM 911a) dissolved in chloroform was used to obtain the reference spectrum and to calibrate the 525 nm maximum in mg/dl.mdeg. HDL secondary standards from Sigma Chemical Co. were used to calibrate the signal at 390 nm. The (CR) reaction is non-enzymatic but does require an incubation period. The colour is stable for almost 2 h. In the serum cholesterol tests, the reagents are added to a 50 μl aliquot of serum, incubated for 8 min, diluted with a 1 ml aliquot of chloroform, and centrifuged at high speed for 2 min. The supernatant is transferred to a 1 cm cuvette and the CD spectrum measured from 625–325 nm. Cell blank and baseline corrections are made daily. The (VLDL + LDL) and HDL fractions

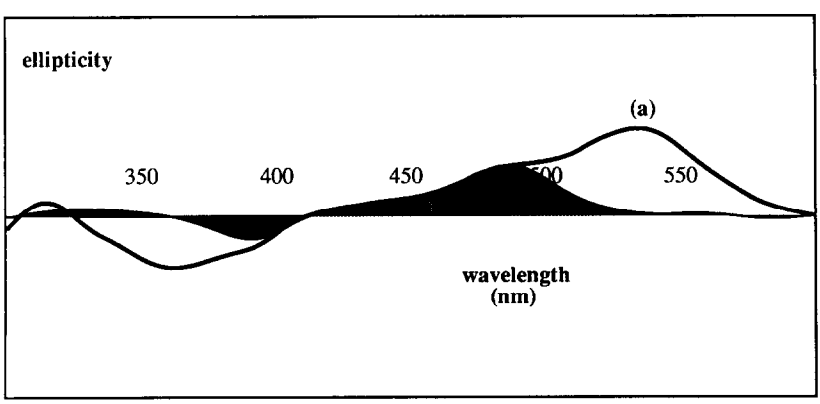

Figure 3
CD spectrum for the coloured product of the cholesterol reaction. Curve (a) is representative of total cholesterol, while the shaded area corresponds to the spectrum after the addition of LDL precipitating reagent and is therefore representative of the HDL fraction only.

are both measured from the same spectrum in a single experiment and their sum gives the total serum cholesterol level. The time for a single determination is approximately 15 min. The stability of the colour means that several samples can be incubated concurrently, and other strategies can be introduced to reduce the time per sample for large scale screening purposes.

Results for almost 200 serum samples have been compared with those from two independent laboratories which use two different commercially developed cholesterol screening tests. The correlation between the total cholesterol levels for all three laboratories is excellent with a slope of 1.0055 and an intercept of -10.2 mg dl^{-1}. Results for the (VLDL + LDL) and HDL correlations are poor by comparison. This can be explained in part by the fact that in the commercial methods only the total cholesterol and the HDL fraction are measured in a direct manner. The VLDL fraction is presumed to be one fifth of the total triglyceride (TGL), which is measured in a third test, and the LDL therefore is obtained by difference, equal to (Total − HDL − 0.2 TGL).

The three sets of laboratory results were then evaluated individually. For the two commercial procedures, (VLDL + LDL) was taken to be the simple numerical difference between the experimentally measured total and HDL levels. Slopes and intercepts for the three [total cholesterol vs (VLDL + LDL) cholesterol] linear correlations treated separately are: (0.85 and -5.0 mg dl^{-1}); (0.98 and -47.7 mg dl^{-1}); and (0.99 and -46.5 mg dl^{-1}), for the CD procedure and the two commercial procedures, respectively. For the corresponding [total cholesterol vs HDL cholesterol] linear correlations, the equivalent parameters are: (0.15 and 5.2 mg dl^{-1}); (0.012 and 47.6 mg dl^{-1}); and (0.013 and 46.5 mg dl^{-1}), respectively. The comparisons imply that there is a bias of almost 50 mg dl^{-1} in the data obtained by commercial procedures. Research is being continued in this area in the hope of finding a direct way to distinguish the VLDL fraction from the LDL fraction.

Summary

The work with CD as a stand-alone analytical detector has been shown to have broad applications. It has unique features that should make it the detector of choice whether it is used in the stand-alone mode or as an on-line LC detector, some of which have been included here. The Authors believe the time has come when its utility should be exploited for quality assurance testing in the pharmaceutical industry, and for screening tests in clinical chemistry.

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