

Chapter 2

UV AND VISIBLE ABSORPTION TECHNIQUES

I. INTRODUCTION

Ultraviolet (UV) and visible absorption techniques encompass analytical methods based upon measurement of light absorption by substances in the wavelength (λ) region from approximately 190 to 900 nm. The region from 190 to 380 nm is the UV region and from 380 to 900 nm the visible region of the spectrum. Absorption in both regions arises from electronic transitions within the molecule. The relation of the UV and visible to other regions of the electromagnetic spectrum is given in Table 1.

Absorption in the UV/visible region is often strong and therefore allows many substances to be measured in the low part per million range in nonabsorbing media. Good sensitivity combined with ready availability of simple, accurate, and inexpensive spectrophotometers has made UV/visible spectrophotometry one of the most widely used instrumental techniques in organic analysis.¹⁻⁹

The absorptivity of a substance varies with the wavelength of the light. Therefore light absorption is discussed in terms of the absorption spectrum of the substance, which is a plot of absorptivity, molar absorptivity, or absorbance (see Section II.C. for definitions of these terms) as a function of the wavelength or frequency of the incident light. The most common practice in UV/visible spectroscopy is to plot absorbance versus wavelength in nanometers. In tabular presentations of data it is customary to include the maximum values of the molar absorptivity, ϵ , and the wavelengths, λ_{MAX} , where the maxima occur.

II. PRINCIPLES OF UV/VISIBLE SPECTROPHOTOMETRY

A. The Origin of Absorption

Absorption in the UV/visible region arises from electronic transitions within the molecule. The highest energy electrons in the ground state of an organic molecule may be in σ , π , or n (nonbonding) orbitals. The corresponding excited states of lowest electronic energy are the σ^* , π^* and n^* states, respectively. The transitions that are responsible for UV/visible absorption are $\sigma \rightarrow \sigma^*$, $n \rightarrow \sigma^*$, $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$. The part of a molecule that is responsible for the transition is called a "chromophore". The simplest chromophore is a single bond which gives rise to $\sigma \rightarrow \sigma^*$ transitions. As $\sigma \rightarrow \sigma^*$ transitions are usually very high energy, they are observed only in the vacuum UV ($\lambda < 190$ nm) and are of little interest in pharmaceutical analysis.

Compounds containing halogen, ether, thioether, amino, hydroxy, and other functional groups with unshared pairs of electrons will absorb light in the short wavelength UV region due to $n \rightarrow \sigma^*$ transitions. Examples of the wavelength of maximum absorption, λ_{MAX} , for $n \rightarrow \sigma^*$ transitions in several simple organic compounds are presented in Table 2. The absorption due to $n \rightarrow \sigma^*$ transitions is of very limited utility in pharmaceutical analysis because the absorption is weak and in most cases occurs at wavelengths too short to be easily measured.

Transitions to π^* orbitals are necessarily associated with unsaturated centers in the molecule such as alkenyl, carbonyl, imino, and azo groups. $n \rightarrow \pi^*$ transitions are lower in energy than $n \rightarrow \sigma^*$ transitions and therefore result in absorption at longer wavelengths. $\pi \rightarrow \pi^*$ transitions lie between $n \rightarrow \sigma^*$ and $n \rightarrow \pi^*$ in energy. Several examples of $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions are given in Table 3. A list of common functional groups and their typical spectral properties is given in Table 4. The characteristics of a number of unsubstituted ring systems are given in Table 5.

Table I
REGIONS OF THE ELECTROMAGNETIC SPECTRUM

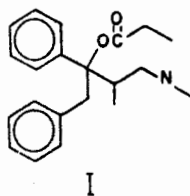
	Wavelength	Frequency		Energy*		Physical basis of absorption
		Hz	cm ⁻¹	eV	Kcal/mol	
Gamma & hard X-rays	< 0.1 nm	> 3 × 10 ¹⁸	> 10 ⁸	> 1.2 × 10 ⁴	> 2.8 × 10 ⁵	Nuclear transitions
Soft X-rays	0.1—1.0 nm	3 × 10 ¹⁷ —3 × 10 ¹⁸	10 ⁷ —10 ⁸	1.2 × 10 ³ —1.2 × 10 ⁴	2.8 × 10 ⁴ —2.8 × 10 ⁵	Inner shell electron transitions
Vacuum UV	1.0—190 nm	1.6 × 10 ¹⁵ —3 × 10 ¹⁷	5.3 × 10 ⁴ —10 ⁷	6.5—1.2 × 10 ³	1.5 × 10 ⁴ —2.8 × 10 ⁵	Valence electron transitions
Near UV	190—380 nm	7.8 × 10 ¹⁴ —1.6 × 10 ¹⁵	2.6 × 10 ⁴ —5.3 × 10 ⁴	3.2—6.5	74—1.5 × 10 ⁴	Valence electron transitions
Visible	380—900 nm	3.3 × 10 ¹⁴ —7.8 × 10 ¹⁴	1.1 × 10 ⁴ —2.6 × 10 ⁴	1.4—3.2	32—74	Valence electron transitions
Near IR (Overtone region)	900—2500 nm (0.9—2.5 μ)	1.2 × 10 ¹⁴ —3.3 × 10 ¹⁴	4 × 10 ³ —1.1 × 10 ⁴	4.9 × 10 ⁻¹ —1.4	11—32	Intramolecular vibrational overtone & combination bands
IR (fundamental region)	2.5—25 μ	1.2 × 10 ¹³ —1.2 × 10 ¹⁴	4 × 10 ² —4 × 10 ³	4.9 × 10 ⁻² —4.9 × 10 ⁻¹	1.1—11	Intramolecular vibrations
Far IR	25—400 μ (0.0025—0.04 cm)	7.5 × 10 ¹¹ —1.2 × 10 ¹³	25.0—4 × 10 ²	3.1 × 10 ⁻³ —4.9 × 10 ⁻²	7.2 × 10 ⁻² —1.1	Intramolecular skeletal and lattice deformations
Microwave	0.04—25 cm	1.2 × 10 ⁹ —7.5 × 10 ¹¹	0.040—25	4.9 × 10 ⁻⁶ —3.1 × 10 ⁻³	1.1 × 10 ⁻⁴ —7.2 × 10 ⁻²	Inter- and intramolecular rotations, electron spin reorientation
Short wave radio	0.25 m—18.5 m	6.2 × 10 ⁶ —1.2 × 10 ⁹	4.0 × 10 ⁻² —5.4 × 10 ⁻³	6.7 × 10 ⁻⁶ —4.9 × 10 ⁻⁶	1.6 × 10 ⁻⁶ —1.1 × 10 ⁻⁶	Nuclear and electronic spin reorientations
Broadcast radio	3030 m—18.5 m	10 ⁴ —6.2 × 10 ⁶	3.3 × 10 ⁻⁷ —5.4 × 10 ⁻⁵	4.1 × 10 ⁻¹⁰ —6.7 × 10 ⁻⁸	9.5 × 10 ⁻⁹ —1.6 × 10 ⁻⁶	
Long wave radio	>3030 m	<10 ⁴	<3.3 × 10 ⁻⁷	<4.1 × 10 ⁻¹⁰	<9.5 × 10 ⁻⁹	

* Atomic first ionization potentials range from 3.7—24.6 eV. Bond energies in organic substances range from 50—200 Kcal/mol (C-I to C≡N, respectively).

Table 2
 ABSORPTION CHARACTERISTICS: $n \rightarrow \sigma^*$
 TRANSITIONS

Compound	λ_{MAX} , nm	ϵ , l mol ⁻¹ cm ⁻¹	Solvent	Ref.
Methanol	183	150	Gas	10
Ethanol	181	324	Gas	11
Ethane thiol	195	1413	Ethanol	11
Dimethyl ether	184	2512	Gas	11
	163	3981	Gas	11
Diethyl ether	188	1995	Gas	11
	171	3981	Gas	11
Diethyl sulfide	215	1585	Heptane	11
	194	4786	Heptane	11
Trimethylamine	227	900	Gas	10
Chloromethane	173	~100	Gas	10
Bromomethane	202	263	Heptane	11
Iodomethane	258	378	Hexane	10
Dibromomethane	218	1148	Methanol	11
Diiodomethane	290	1318	Methanol	11
	255	631	Methanol	11
Tribromomethane	224	2138	Heptane	11
	205	2138	Heptane	11
Triiodomethane	336	2042	Methanol	11
	296	1738	Methanol	11
	266	1413	Methanol	11

The spectra of unsubstituted aromatic ring systems exhibit a number of sharp bands due to vibrational effects on the $\pi \rightarrow \pi^*$ transition. The bands are broadened and their intensity reduced in solution and may be eliminated entirely in polar solvents such as ethanol. Substitutions on the ring also reduce the complexity of the fine structure bands. The spectrum of propoxyphene(I) in Figure 1 shows the vibrational fine structure clearly, although the bands are relatively broad, and the overall pattern quite simple.



B. Factors Determining λ_{MAX}

1. Solvent

The exact wavelength of a particular electronic transition depends not only on the chromophore, but also on the solvent, substituents present on the chromophore, and chromophore geometry. The solvent effect arises because solvation alters the electronic energy levels of a chromophore, and the degree of solvation is frequently different for the ground and excited states. If the ground state is solvated more strongly than the excited state, the energy difference between the levels is increased. The increase in energy difference is reflected in a shift of the absorbance to shorter wavelengths (hypsochromic or blue shift) than those observed in the gas phase where there is no solva-

Table 3
ABSORPTION CHARACTERISTICS: $n \rightarrow \pi^*$ AND $\pi \rightarrow \pi^*$
TRANSITIONS

Compound	$\pi \rightarrow \pi^*$			$n \rightarrow \pi^*$			Ref.
	λ_{MAX} nm	ϵ^a	Solvent	λ_{MAX} nm	ϵ^a	Solvent	
Ethylene	171	15,530	Gas				10
1-Butene	187	12,589	Gas				11
	175	15,849	Gas				11
1,2-Butadiene	186	3,981	Gas				11
	178	19,953	Gas				11
	217	20,900	Hexane				10
Acetaldehyde	180	10,000	Gas	290	17	Hexane	10
Acetone	189	900	Hexane	279	15	Hexane	10

^a Extinction coefficient in $1 \text{ mol}^{-1} \text{cm}^{-1}$.

tion. If the excited state is solvated more strongly, the energy difference decreases and the absorbance is shifted to longer wavelength (bathochromic or red shift). Absorption due to $n \rightarrow \sigma^*$ and $n \rightarrow \pi^*$ transitions is especially sensitive to solvent effects because the unshared pair of electrons responsible for the transition also participate directly in solvation. It is frequently observed that $n \rightarrow \sigma^*$ and $n \rightarrow \pi^*$ transitions are shifted to *shorter* wavelengths in more polar solvents. Typical values for the solvent shifts observed for α, β -unsaturated ketones are presented in Table 6. $\pi \rightarrow \pi^*$ transitions are usually shifted to *longer* wavelengths in more polar solvents, but the magnitude of the shifts are smaller than those observed for transitions originating in an n state.

2. Geometry of the Chromophore

Differences in steric interactions within a pair of closely related molecules can alter the geometry of the chromophore, thereby causing changes in orbital overlap and hybridization. The resulting changes in electronic structure are reflected in changes in the absorption spectrum of the chromophore. The effects of geometry are seen most clearly by comparing the spectra of pairs of geometrical isomers because there is no possibility in this case that the differences arise from direct substituent effects. Two examples are presented in Table 7. The *cis* isomers are twisted slightly out-of-plane by steric interactions so that the degree of conjugation in the π system is slightly less than in the *trans* isomer, resulting in greater energy (shorter wavelength) for the transitions. The effect is much larger in stilbene than in 2-butene because the phenyl group is much larger than the methylene group resulting in greater steric interactions. Phenanthrene represents an intermediate case between *cis* and *trans* stilbene in which the distortion normal to the *cis* form is partially removed by linking the two phenyl rings together.

3. Substituents

Placing a substituent on a chromophore may change the absorption spectrum of the chromophore by two different mechanisms: introduction of an entirely new transition and/or shifting the wavelengths of existing transitions. A new transition most frequently arises when an atom or group with electrons in an n orbital is added to a chromophore with only σ and π orbitals. A long wavelength $n \rightarrow \sigma^*$ or $n \rightarrow \pi^*$ may then appear in the spectrum in addition to the transitions, possibly shifted in wavelength, that were present in the unsubstituted chromophore. For example, the longest wave-

Table 4
TYPICAL uv/VISIBLE ABSORPTION CHARACTERISTICS OF
ISOLATED FUNCTIONAL GROUPS



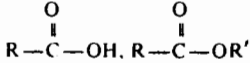
Functional group	Transition	Typical λ_{MAX} , nm	Typical ϵ , l moles ⁻¹ cm ⁻¹
C=C	$\pi \rightarrow \pi^*$	170--178	10,000--20,000
	$\pi \rightarrow \pi^*$	185--205	100--12,000
C \equiv C	$\pi \rightarrow \pi^*$	172--178	6,000--10,000
	$\pi \rightarrow \pi^*$	185--195	2,100
	$\pi \rightarrow \pi^*$	223	150
	$\pi \rightarrow \pi^*$	200--210	2,000--8,000
		250--265	150--250
R-OH	$n \rightarrow \sigma^*$	180--185	500
R-SH	$n \rightarrow \sigma^*$	190--200	1,500
	$n \rightarrow \sigma^*$	170 175	300
R ₂ O	$n \rightarrow \sigma^*$	180--185	3,000
R ₂ S	$n \rightarrow \sigma^*$	210--215	1,200
	$n \rightarrow \sigma^*$	235--240	100
RSSR	$n \rightarrow \sigma^*$	250	400
	$n \rightarrow \sigma^*$	150 160	-
	$\pi \rightarrow \pi^*$	180--195	1,000--10,000
	$n \rightarrow \pi^*$	270--295	15
	$n \rightarrow \pi^*$	195--210	20 100

Table 4 (continued)
 TYPICAL uv/VISIBLE ABSORPTION CHARACTERISTICS OF
 ISOLATED FUNCTIONAL GROUPS

Functional group	Transition	Typical λ_{MAX} , nm	Typical ϵ , l moles ⁻¹ cm ⁻¹
$\begin{array}{c} \text{O} \\ \\ \text{R}-\text{C}-\text{NH}_2 \end{array}$	$n \rightarrow \pi^*$	175	7,000
$\begin{array}{c} \text{O} \\ \\ \text{R}-\text{C}-\text{Cl} \end{array}$	$n \rightarrow \pi^*$	280	15
$\begin{array}{c} \text{O} \qquad \text{O} \\ \qquad \\ \text{R}-\text{C}-\text{NH}-\text{C}-\text{R} \end{array}$	$n \rightarrow \pi^*$ $\pi \rightarrow \pi^*$	230-240 190-200	90 10,000-15,000
$\text{R}-\text{C} \equiv \text{N}$	-	< 170	-
$\text{RNH}_2, \text{R}_2\text{NH}, \text{R}_3\text{N}$	-	185-200	2,500-4,500
$\text{R}-\text{NO}_2$	$n \rightarrow \pi^*$ $\pi \rightarrow \pi^*$	270-280 200-210	25 15,000
$\text{R}-\text{ONO}$	$n \rightarrow \pi^*$ $\pi \rightarrow \pi^*$	350 220	150 1,000
$\begin{array}{c} \text{R}-\text{S}-\text{R} \\ \\ \text{O} \end{array}$	$n \rightarrow \pi^*$	210-230	1,400-2,600
$\text{R}-\text{SO}_2-\text{R}$	-	< 180	-
$\text{R}-\text{Cl}$	$n \rightarrow \sigma^*$	170-175	300

R—Br	n → σ*	200–210	400
R—I	n → σ*	255–260	500
C=NOH		< 220	—
$\text{>C}=\overset{\text{I}}{\text{C}}-\text{NOH}$		235	15,000
$\text{>C}=\text{N}-\overset{\text{O}}{\parallel}\text{C}-\text{NH}_2$		225–230	11,000
$\text{>C}=\text{C}=\overset{\text{O}}{\parallel}\text{C}=\text{N}-\text{N}-\text{C}-\text{NH}_2$		265	25,000
$\text{>C}=\text{N}-\overset{\text{S}}{\parallel}\text{C}-\text{NH}_2$		230 280	7,000 20,000
$\text{>C}=\text{C}=\overset{\text{S}}{\parallel}\text{C}=\text{N}-\text{NH}-\text{C}-\text{NH}_2$		245 300	10,000 30,000
$\text{>C}=\overset{\text{O}}{\parallel}\text{C}-\text{C}-$		210 333	12,000 20

Table 5
ABSORPTION OF UNSUBSTITUTED RING SYSTEMS^a


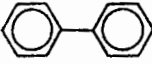

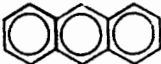
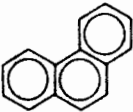

Compound	λ_{MAX} , nm	ϵ , l mole ⁻¹ cm ⁻¹	Solvent
 Benzene	268	-	cyclohexane
	260	-	
	254	-	
	248	-	
	243	-	
 Biphenyl	247	19,300	methanol
 Naphthalene	334	-	methanol
	320	-	
	311	239	
	304	224	
	301	294	
	297	313	
	286	3,760	
	283	3,710	
	275	5,530	
	266	4,990	
	258	3,470	
221	10,600		
 Anthracene	376	7,590	methanol
	355	7,770	
	338	5,290	
	322	2,750	
	309	1,230	
	296	531	
	250	20,000	
	220	11,800	
	218	11,700	
 Phenanthrene	291	12,800	methanol
	250	64,000	
	211	34,000	
 Naphthacene	471	14,000	
	442	10,400	
	415	5,300	
	395	2,200	
	375	1,050	
	356	445	
	339	240	
	294	29,500	
	275	410,000	
	265	92,000	
	239	3,900	
	228	5,700	
	223	8,600	
	220	10,600	
215	31,500		

Table 5 (continued)
 ABSORPTION OF UNSUBSTITUTED RING SYSTEMS^a

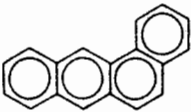
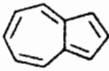

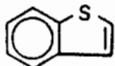

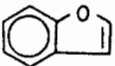
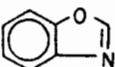

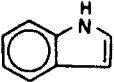
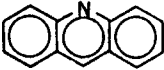



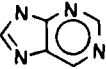
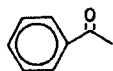
Compound	$\lambda_{\text{MAX.}}$ nm	ϵ , l mole ⁻¹ cm ⁻¹	Solvent
	341		methanol
	300		
	288		
	277		
Benanthracene			
	700	95	methanol
	633	257	
	580	316	
	352	501	
	341	3,981	
	326	2,818	
	295	3,548	
	279	43,652	
273	47,863		
238	14,791		
	237	~4,500	methanol
	233		
Thiophene			
	437	47	methanol
	297	2,870	
	288	2,030	
	256	5,420	
	226	27,900	
	208	7,943	ethanol
Furan			
	281	2,630	ethanol
	274	2,455	
	244	10,715	
Benzofuran			
	276	3,236	ethanol
	270	3,388	
	263	2,399	
	231	7,943	
Benzoxazole			
	350	300	methanol
	209	6,730	
Pyrrole			

Table 5 (continued)
 ABSORPTION OF UNSUBSTITUTED RING SYSTEMS^a

Compound	λ_{MAX} nm	$\text{l mole}^{-1} \text{cm}^{-1}$	Solvent
 Indole	287	—	cyclohexane
	279	—	
	266	~6,300	
	261	—	
	214	25,000	
 Acridine	348	~8,000	methanol
	339	—	
	250	~170,000	
	212	—	
 Pyridine	261	—	methanol
	256	—	
	250	—	
 Pyrazine	311	682	methanol
	267	4,560	
	261	6,420	
	255	5,400	
 Pyrimidine	280	298	methanol
	243	2,360	
	238	2,220	
 Purine	265	—	methanol

length transition in benzene in solution in ethanol is a $\pi \rightarrow \pi^*$ transition at 246 nm ($\epsilon = 19,950$), but the longest wavelength transition in acetophenone (II) is the $n \rightarrow \pi^*$ at 319 nm ($\epsilon = 50$), arising from the n orbital of the carbonyl group. The $\pi \rightarrow \pi^*$ transition in acetophenone is shifted to 278 nm ($\epsilon = 1096$). The effects of several substituent groups on the spectrum of benzene is shown in Tables 8 and 9.



II

Existing transitions are shifted by substitution due to the inductive and resonance

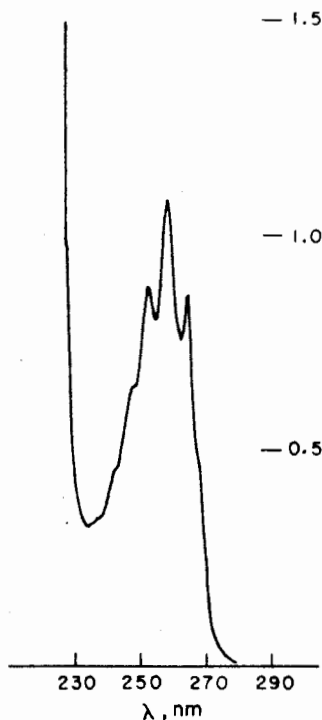


FIGURE 1. Ultraviolet spectrum of *d*-propoxyphene. Spectrum obtained using a 1 mg/ml solution of *d*-propoxyphene hydrochloride in dilute hydrochloric acid. The vibrational fine structure characteristic of phenyl groups is clearly seen in the 257-nm band.

effects of the substituent on the chromophore. The shifts are fairly systematic and empirical rules have been formulated that allow one to predict λ_{MAX} for several important chromophores with a variety of substituents. The basic relation is ¹²⁻¹⁷

$$\lambda_{MAX} = \lambda_{REF} + \sum_i \delta_i \quad (1)$$

where λ_{REF} = wavelength of maximum absorption for the unsubstituted chromophore and δ_i = wavelength increment for the i^{th} substituent or structural feature.

Values of λ_{REF} and δ_i for chromophores derived from butadiene, butenone, benzaldehyde and benzonitrile are given in Table 10. The sum in Equation 1 includes all groups extending the conjugation of the basic chromophore and all groups other than hydrogen that are directly bonded to the conjugated system. The use of these rules to estimate λ_{MAX} is illustrated with three examples in Figure 2. The rules provide surprisingly good estimates of λ_{MAX} for a wide variety of molecules.

C. Quantitative Description of Light Absorption

1. Beer's Law

Light absorption in the UV/visible region causes the transition of an electron from a ground state to an excited state. The lifetimes of excited electronic states (singlet) for most organic molecules in solution is 10^{-9} to 10^{-12} sec with the energy being lost

Table 6
SOLVENT
SHIFTS OF λ_{MAX}
FOR α, β -
UNSATURATED
KETONES AND
ALDEHYDES
RELATIVE TO
ETHANOL*

Solvent	$\Delta\lambda_{MAX}$
Hexane	+ 11
Cyclohexane	+ 11
Diethyl ether	+ 7
Dioxane	+ 5
Chloroform	+ 1
Methanol	0
Water	- 8

$$\begin{aligned} \lambda_{MAX} \text{ (ethanol)} &= \\ \lambda_{MAX} \text{ (listed solvent)} & \\ + \Delta\lambda_{MAX} & \end{aligned}$$

through transfer to vibrational, rotational, and translational motion of the molecules in the solution. In some cases loss by radiation is also important, as is discussed in more detail in Chapter 4. An important consequence of rapid relaxation of the excited state is that the populations of the ground and excited states are not appreciably disturbed by absorption of light energy from any but the most intense sources (such as lasers). Since the populations are not disturbed, the fraction of light absorbed from an incident beam is independent of the intensity of the beam. Equation 2 is a quantitative expression of this fact.

$$\frac{dI}{I_0} = -acdx \quad (2)$$

where I_0 = incident beam intensity, dI = amount of light absorbed as the beam traverses the small distance dx (centimeters), a = absorptivity of the molecule at the wavelength of the beam, and c = concentration of the solution in grams per liter.

Equation 2 can be integrated to obtain Beer's Law,

$$A = \log \frac{I_0}{I} = abc \quad (3)$$



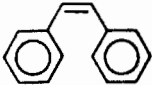
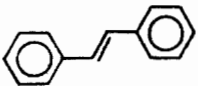
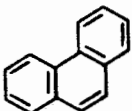
where b = length of the path of the beam through the solution, I = the intensity of the beam after passing through the solution, and A = absorbance of the solution at the particular wavelength of the light beam.

The ratio, I_0/I , is called the transmittance and is denoted by T . Beer's Law is often written in terms of moles rather than mass,

$$A = \epsilon Mb \quad (4)$$

where ϵ = molar absorptivity at the wavelength used to measure A and M = molarity of the solution.

Table 7
 EXAMPLES OF THE EFFECTS OF GEOMETRY
 ON THE ABSORPTION SPECTRUM^a

Compound	λ_{MAX} , nm	ϵ , l moles ⁻¹ cm ⁻¹	Solvent
<i>cis</i> -2-butene 	200	501	gas
	196	1,000	
	175	19,953	
	160	12,589	
<i>trans</i> -2-butene 	202	501	gas
	187	6,310	
	177	12,589	
	163	7,943	
<i>cis</i> -stilbene 	276	10,900	methanol
	223	20,600	
	203	—	
<i>trans</i> -stilbene 	307	32,100	methanol
	294	33,200	
	227	21,000	
phenanthrene 	291	12,800	methanol
	250	64,600	
	211	34,000	

^a Data from reference 11.

Typical values of ϵ for a variety of chromophores may be found in Tables 2-5 and 7-9. The relation between a and ϵ is

$$a = \epsilon \cdot MW \quad (5)$$

where MW = molecular weight of the absorbing specie.

$E_{1\%}^{1\text{ cm}}$ values are sometimes used instead of absorptivity or molar absorptivity, particularly in cases where the molecular weight is unknown or not clearly defined (e.g., polymer solutions). $E_{1\%}^{1\text{ cm}}$ is the absorbance of a 1% solution in a 1-cm cell and is therefore a directly measurable quantity. It is related to absorptivity and molar absorptivity by

$$E_{1\%}^{1\text{ cm}} = \frac{\epsilon \times 10}{MW \times \rho} = \frac{a \times 10}{\rho} \quad (6)$$

where ρ = density of the solution in grams per cubic centimeters. It should be noted that other names are often used for the quantities defined above, e.g., optical density, absorbancy or extinction for absorbance, and absorbancy index or extinction coefficient for absorptivity. The nomenclature used in this chapter is that recommended by

Table 8
NEAR UV ABSORPTION SPECTRA OF SOME MONOSUBSTITUTED
BENZENE DERIVATIVES (Ar-R)*

R	200 m μ band (K or E or primary band)		260 m μ band (B or secondary band)		Solvent
	λ_{MAX} , m μ	ϵ_{MAX} , l mol ⁻¹ cm ⁻¹	λ_{MAX} , m μ	ϵ_{MAX} , l mol ⁻¹ cm ⁻¹	
H	203.5	7,400	254	204	2% methanol in water
CH ₃	206.5	7,000	261	225	2% methanol in water
Cl	209.5	7,400	263.5	190	2% methanol in water
Br	210	7,900	261	192	2% methanol in water
OH	210.5	6,200	270	1,450	2% methanol in water
OCH ₃	217	6,400	269	1,480	2% methanol in water
CN	224	13,000	271	1,000	2% methanol in water
COOH	230	11,600	273	970	2% methanol in water
NH ₂	230	8,600	280	1,430	2% methanol in water
NO ₂	268.5	7,800	—	—	2% methanol in water
CH=CH ₂	244	12,000	282	450	Alcohol
C \equiv CH	236	12,500	278	650	Heptane
Ph	246	20,000	—	—	Heptane
COCH ₃ [†]	240	13,000	278	1,100	Heptane
N=NPh [†] (trans)	319	19,500	—	—	Chloroform

* Reprinted from Rao, C. N. R., *Ultra-Violet and Visible Spectroscopy*, Butterworths, London, 1961, 40. With permission.

[†] In addition, these compounds exhibit weak bands (R-bands) due to $n \rightarrow \pi^*$ transitions of the chromophores at longer wavelengths: acetophenone at 329 m and azobenzene at 445 m μ .

the editors of *Analytical Chemistry*⁸ and is consistent with usage in the *United States Pharmacopeia XIX* and *National Formulary XIV*.

Quantitative analysis by UV/visible spectrophotometry requires measurement of I/I_0 , which is the fraction of an incident beam of light that passes through the solution. Substituting I/I_0 into Equation 3 along with values of the path length and absorptivity allows one to calculate the concentration of the unknown solution directly. However, the measured values of the absorptivity may deviate significantly from the true value due to the limited resolution and level of stray light in the spectrometer. In order to avoid the systematic error that would result from using an improper value for the absorptivity, standard solutions of known concentration are usually measured at the same time as the unknown samples. The concentration of the unknown is then obtained from the concentration of the known by use of Equation 7 which is derived from Equation 4.

$$C_u = \left(\frac{A_u}{A_s} \right) C_s \quad (7)$$

The subscript u refers to the unknown solution and s to the standard solution. The derivation of Equation 7 also assumed that the path lengths used to measure A_u and A_s were identical.

2. Deviations from the Beer's Law

Four conditions must be met if a set of absorbance measurements are to be accurately described by the Beer-Lambert Law:

Table 9
 ABSORPTION SPECTRA OF DISUBSTITUTED BENZENE DERIVATIVES^{a,b}(R₁-C₆H₄-R₂)

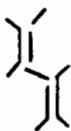

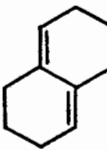
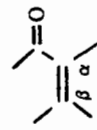
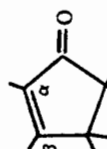
R ₁	R ₂	para-Derivatives			meta-Derivatives			ortho-Derivatives				
		Observed band			200 mμ Band		260 gmμ Band		200 mμ Band		260 mμ Band	
		λ _{max} , mμ	ε _{max}	λ _{max} , mμ	ε _{max}	λ _{max} , mμ	ε _{max}	λ _{max} , mμ	ε _{max}	λ _{max} , mμ	ε _{max}	λ _{max} , mμ
CH ₃	CN	237	17,200 ^c	229.5	11,000	276	1,280	228.5	11,100	276.5	1,440	
Cl	COOH	241	16,300	231.5	9,100	283	1,080	229	5,900	280	870	
Cl	NO ₂	280	10,300	264	7,100	313	1,300	260	4,000	310	1,400	
NO ₂	CH ₃	285	9,250	273	7,300	315	1,300	266	5,300	325	1,300	
NO ₂	OH	317.5	10,000	273.5	6,000	333	1,960	278.5	6,600	351	3,200	
NO ₂	NH ₂	381	13,500	280	4,800	358	1,450	282.5	5,400	412	4,500	
NO ₂	COOH	264.5	12,400	—	—	—	—	—	—	—	—	
NO ₂	NO ₂	266	14,500	241.5	16,300	305	1,100	—	—	—	—	
NH ₂	COOH	284	14,000	250	2,400	310	650	248	3,900	327	1,940	
NH ₂	COCH ₃	311.5	17,100	—	—	—	—	—	—	—	—	
NH ₂	CN	270	19,800	236.5	8,200	308	2,400	—	—	—	—	
OH	CHO	283.5	16,000	254.5	10,100	314	2,580	256	12,600	324	3,400	
OH	COOH	255	13,900	236.5	7,500	296	2,500	237	9,000	302.5	3,600	
OH	COCH ₃	275	14,300	250.5	9,100	308	2,300	252.5	10,900	324	3,300	

^a Reprinted from Rao, C. N. R., *Ultra-Violet and Visible Spectroscopy*, Butterworths, London, 1961, 46. With permission.

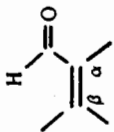
^b In 1 mol⁻¹cm⁻¹.

^c There is another band at 268 mμ (ε_{max} = 750).

Table 10
EMPIRICAL RULES FOR ESTIMATING λ_{MAX} IN ETHANOL

Parent chromophore	λ_{REF}	Structural element	δ , nm
Acyclic diene 	214	double bond extending conjugation	+30
Homoannular diene 	253	alkyl substituent	+5
Heteroannular diene 	217	exocyclic double bond RCO ₂ — RO— RS— Cl— Br— R ₂ N—	+5 0 +6 +30 +17 +17 +60
Acyclic or large ring (> 6 atoms) 	215	double bond extending conjugation	Substitution position α +30 β γ > δ
Cyclopentanone 	205	homoannular diene component exocyclic double bond alkyl substituent	+39 +5 +10 +12 +18 +18

α, β unsaturated aldehydes



210

HO—
RO—
RS—
CH₃CO₂—
Cl—
Br—
R₂N—

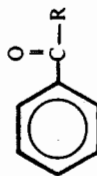
+35 +30 +50
+35 +30 +17 +31
— +85 —
+6 +6 +6
+15 +12 —
+25 +30 —
— +95 —

Derivatives of benzaldehyde and benzonitrile



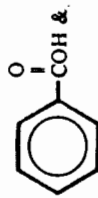
250 nm

alkyl substituent
HO— or RO—



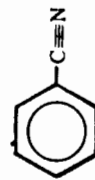
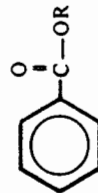
246 nm

—O⁻



230 nm

Cl⁻
Br⁻



224 nm

H₂N—
R—NH—
R₂N—

CH₃—C(=O)—NH—

ortho meta para

+3 +3 +10
+7 +7 +25

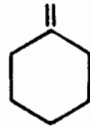
+11 +20 +78

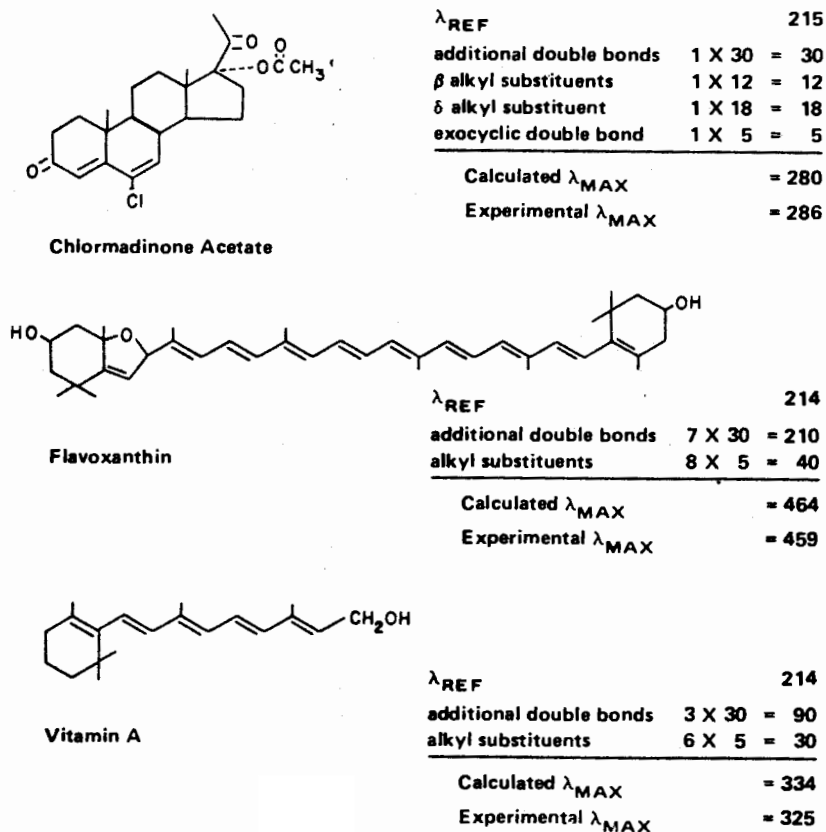
0 0 +10
+2 +2 +15

+13 +13 +58
— — +73
+20 +20 +85

+20 +20 +45

Definitions:
homoannular diene — 2 conjugated double bonds within the same ring
heteroannular diene — 2 conjugated double bonds in adjacent rings
exocyclic double bond — double bond including a ring carbon but not inside the ring, e.g.,



FIGURE 2. Three examples of the procedure for estimating λ_{MAX} .

1. The intensity of the light beam must not be excessive.
2. The instrument used must accurately measure the fraction of light the sample absorbs from the beam.
3. The light beam must be monochromatic.
4. The concentration used in Equation 3 must be the concentration of the absorbing species.

The first two of these requirements are relatively trivial. Only laser light sources achieve intensities great enough to cause deviations from Beer's Law, and most analytical spectrometers will be accurate enough if the absorbance values lie within the recommended range of operation of the instrument. The effect of several instrument parameters including stray light level, band pass, and optimum absorbance range on accuracy are discussed in more detail in Sections IVB and IVC.

The derivation of Beer's Law assumes that a single absorptivity describes the absorption of all light in the incident beam, i.e., that ϵ is a constant. If the light beam contains a broad enough range of wavelengths that this condition is not met, significant deviations from Beer's Law may result as is discussed in more detail in Section IVB. These deviations are a very practical problem because the bandwidth is under operator control via slit adjustments on most instruments and must be adjusted according to the width of the spectrum of the analyte.

Whenever the analyte is involved in an equilibrium such as protonation or deprotonation, tautomerism, dimerization, or complex formation, the material added to the solution will be distributed among several forms and the apparent concentration (amount material dissolved/volume) will not be proportional to the actual concentration of the parent substance. A deviation from Beer's Law will be observed under these circumstances unless the absorptivity is identical for all the species present or the equilibrium is controlled in some manner. If only two species are present and their spectra are not too different, useful measurements following Beer's Law can be made by measuring at the isobestic point rather than at λ_{MAX} . The isobestic point is the wavelength at which the UV spectra of the two species cross when measured at equal molarities or, equivalently, the wavelength at which their molar absorptivities are equal. Deviations arising from acid-base equilibria can be avoided by carefully buffering the solutions since the ratio of protonated to deprotonated analyte will be constant at constant pH. The percent ionized at a given pH can be estimated from the nomogram in Figure 5 of Chapter I. A variety of other equilibria can be controlled in a similar manner.

III. UV/VISIBLE SPECTROPHOTOMETERS

Instruments for measuring light absorption in the UV/visible region of the spectrum consist of four basic components: light source, device for isolating the wavelength of interest, sample holder, and detector. The usual configuration for these components is shown schematically in Figure 3. Proper selection and adjustment of each component is essential if maximum sensitivity, precision, and accuracy are to be achieved. The components will be discussed briefly in the following paragraphs, and the effects of instrument parameters on precision and accuracy will be discussed in Section IV.

A. Light Source

The selection of the light source depends upon the wavelength at which measurements are to be made. Tungsten lamps are most frequently used when measurements are to be made above 380 nm as they provide a continuous spectrum with reasonable intensity in this region. The output of a tungsten lamp (Einsteins per second) is greatest in the 600 to 700 nm region and falls off rapidly on both the long and short wavelength sides of the maximum. High pressure xenon lamps also have a continuous spectrum (with some superimposed lines) in the visible region and can be used from approximately 300 to 800 nm. However, the xenon lamps are expensive and since their high output intensity is not normally needed in visible spectrophotometry, they are rarely used. High pressure mercury lamps also provide adequate continuous output from about 280 to 700 nm, but with very strong superimposed emission lines (see Table 7).

The output of a high pressure deuterium lamp is continuous, and of adequate intensity from 380 nm to 185 nm. Above 380 nm the spectrum is no longer continuous, and below 185 nm the intensity is rapidly attenuated by absorption in the fused silica bulb. A high pressure hydrogen lamp can also be used in this region of the spectrum, but its intensity is only about one third of the intensity of the deuterium lamp under otherwise identical operating conditions. Spectra of several common lamps may be found in References 19, 20, and 21.

The most frequently used lamps in practice are the deuterium lamp for the UV region and the tungsten lamp for the visible. Many spectrophotometers are equipped with both lamps, and the lamp to be used is selected either by rotating a mirror or by physically moving the lamp mounts so that the image of the proper lamp falls upon the entrance slit of the monochromator.

The light source must also contain a power supply for the lamp. Fluctuations in the

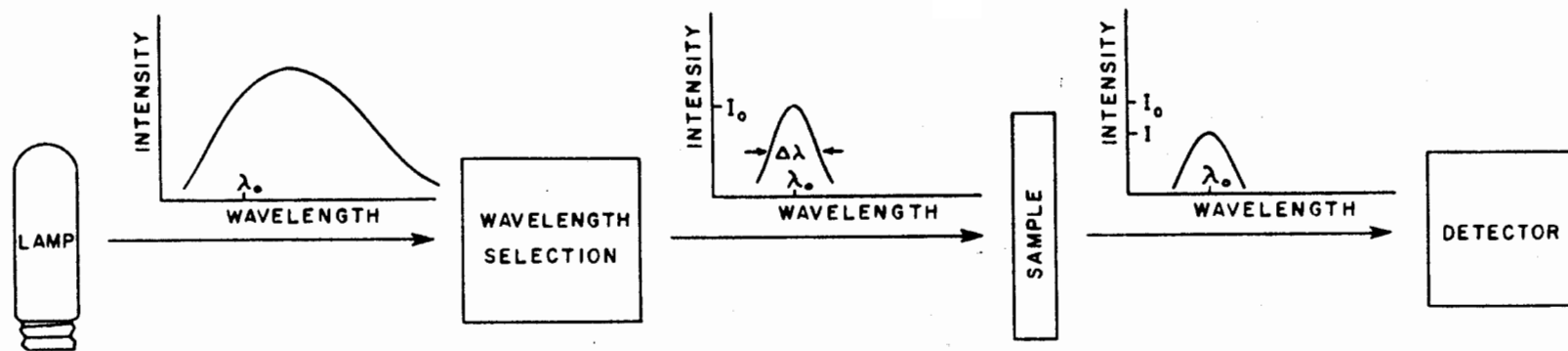


FIGURE 3. Block diagram of a typical UV/Visible spectrophotometer. The lamp produces a broad spectrum of light from which the wavelengths of interest are selected using a combination of filters, prisms, diffraction gratings and slits. The resulting beam has an intensity I_0 at its nominal wavelength, λ_0 , and a bandwidth $\Delta\lambda$. $\Delta\lambda$ is measured at $1/2 I_0$. The beam then passes through the sample where it is attenuated to intensity I before striking the detector.

output voltage of the power supply can cause large fluctuations in the source intensity and corresponding fluctuations in the detector output. This problem can be overcome in two ways. The first is to use a highly stable power supply with fluctuations no larger than a few millivolts. This technique is used in simple, single beam photometers and spectrophotometers. The alternate approach is to monitor the lamp output along a reference path not including the sample and correcting the intensity in the analytical beam for any changes observed in the reference beam. The dual beam technique is used in virtually all research quality spectrophotometers.

B. Wavelength Selection

A beam of light will contain a range of wavelengths, although the range may be very narrow in some cases (e.g., laser beams). The range of wavelengths present is described by the bandwidth of the beam as shown in Figure 3. A narrow bandwidth is desirable in quantitative spectrophotometry because it provides the best sensitivity and selectivity in the analysis, and because deviations from Beer's Law (Equation 3) are avoided. Isolation of the desired wavelengths from the broad emission band of the light source may be accomplished by using filters, prisms, or diffraction gratings.

Filters may be of the absorption or interference type. Absorption filters may pass all light above a certain wavelength (long wavelength filter), below a particular wavelength (short wavelength filter), or within a certain spectral band (band pass filter). Light that is not passed is absorbed by the filter. Absorption filters have band passes on the order of 35 to 50 nm, which is unacceptably broad for many analytical applications. The intensity of the beam at the center of the band is greatly reduced by absorption type band pass filters because their peak transmittances are in the range of only 5 to 25%. Wratten® filters, which consist of a gelatin layer containing organic dyes sandwiched between two glass plates, have the additional disadvantage of deteriorating with use. Characteristics of a number of commercial filters can be found in Reference 22.

Interference filters are band pass filters made by coating both sides of a thin layer of dielectric material, such as magnesium fluoride, with a semitransparent layer of silver. Light rays entering the dielectric are reflected back and forth between the silver layers, leading to interference between the superimposed rays. Only light of nominal wavelength λ where λ is given by Equation 8, interferes constructively and passes through the filter.

$$\lambda = 2 dn/m \quad (8)$$

where d = thickness of the dielectric layer, n = refractive index of the dielectric, and m = a positive integer.

The remainder of the light is reflected back toward the source. A filter made from magnesium fluoride ($n = 1.38$) with a thickness of $0.181 \mu\text{m}$ will have maximum transmittance at 500 nm ($m = 1$) according to Equation 8. It will also pass light of wavelength 250 nm ($m = 2$): light of wavelengths 167 nm ($m = 3$), 125 nm ($m = 4$) and so on will be blocked by absorption in the dielectric rather than by interference. Simple interference filters achieve transmittances of 40 to 60% with a bandwidth of 10 to 20 nm. Multilayer interference filters achieve transmittances of 60 to 95% with bandwidths less than 8 nm. Nonetheless, the broad bandwidth and inconvenience when changing wavelengths limit the use of filters for wavelength selection to inexpensive instruments for routine monitoring functions.

A prism and slit combination may also be used to select a particular wavelength and bandwidth for an absorption measurement. The prism spreads the wavelengths out in

space and the slit is then used to select that portion of the spectrum to be used for the measurement, as shown in Figure 4. Prisms have the advantages of greater transmittance than filters, relatively narrow bandwidths can be obtained by using narrow slits, and the nominal wavelength is continuously variable. A major disadvantage of prisms is the decrease in their dispersion at longer wavelengths, which leads to crowding of the wavelength scale (see Figure 4). Glass prisms can be used into the near infrared ($\lambda = 1000$ nm) without severe crowding, but their absorption is too great below 350 nm for use in the UV region of the spectrum. Quartz and fused silica prisms can be used in the UV, but crowding of the wavelength scale becomes a serious problem above 600 nm. Crowding of the scale also means that a smaller slit must be used at long wavelengths than at short wavelengths in order to achieve the same bandwidth. Diffraction gratings avoid these problems and have almost completely replaced prisms in modern spectrophotometers.

A diffraction grating consists of a large number of fine, parallel grooves machined into a highly reflective surface. The gratings used in analytical spectrophotometers usually have about 15,000 lines/in. Each groove on the grating reflects light, but the phases of the reflected rays are slightly different when the rays originate from different grooves. The rays will interfere destructively except when the condition expressed by Equation 9 holds.

$$\lambda = \frac{d}{m} (\sin i \pm \sin \theta) \quad (9)$$

where d = distance between grooves, m = integer, i = angle of incidence of the light beam with respect to the grating normal, and θ = angle of reflected beam with respect to the grating normal.

Thus different wavelengths of light are reflected at different angles, θ and as long as θ is small the separation of wavelengths will be essentially constant across the spectrum. A spectral bandwidth of 0.2 nm or less can be achieved on most grating spectrophotometers. Furthermore a grating does not attenuate the beam intensity by absorption at shorter wavelengths and may be used from the vacuum UV into the near IR regions of the spectrum. As with any interference device, the light energy reflected from a grating is distributed among several spectral orders. The short wavelength end of the second order spectrum will usually overlap the long wavelength end of the first order spectrum. This problem is overcome in most instruments by cutting the grooves of the grating at an angle (blaze angle) to concentrate the diffracted intensity in the first order spectrum and then using a long wavelength filter to remove light from the higher order spectra when making absorption measurements in the long wavelength region.

C. Detectors

A number of photosensitive devices can be used as detectors in UV/visible absorption instruments. The simplest is the barrier-layer photocell, which consists of two conductors separated by a semiconductor layer, as shown in Figure 5. Light passing through the semitransparent silver layer causes release of an electron which migrates to the collector. The electrons accumulating on the collector result in a potential difference between the base and collector which can be measured by a low resistance galvanometer circuit. The current flow is proportional to the intensity of the incident light provided the intensity is not too great. Several disadvantages of this type of detector are (1) its low impedance, which makes amplification difficult so that relatively high beam intensities are required for measurement, (2) fatigue, which results in a decline in output current with time at a constant level of illumination, particularly at

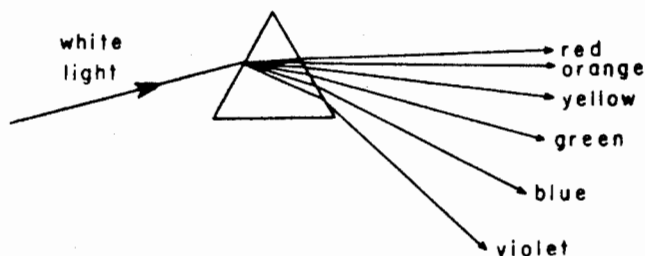


FIGURE 4. Dispersion of light by a prism.

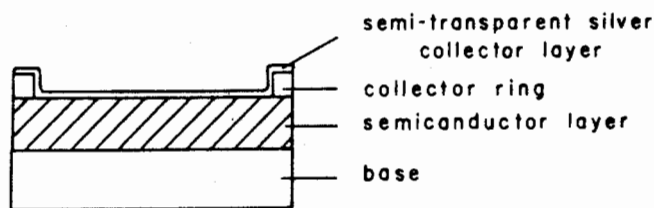


FIGURE 5. Construction of a barrier layer cell.

high levels of illumination, (3) a response that is strongly dependent on temperature, and (4) a response that is limited to the visible region of the spectrum. As a result of these problems, photocells are usually found only in inexpensive filter photometers.

Photoemissive tubes consist of an anode and a cathode sealed in an evacuated glass tube which may have a quartz or silica window for UV measurements. The cathode is coated with a layer of light sensitive material that emits electrons upon absorption of photons. A power supply maintains the anode positive with respect to the cathode so that the photoelectrons are collected at the anode. With a sufficiently high voltage, the photocurrent is directly proportional to light intensity. Phototubes have a relatively high impedance so their output current is easily amplified. Phototubes can measure light fluxes 50 to 100 times lower than can be measured with photocells, and the photocurrent is relatively independent of temperature. Phototubes are available for use over the entire UV/visible region of the spectrum, but no single tube covers the entire range satisfactorily. Therefore many instruments with phototube detectors employ interchangeable blue and red sensitive phototubes in order to provide sufficient sensitivity over the entire spectrum.

Photomultiplier tubes are very sensitive detectors with very short response times. Photomultipliers will measure light fluxes two orders of magnitude less than can be measured using a phototube. A photomultiplier contains a photocathode and a series of plates called dynodes which are also photosensitive. A potential of 30 to 100 V is maintained between the photocathode and first dynode, and between each pair of dynodes. A photoelectron released from the photocathode is accelerated toward the first dynode by their voltage difference, where it strikes with energy sufficient to release several electrons. The secondary electrons are then accelerated toward the second dynode where the process repeats. This may be repeated at 10 to 20 dynode stages. In this way each electron released from the photocathode results in a large number of electrons being collected at the anode. The amplification obtained from a photomultiplier tube can be controlled by controlling the potential difference between the dynodes, but is limited by the inherent background current arising from thermionic elec-

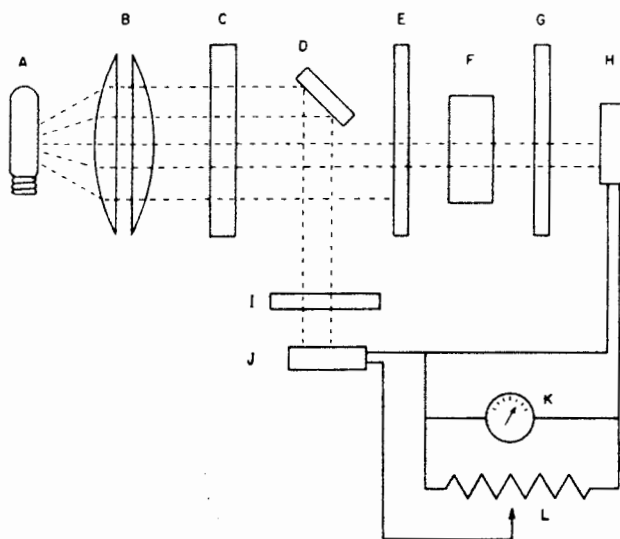


FIGURE 6. Typical double beam filter spectrophotometer. A — lamp; B — light collimating lenses; C — filter; D — reference beam mirror; E, G, and I — slits; F — sample cell; H — sample beam detector; J — reference beam detector; K — galvanometer; L — %T slidewire.

one shown in Figure 6 except for deletion of the reference photocell and corresponding changes in the electronics.

A schematic diagram of a double beam grating spectrophotometer of the dual beam in time type is presented in Figure 7. Tungsten halogen and deuterium lamps are provided for operation in the visible and UV regions, respectively. The collimated beam from the lamp is passed through a beam chopper and into the monochromator where the desired wavelength is selected by rotating the grating until that wavelength falls upon the exit slit. Light from the exit slit falls upon the beam splitter which alternately reflects it along the reference and sample paths. Two detectors are provided, one for short and one for long wavelength work. Only a single detector tube would be required if a photomultiplier were used.

A large number of variations can be made on the basic instrument designs described here. More detailed discussions of spectrophotometers, including commercially available models, may be found in References 21 and 25 and in the literature of the manufacturers.

IV. SELECTION OF CONDITIONS FOR SPECTROPHOTOMETRIC MEASUREMENTS

A. Wavelength

The absorption spectrum of the analyte sets limits on the range of wavelengths that can be used in its analysis, but any wavelength within that range can be used for analysis since Beer's Law is obeyed at all points on the absorption curve. The most frequently used wavelength for spectrophotometric analyses is the λ_{MAX} of the analyte, but other wavelengths may offer distinct advantages in special cases. If a substance with an absorption spectrum overlapping the spectrum of the analyte is likely to be present in the solutions at the time of measurement, the optimum wavelength for the measurement, λ , will be the one that gives the largest positive value of the difference

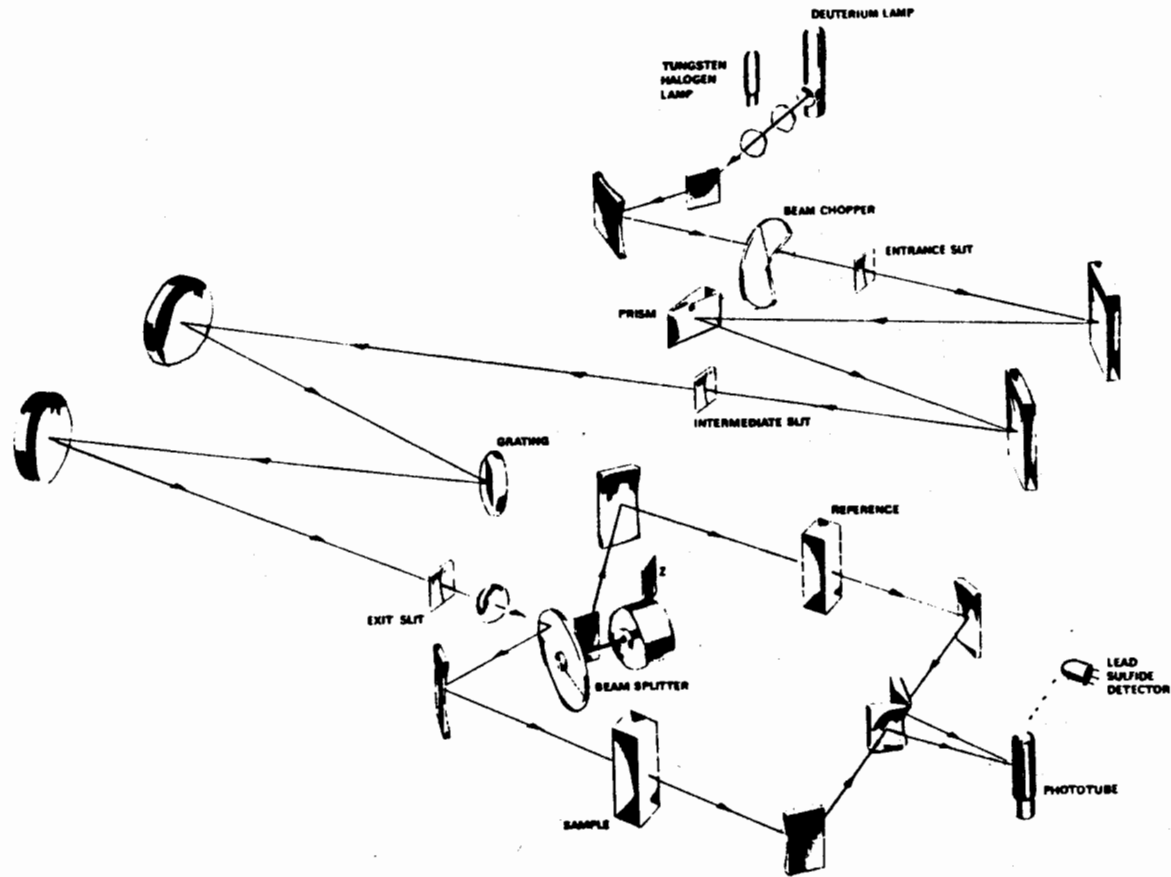


FIGURE 7. Cary 17 optical diagram. The Cary 17 is a double beam in time grating spectrometer. Reprinted with permission from Varian Associates, Palo Alto, California.

$\epsilon_A(\lambda) - \epsilon_s(\lambda)$, where $\epsilon_A(\lambda)$ and $\epsilon_s(\lambda)$ are the molar absorptivities of the analyte and other substance at λ , respectively. This wavelength will minimize systematic bias in the analysis due to the presence of the second substance.

If the analyte is involved in a simple equilibrium between two forms, $A \rightleftharpoons B$, and the absorbance of A and B are similar, then it may be advantageous to measure the absorbance at the isobestic point, λ_i . λ_i is the wavelength at which the molar absorptivities of A and B are equal, and therefore the position of the equilibrium does not affect absorbances measured at this wavelength.

It may also be advantageous to use a wavelength other than λ_{MAX} when the analyte is presented as a relatively concentrated solution with a high absorbance. The most accurate measurements are usually obtained when the absorbance is between 0.4 and 1.0 (see Section IVB) and an absorbance in this range may be achieved either by serial dilution of the sample or by working at a wavelength where the molar absorptivity is low. The latter technique has the advantage of avoiding the additional component of variability introduced by the dilutions, as well as being faster and requiring less solvent.

The slope of the absorption curve of a substance, $d\epsilon(\lambda)/d\lambda$, is much greater at wavelengths other than λ_{MAX} than it is at λ_{MAX} . The difference in slope affects the measurement of absorbance in two ways. First, greater values of $d\epsilon/d\lambda$ necessitate using narrower bandwidths in the measurement in order to have ϵ approximately constant across the band. A narrower bandwidth means less total light reaching the detector and therefore a lower signal to noise ratio. Secondly, small shifts in the absorption spectrum will have a much greater effect on an absorbance measured at a steeper part of the spectrum than at the relatively flat region about λ_{MAX} . Therefore greater care must be taken to control solvent composition, temperature, pH, and other factors that may affect the absorption spectrum of the analyte.

B. Bandwidth

The bandwidth of most spectrometers is controlled by the operator. In prism and grating instruments the bandwidth is controlled by adjusting the slit, wider slits giving broader bandwidths than narrow slits. The bandwidth in filter instruments is set by selecting a combination of long and short wavelength filters or an interference filter that passes only a limited portion of the spectrum. The most obvious problem arising from too great a bandwidth is loss of assay specificity: the wider the range of wavelengths used, the more likely it becomes that significant contributions to the measured absorbance will be due to extraneous substances in the sample.

Selection of too broad a bandwidth also adversely affects the sensitivity and linearity of an analysis. The reason for this can be seen qualitatively by considering Figure 8, which shows a typical absorption curve plotted as percent transmittance. If a very wide bandwidth ranging from λ_1 to λ_4 were used in a measurement, the amount of light represented by the crosshatched area under Curve a would be absorbed. Assuming the absorbed light is 40% of the total light admitted through the slit, then a 10% change in the concentration of the sample would result in only a 4% (10% of 40%) change in the intensity of the light reaching the detector. If a narrow slit admitting wavelengths between λ_2 and λ_3 were used instead, 70% of the light from the slit would be absorbed initially so that a 10% change in sample concentration now yields a 7% (10% of 70%) change in the light reaching the detector. The sensitivity is therefore almost doubled by using the narrower slit in this example. Maximum sensitivity will be achieved in any case by selecting a bandwidth narrow enough that the absorbance of the sample is essentially constant over the range of wavelengths admitted to the cell. This condition is also necessary if the measured absorbances are to follow the Beer-Lambert Law, since the integration of Equation 2 to obtain Equation 3 assumes that the absorptivity

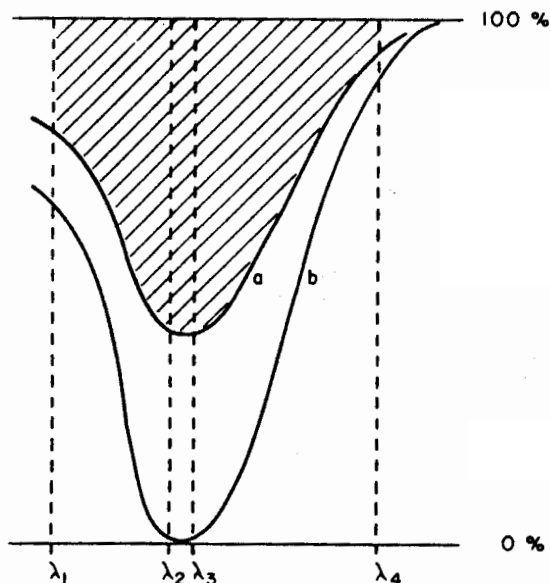


FIGURE 8. Effect of bandwidth on absorption measurements. Vertical scale is percent transmittance. (λ_1, λ_4) is the range of wavelengths admitted to the detector using a very wide slit (large bandwidth). (λ_2, λ_3) is the range admitted when using a narrow slit (narrow bandwidth).

is independent of wavelength. In practice, use of too great a bandwidth results in obvious nonlinearity in the plot of absorbance vs. concentration.

The effect of stray light on absorbance measurements is very similar to the effect of too great a bandwidth. Stray light is light which reaches the detector beam outside the spectral band selected by the filters or monochromator. It arises from imperfections in the optics, unwanted reflections within the instrument and even leakage from outside the instrument. Stray light effectively broadens the bandwidth used in the measurement and can result in significant errors in the measured absorbance, particularly at high absorbance values. Figure 9 gives the percent error in an absorbance measurement as a function of the stray light level as estimated by Cook and Jankow.²⁶

C. Concentration and Optimum Absorbance Value

The measured intensity, I , of a light beam passing through a sample will fluctuate randomly as a result of source flicker, vibration in the optical system, dust in the light beam, detector noise, amplifier noise, etc. If the magnitude of the fluctuation is ΔI , then the greatest spectrophotometric precision will be obtained when $\Delta I/I$ is minimum. Experimentally this means adjusting sample concentrations so that the measured values of I will be near the optimum, or equivalently, so that the absorbance of the sample is in the optimum range. The optimum absorbance can be estimated easily for two special but realistic cases.

In the first case the assumption is made that the noise level is independent of the intensity of the light falling on the detector. This would be the case for a spectrophotometer using a photoconductive detector, e.g., a lead sulfide detector, under conditions where the detector is the principal source of noise. The relation between a change in measured intensity, ΔI , and the resulting change in apparent concentration, ΔC , can be obtained by differentiating Equation 3:

$$\Delta C = \frac{1}{C} \cdot \frac{1}{I} \frac{\Delta I}{I} \quad (10)$$

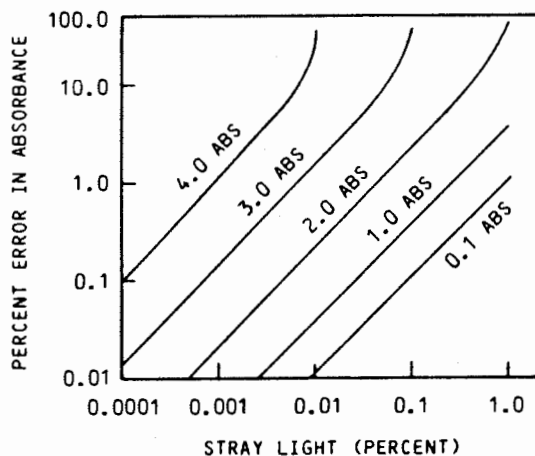


FIGURE 9. Percent error in absorbance measurements as a function of the percent stray light. Reprinted from Cook, R. B. and Jankow, R., *J. Chem. Ed.*, 49, 405, 1972. With permission.

The relative noise level is obtained by dividing through by C , again using Equation 3:

$$\frac{\Delta C}{C} = -\frac{0.43}{A} \frac{\Delta I}{I} \quad (11)$$

The minimum value of $\Delta C/C$ can be obtained by differentiating with respect to I and setting the derivative equal to zero:

$$\frac{d}{dI} \left(\frac{\Delta C}{C} \right) = -0.43 \Delta I \left(I \log \frac{I_0}{I} \right)^{-2} \left(\log \frac{I_0}{I} - 0.43 \right) \quad (12)$$

The form of the derivative depends upon the assumption that the noise level, ΔI , is independent of I . The minimum in $\Delta C/C$ occurs for $A = 0.43$. This result is frequently quoted, but the assumption concerning the nature of the noise is not often clearly stated.

The second case is also a spectrophotometer in which detector noise is limiting, but in this case the detector is a phototube so the noise level is not independent of signal level but rather $\Delta I = kI^{1/2}$ where k is a proportionality constant. Using the $I^{1/2}$ dependence of the noise in differentiating Equation 11, the minimum in $\Delta C/C$ is found to occur at $A = 0.86$. Since most modern UV/visible spectrophotometers use phototube detectors and electronics with relatively low noise, the relative error in a spectrophotometric analysis will be minimum when the absorbance is near 0.86.²⁷ Treatments of more complex cases can be found in the literature.^{28,29}

D. Solvent

The choice of solvents for use in a spectrophotometric analysis is limited primarily by the solubility of the analyte. However, the absorbance of the solvent at the analytical wavelength is an important limiting factor when the analytical wavelength is in the UV region of the spectrum. The "solvent cutoff" is the lowest wavelength at which a solvent is useful under normal analytical conditions and is usually taken as the wave-

length at which the solvent has an absorbance of 1.0 in a 1.0-cm cell. The absorption characteristics of several common solvents are listed in order of increasing cut-off in Table 11.

The final choice of solvent from among those with adequate solubility and low enough cut-off is made by considering several factors. The solvent should be readily available in suitable purity, where suitable purity means the absence of extraneous materials that absorb at the analytical wavelength and, even more important, the absence of fluorescent impurities as these contribute significantly to the "stray light" reaching the detector. If the analyte is to be recovered after measurement, a solvent that evaporates quickly might be desirable (see Table 4, Chapter 1). Moisture sensitive compounds will require use of solvents which can be dried easily and which have little tendency to pick up water.

If a substance is involved in acid-base equilibria, tautomerism, complex formation, or other equilibria, it will also be necessary to select a solvent system which provides adequate control of the equilibrium. Acid-base and tautomeric equilibria are most frequently encountered. These can usually be controlled adequately by using a strongly acidic or strongly basic solvent so that essentially all of the analyte will be present in a single form (see the nomogram in Figure 5, Chapter 1). A buffer may also be used if it is prepared from salts that do not absorb appreciably at the wavelength used in the analysis: acetate, phosphate, and carbonate buffers are often suitable (see Table 3, Chapter 1) for UV work.

V. APPLICATIONS

A. Scope

The applications discussed in this section are limited to methods applicable to a wide range of compounds, e.g., methods applicable to all substances containing an activated aromatic system, or to a special group of compounds characterized by a complex structure, such as barbiturates, penicillins and cephalosporins.

The applications are divided into four groups. The "UV" methods involve measurements below 380 nm and "visible" methods above 380 nm. UV and visible methods are further divided into "direct" and "indirect" methods. "Direct" spectrophotometric methods are those in which the absorbance of the substance itself is measured after suitable separations, dilutions, etc. In "indirect" methods, the absorbance of a second substance, usually a chemically modified form of the analyte, is measured and related back to the concentration of analyte originally present. Indirect spectrophotometric assays employing measurements in the visible region of the spectrum are better known as "colorimetric" methods.

B. UV Spectrophotometry

1. Direct Methods

Essentially all compounds containing conjugated double bond or aromatic rings, and many inorganic species as well, absorb light in the accessible portion of the UV spectrum. Since UV absorption measurements are simple, accurate, and sensitive, UV methods can be used for most substances encountered by the pharmaceutical analyst. The most important limitation on these methods is the potential for interference from other UV absorbing species in the sample. This lack of specificity can be overcome in most cases by including a separation step in the analytical scheme prior to the absorbance measurement. The use of a separation step followed by a UV measurement is probably the most widely used analytical procedure in pharmaceutical analysis today. The applications are so numerous that only a few selected examples can be pre-

Table 11
UV ABSORPTION OF COMMON SOLVENTS

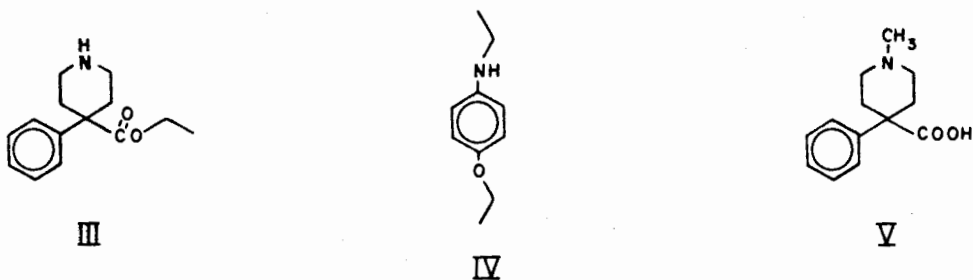
Solvent	Wavelength (nm) for absorbance of*		
	0.1	0.5	1.0
Acetic acid			
Acetone	337	331	328
Acetonitrile	201	193	191
Benzene	292	279	277
Benzonitrile	308	300	298
Bis(2-methoxyethyl)ether	254	228	218
Bromoform	347	335	330
1-Butanol	215	205	203
2-Butanol	229	208	205
Butyl acetate	260	255	253
n-Butyl ether	239	217	210
Carbon disulfide	393	383	378
Carbon tetrachloride	273	266	263
1-Chloro-2,2-dimethylpropane	235	223	220
Chloroform	254	245	242
Cyclohexane	229	213	205
Cyclopentane	217	202	195
1,2-Dichloroethane	235	227	223
Dichloromethane	240	237	230
N,N-Dimethylacetamide	289	272	268
N,N-Dimethylformamide	290	271	268
Dimethylsulfoxide	275	265	261
p-Dioxane	220	211	210
Ethanol			
Ethyl acetate	261	255	253
Ethyl ether	243	218	215
Ethyl formate	265	262	259
Ethyl propionate	260	255	253
Formic acid			
Glycerol	220	205	202
Heptane	218	204	194
Hexadecane	239	217	208
Hexane	215	203	194
Methanol	210	204	202
2-Methylbutane	216	201	194
Methylcyclohexane	230	216	208
Methyl Formate	263	259	258
3-Methyl-1-pentanol	219	208	205
1-Methyl-2-pyrrolidinene	286	264	261
Nitromethane	396	382	367
Pentane	208	190	190
2-Propanol	210	205	202
Pyridine	311	309	306
Tetrachloroethylene	299	292	288
Tetrahydrofuran			
Toluene	300	286	284
1,1,2-Trichloro-1,2,2-trifluoroethane	242	233	230
2,2,4-Trimethylpentane	220	205	196
Water			
m-Xylene	310	292	288

* 1 cm pathlength. The "UV cutoff" is usually taken to be the wavelength at which $A = 1.0$.

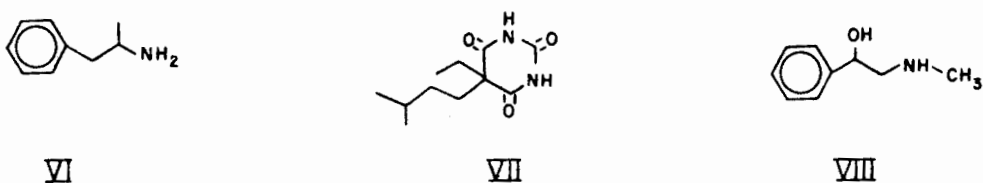
sented here. A more complete list of the applications of UV in pharmaceutical analysis may be found in the reviews, "Pharmaceuticals and Related Drugs", published in odd numbered years in the review issue of *Analytical Chemistry*.

Drugs containing only a simple phenyl chromophore have a long wavelength absorption band consisting of several maxima between 250 and 270 nm. Examples are given in Figure 1 and in Table 12. The molar absorptivities for the components of this band are quite small, as can be seen from the values in Table 12.

A number of UV assays for meperidine hydrochloride (III) have been reported. Marozzi and Falzi³⁰ separated meperidine from other substances by paper chromatography, eluted the meperidine zone with hydrochloric acid, and measured the absorbance at 257 nm. Pro and Nelson³¹ determined meperidine in formulations with phenacetin(IV) by steam distilling the meperidine and then measuring its absorbance at 257 nm in water. Meperidine can also be isolated by extraction. The UV identity test in *U.S.P. XIX* specified extracting the drug from an alkalized solution with ether prior to the spectral measurement. *N.F. XIV* specified a similar procedure, but used hexane in place of ether. Extraction from a basic solution also eliminates possible interference from 1-methyl-4-phenyl-4-piperidine carboxylic acid(V) which is the principal degradation product of the formulated drug.³²

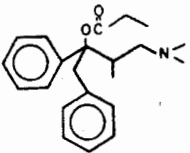
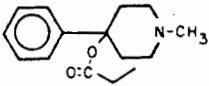
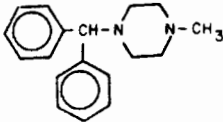


A number of UV methods for dextroamphetamine(VI) have been reported in the literature.³³⁻³⁵ Fabrizio³³ used an alginic acid column to separate dextroamphetamine from amobarbital(VII) in timed-release capsules, and then quantitated both drugs by UV. The column was prepared in acid form, the sample added, and amobarbital eluted with ethanol. Amobarbital was determined by measuring its absorbance at 240 nm in a pH 10 borate buffer. Dextroamphetamine was then eluted from the column with 0.5 *N* sulfuric acid and its absorbance at 257 nm was measured using 0.5 *N* sulfuric acid as the blank. Fabrizio³⁶ also used an alginic acid column to separate ephedrine(VIII) from amobarbital(VII) and aminophylline(IX) in antiasthma capsules. Ephedrine was then quantitated by measuring its absorbance at 257 nm in 0.1 *N* hydrochloric acid in 4 cm cells. Aminophylline and amobarbital were then determined by differential UV using absorbance measurements at 240 nm and 285.5 nm in pH 4.0 and pH 10.0 buffer solutions.



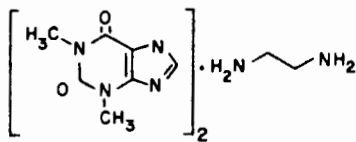
A drug whose principal chromophore is a phenyl ring with one or more heteroatom

Table 12
THE UV SPECTRA OF SEVERAL DRUGS WITH A SIMPLE PHENYL CHROMOPHORE

Drug compound	λ_{MAX}^a nm	ϵ , l moles ⁻¹ cm ⁻¹	Solvent	Ref.
Propoxyphene·HCl 	242 (SH)	137	95% ethanol	
	247 (SH)	200		
	252	279		
	258	360		
	264	288		
	267 (SH)	156		
Meperidine·HCl 	251	176	water	31
	257	217		
	263	174		
Cyclizine·HCl 	269	540	0.1N HCl	
	263	742		
	258	694		
	253 (SH)	548		

^a SH = shoulder

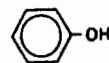
substituents generally absorbs at a longer wavelength and with a greater absorptivity than a drug with only a simple phenyl chromophore. Substitution also reduces the intensity of the vibrational fine structure characteristic of the phenyl bands, and often eliminates the fine structure entirely. A large number of drug substances belong to this group, including the derivatives of catechol(X), phenol(XI), salicylic acid(XII), aniline(XIII), etc. The UV characteristics of several common drugs in this group are presented in Table 13.



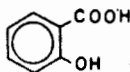
IX



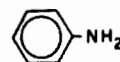
X



XI



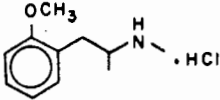
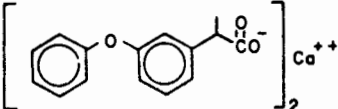
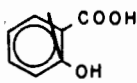
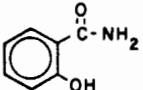
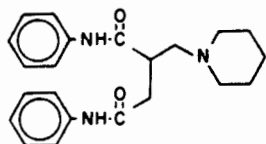
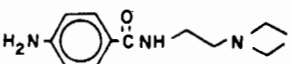
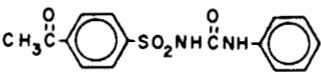
XII



XIII

Soliman and Salaheldin³⁷ determined salicylamide(XIV) in analgesic tablets by UV in the presence of acetaminophen(XV), phenobarbital(XVI), caffeine(XVII), codeine

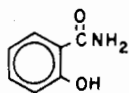
Table 13
 UV ABSORPTION CHARACTERISTICS OF DRUGS WITH
 HETEROATOM-SUBSTITUTED PHENYL CHROMOPHORES

Compound	λ_{MAX} , nm	ϵ^a	Solvent	Ref.
 Methoxyphenamine hydrochloride	275	—	—	180
 Fenoprofen calcium	278 272 266	3,530 3,910 3,424	methanol	66
 Salicylic acid	300 233	794 1,259	water	57
 Salicylamide	308	4,280	chloroform	37
 Diperodon	233	26,000	aq. HCl	371
 Procainamide	291	18,754	methanol	372
 Acetohexamide	247 284		ethanol	373

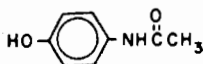
^a Extinction coefficient in $l \text{ moles}^{-1} \text{ cm}^{-1}$

phosphate(XVIII), prednisone(XIX), ascorbic acid(XX), and chloroquine phosphate (XXI). Salicylamide was dissolved by shaking the powdered tablets with chloroform. An aliquot of the filtered chloroform was then diluted to obtain a solution containing between 10 and 30 $\mu\text{g/ml}$ and the absorbance of the dilute solution was measured at

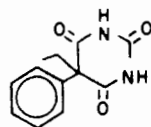
308 nm. Although salicylamide was not separated from the other drugs before measurement, the method was sufficiently specific because (1) salicylamide absorbs at a longer wavelength than the other drugs and (2) it is a major component of the formulation. Even so, a small residual absorbance due to the other species was apparent in the calibration curve (Absorbance = $0.0130 + 0.03043C$, where C is the concentration in $\mu\text{g}/\text{ml}$), and the effect of degradation products of the drugs on the analysis was not determined. Other literature methods for salicylamide include a differential UV measurement applicable in the presence of aspirin (XXII), acetaminophen (XV), and caffeine (XVII);³⁸ and simultaneous determination of salicylamide (XIV), aspirin (XXII), phenacetin (IV), caffeine (XVII), and salicylic acid (XII) by measuring the absorbance of three different solutions (acidic, basic, and hydrolyzed) at several wavelengths.³⁹



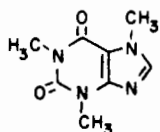
XIV



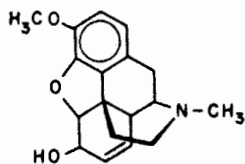
XV



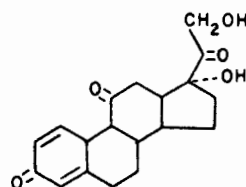
XVI



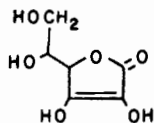
XVII



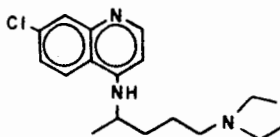
XVIII



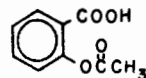
XIX



XX



XXI



XXII

Many workers have reported UV spectrophotometric methods for aspirin (XXII) and for salicylic acid (XII) in aspirin products. The literature on this subject has been reviewed by Kelly.⁴⁰ Salicylic acid is the principal degradation product of aspirin in formulations, so the determination of small amounts of salicylic acid in the presence of large amounts of aspirin is an important problem in quality control of these products. Aspirin and salicylic acid can be determined simultaneously by UV spectrophotometry because their λ_{MAX} values are quite different. λ_{MAX} values for aspirin and salicylic acid are 278 nm and 308 nm,⁴¹ respectively, in chloroform solution, and 226 and 235 nm, respectively, in ethanol.⁴² Most methods in use today, however employ column separation prior to UV or colorimetric measurement.^{43,44}

The absorption characteristics of several cephalosporins are summarized in Table 14. The absorption is due to the fused ring system containing the

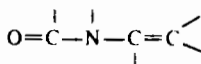
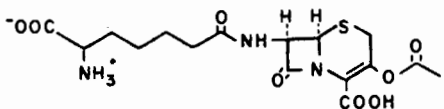
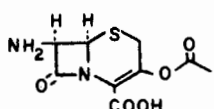
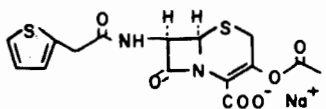
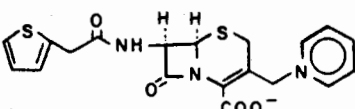
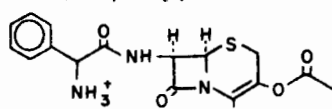
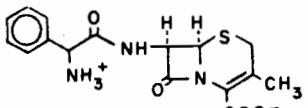
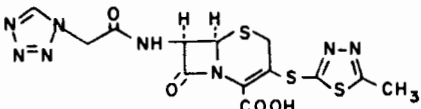


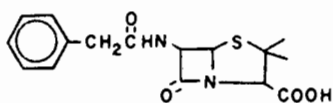
Table 14
UV ABSORPTION CHARACTERISTICS OF SEVERAL CEPHALOSPORINS^a

Compound	λ_{MAX} , nm	ϵ , l moles ⁻¹ cm ⁻¹	Ref.
Sodium cephalosporin C	260	8,300	45
			
7-Aminocephalosporanic acid	265	8,006	45
			
Sodium cephalothin	237 265	14,060 8,536	45
			
Cephaloridine	240 255	15,830 14,542	45
			
Cephaloglycin	260	8,532	45
			
Cephalexin	262	8,198	45
			
Cefazolin	270	13,100	46
			

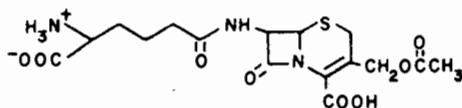
^a 25 $\mu\text{g/ml}$ in water

grouping^{47, 48} and is eliminated when the β -lactam ring is opened. That the thiazine ring also participates in the chromophore is indicated by the absence of absorption above 210 nm in the corresponding penicillins (except when the absorption is due to a chromophore in the side chain, e.g., benzylpenicillin(XXIII)). The loss of absorption with cleavage of the β -lactam was used by Marrelli to analyze for low concentrations of cephalosporin C(XXIV) in the presence of other UV absorbing species.⁴⁹ The amount of cephalosporin C present was determined from the loss in UV absorption at 260 nm following enzymatic hydrolysis with β -lactamase under carefully controlled conditions.

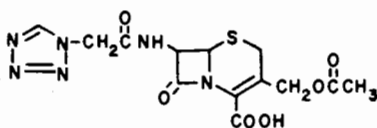
Zappala et al.⁴⁶ reported a high pressure liquid chromatographic (HPLC) procedure for cefazolin(XXV) using a strong anion exchange resin with 0.02 M, pH 6.2 phosphate buffer as the mobile phase and UV detection at 254 nm. High pressure liquid chromatography (HPLC) techniques have also been developed for most of the commercial cephalosporins⁵⁰⁻⁵⁴ (also see Volume III, Chapter 2, Section N). HPLC offers significant advantages over other UV spectrophotometric methods because (1) the high efficiency of the column reduces the likelihood of interference from chemically related impurities to a minimum, (2) the presence of UV-absorbing impurities is clearly shown on the chromatogram, and (3) the analysis can be done on small samples.



XXIII

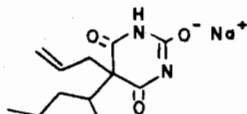


XXIV



XXV

The anionic forms of barbituric acid derivatives absorb strongly in the UV and can be analyzed spectrophotometrically. The UV absorption characteristics of several common barbiturates in alkaline solution are presented in Table 15. The barbiturates may be isolated before measurement by extracting them into ether or chloroform from neutral or very slightly alkaline aqueous solutions. Rotondaro, for example, extracted sodium secobarbital(XXVI) from formulations and then quantitated them by absorbance measurements at 240 nm in 0.01 N sodium hydroxide.⁵⁸ Secobarbital has also been separated from other substances in formulations by column chromatography on an Amberlite IRC-50 cation exchange resin eluted with dimethyl formamide⁵⁹ and a Dowex 2-X8 anion exchange resin eluted with 50% acetic acid in ethanol.^{60, 61} Care must be exercised in any quantitative procedure where barbiturates are eventually measured in alkaline solution because they are subject to alkaline hydrolysis as shown in Figure 10.⁶²⁻⁶⁵



XXVI

Table 15
UV ABSORPTION CHARACTERISTICS OF SEVERAL BARBITURATES

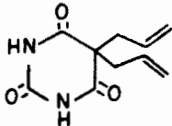
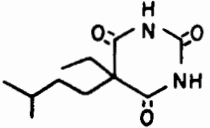
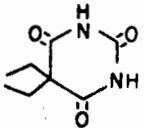
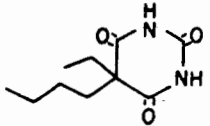
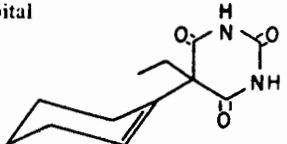
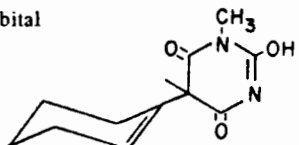
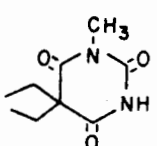
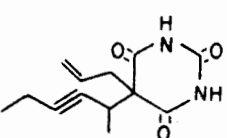
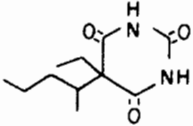
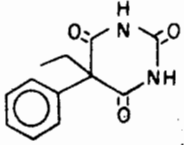
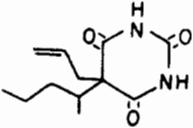
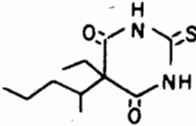
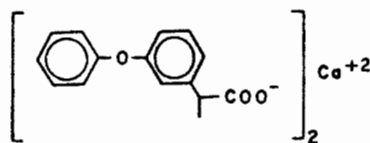
Compound	λ_{MAX} , nm	ϵ , l moles ⁻¹ cm ⁻¹	Ref.
Allobarbital 	241	9,120	57
Amobarbital 	245	6,630	55
Barbital 	244	5,970	55
Butethal 	246	7,005	55
Cyclobarbital 	251	6,995	55
Hexobarbital 	244	3,162	56
Metharbital 	218	7,430	56
Methohexital 	247	—	

Table 15 (continued)
 UV ABSORPTION CHARACTERISTICS OF SEVERAL BARBITURATES

Compound	λ_{MAX} nm	ϵ , l moles ⁻¹ cm ⁻¹	Ref.
Pentobarbital 	242	7,970	55
Phenobarbital 	253	7,430	55
Secobarbital 	241	9,422	56
Thiopental 	304	—	57

Direct UV spectrophotometric methods are easily automated and can provide very rapid, inexpensive analyses. Methods of this type are especially useful in content uniformity studies, dissolution rate measurements, and other applications where large numbers of samples must be analyzed and bias due to impurities is not critical. For example, Fenoprofen calcium (XXVII) has been determined in dissolution samples in pH 7.5 phosphate buffer by measuring the absorbance of the samples at 270 nm on a Technicon Auto-Analyzer® system.⁶⁶ Hanna et al.⁶⁷ reported a completely automated analysis for warfarin tablets based upon the semi-automated procedure recognized by the Association of Official Analytical Chemists^{68,69} and the manual procedure specified in *U.S.P. XIX*. Hanna et al.⁶⁷ noted that the coefficient of variation for the automated procedure was significantly smaller than that of the U.S.P. procedure. An improvement in precision is frequently observed when a spectrophotometric method is automated because variations due to repositioning of the cell, cleanliness of the external surfaces of the cell, carryover from sample to sample, and dilution of the sample prior to measurement are minimized. The precision of the absorbance measurement itself is usually very good, so reduction in variability from other sources has a significant impact on the overall precision of the analysis.

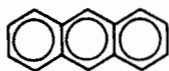
XXVII



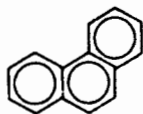
2. Indirect Methods: Chemical Production or Modification of a Chromophore

Direct spectrophotometric analysis of a drug may not be possible for several reasons. The natural absorption of the drug may occur at too low a wavelength to be useful, the molar absorptivity may be too small to give the required sensitivity, or other materials occurring with the drug may absorb at the same wavelength. These problems can be overcome in many cases by chemical modification of the drug in order to change its absorption characteristics.

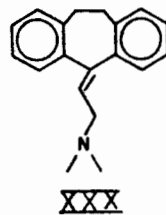
The data in Table 12 show that absorption due to a simple phenyl group is relatively weak and occurs at short wavelengths. The absorption characteristics for this group of compounds may be improved by oxidizing the side chain and measuring the absorption of the oxidation products. Oxidation of an alkyl group on an aromatic ring may be accomplished with a number of strong oxidizing agents, including alkaline permanganate, acid dichromate, and acid cerium(IV). Straight chain and secondary alkyl substituents are oxidized to carboxylic acids. Functional groups may be present in the side chain and, with the exception of the phenyl group, greatly accelerate the rate of reaction when present in the α position. In the presence of an α phenyl group, the reaction usually stops at the diaryl ketone stage. Tertiary alkyl groups are resistant to oxidation and conditions strenuous enough to oxidize them generally result in ring cleavage.⁷⁰ Fuse aromatic ring systems, such as anthracene(XXVIII) and phenanthrene(XXIX), are more easily oxidized than benzene and attempts to oxidize side chains on these ring systems frequently result in ring cleavage.



XXVIII

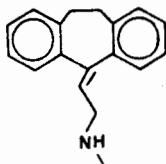


XXIX

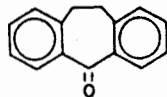


XXX

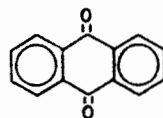
Oxidation followed by spectrophotometric measurement has been used extensively in the analysis of amitriptyline(XXX) and nortriptyline(XXXI). The procedure in *U.S.P. XIX* for amitriptyline specifies direct dilution of preparations for injection or methanol extraction of tablets followed by oxidation with potassium permanganate in an aqueous bicarbonate buffer. Oxidation is complete within 15 min at room temperature after which the reaction is quenched with acid, the products extracted into isoocctane, and the absorbance of the product at 265 nm is measured. The molar absorptivities given in Figure 11 show that a fourfold gain in assay sensitivity results from measuring the oxidation product at 251 nm in hexane instead of the parent compound at 238 nm. Wallace and Dahl⁷¹ described application of this procedure to the analysis of amitriptylene in biological samples and tentatively identified the oxidation product as dibenzosuberone(XXXII), but Bouche⁷² later showed it to be anthraquinone(XXXIII).



XXXI

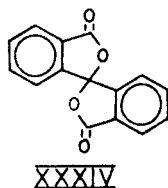


XXXII

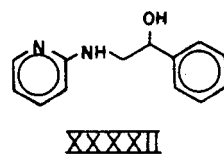
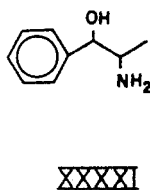
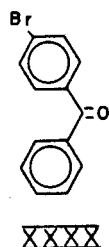
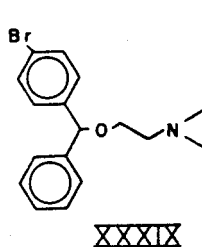
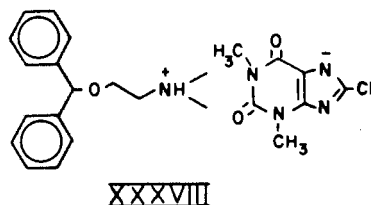
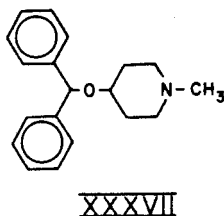
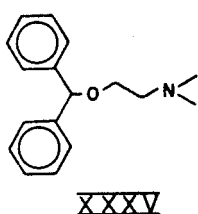


XXXIII

It is often difficult to find conditions where an oxidation reaction of this type will be complete and relatively free of side reactions. Hamilton et al.⁷³ investigated several oxidizing agents including acidic dichromate, alkaline periodate, barium peroxide, and acidic cerium(IV) in an attempt to improve the analysis of amitriptylene. Only cerium(IV) yielded satisfactory results. Missen and Stone⁷⁴ showed that the product of the cerium(IV) and dichromate oxidations was dibenzosuberone rather than anthraquinone. Missen and Stone⁷⁴ also demonstrated that dichromate was not a satisfactory oxidizing agent because 3,3'-spiro-biphthalide(XXXIV) was produced in variable amounts in addition to dibenzosuberone. Dibenzosuberone gives much better response than amitriptylene in an electron capture detector, so gas chromatography with an electron capture detector can be used in place of spectrophotometric measurement when extreme sensitivity is required (25 to 50 pg on column).^{74, 75}



A variety of oxidizing agents have been used in the analysis of other drugs. Wallace et al.⁷⁶ used 0.1 *N* potassium dichromate in 10 *N* sulfuric acid to oxidize diphenhydramine(XXXV) to benzophenone(XXXVI) which was steam distilled and measured at 257 nm. The method was also applicable to diphenylpyraline(XXXVII), dimenhydrinate(XXXVIII), and bromodiphenhydramine(XXXIX), although the latter compound yields 4-bromobenzophenone(XXXX) which is measured at 267 nm rather than 257 nm. The yield of benzophenone was estimated to be 96% in the case of diphenhydramine.



Heimlich et al.⁷⁷ used periodate oxidation for the measurement of phenylpropanolamine(XXXXXI) in urine, and Wallace⁷⁸ used the same oxidant for ephedrine (VII), pseudo-ephedrine and phenylamidol(XXXXXII), as well as phenylpropanolam-

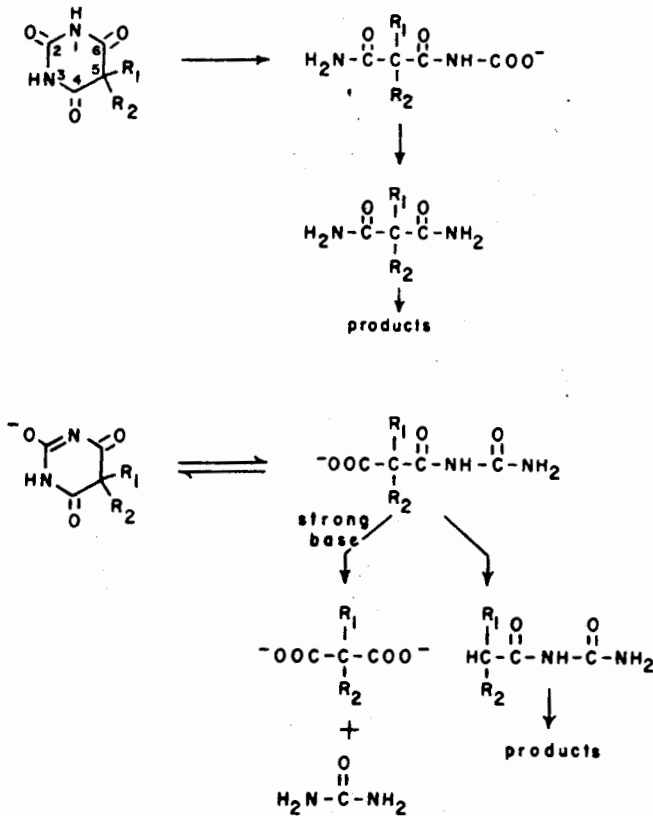
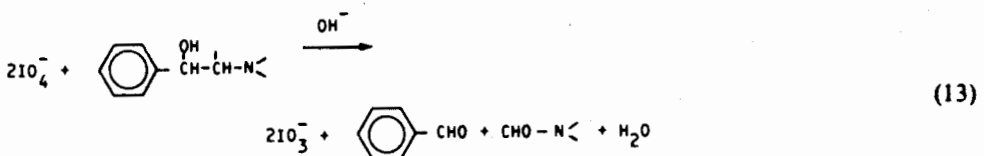
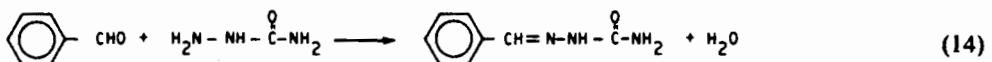


FIGURE 10. Proposed scheme for hydrolysis of barbituric acid derivatives.⁶³ Cleavage of unionized barbituric acid at the 1-6 or 3-4 bonds may also occur, but has not yet been demonstrated.⁶³

ine in biological samples. The product of the reaction in all cases is benzaldehyde, which can be measured at 240 nm in hexane:



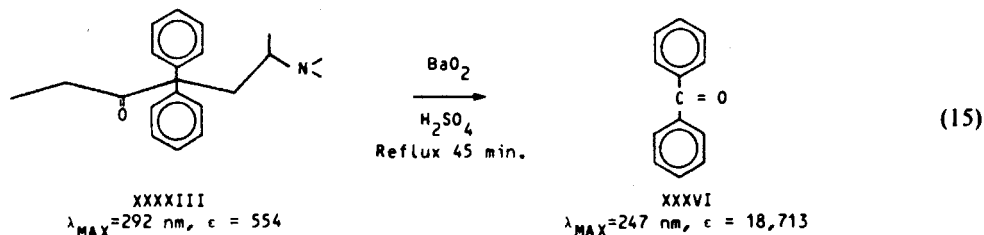
Wallace⁷⁸ increased the sensitivity of the spectrophotometric measurement approximately tenfold by reacting the benzaldehyde with semicarbazide before measurement to obtain the semicarbazone:



which can be measured at 278 nm in pH 4.0 acetate buffer. Chafetz⁷⁹ employed periodate oxidation in the analysis of several phenethanolamines in pharmaceutical prepara-

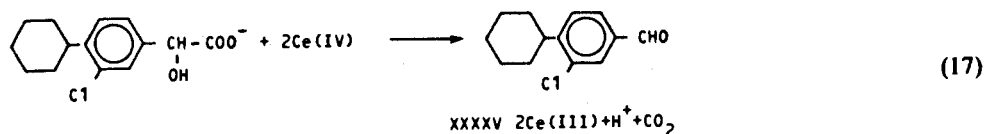
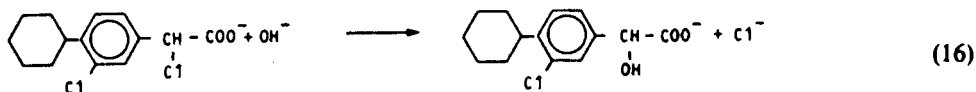
tions. Ephedrine has also been analyzed spectrophotometrically after oxidation with acidic dichromate.⁸⁰

Methadone(XXXXIII) has been analyzed by oxidation with barium peroxide in 4.7 *M* sulfuric acid:⁸¹

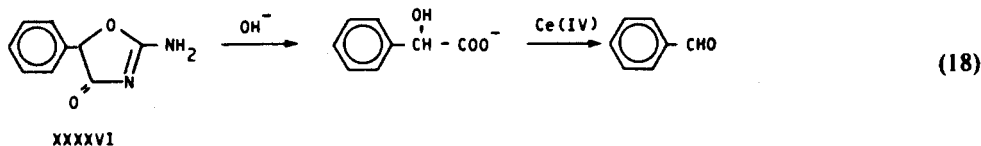


The reflux is carried out in a two-phase system composed of the sulfuric acid/ BaO_2 /methadone solution and heptane. Benzophenone is extracted into the heptane layer as the oxidation proceeds.

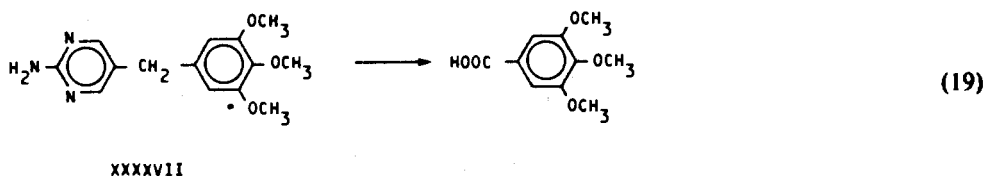
Lee et al.⁸² reported a procedure for Fenclorac(XXXXIV) in serum in which the α -chlorine is first displaced by hydroxide (0.1 *N*) and the resulting α -hydroxyphenyl group is oxidized to a substituted benzaldehyde using 0.2 *M* cerium(IV) in 7 *M* sulfuric acid:



The 3-chloro-4-cyclohexylbenzaldehyde(XXXXV) is then measured at 252 nm. Cummins and Perry⁸³ used a similar procedure to analyze pemoline(XXXXVI) by conversion to benzaldehyde:

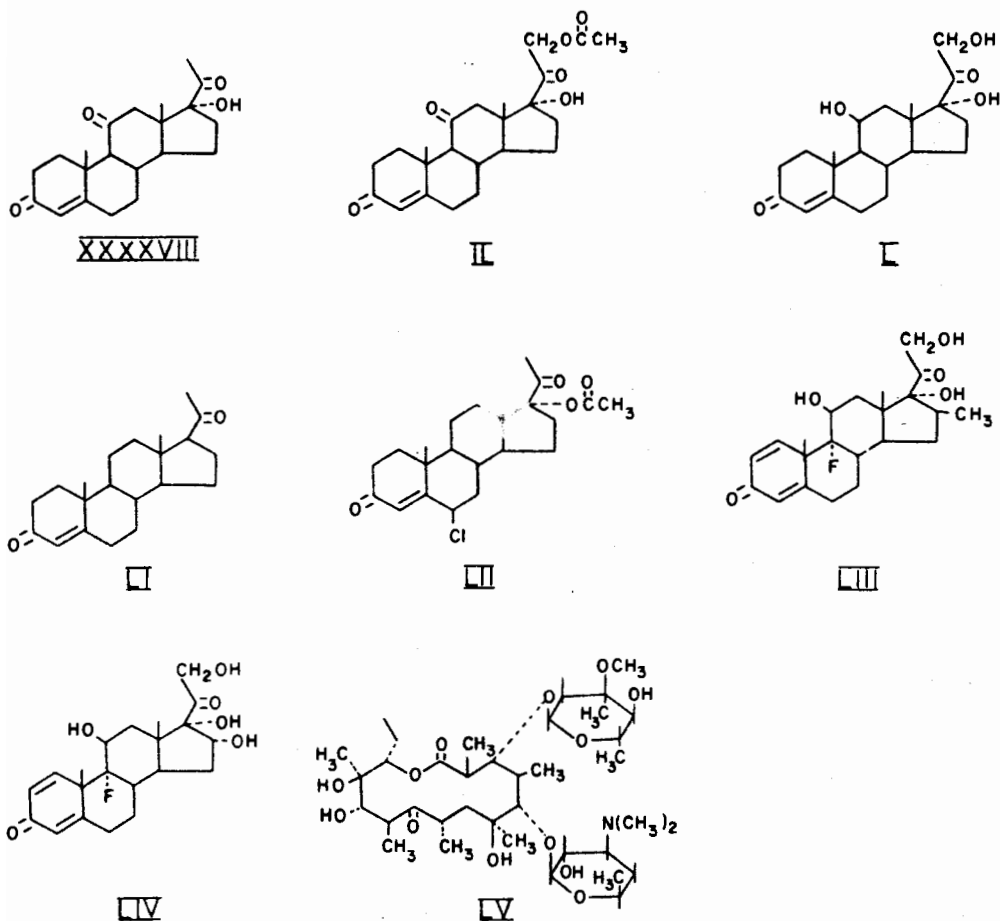


Potassium permanganate has been used to oxidize trimetheprim(XXXXVII) to 3,4,5-trimethoxybenzoic acid^{84,85} for measurement in biological samples:



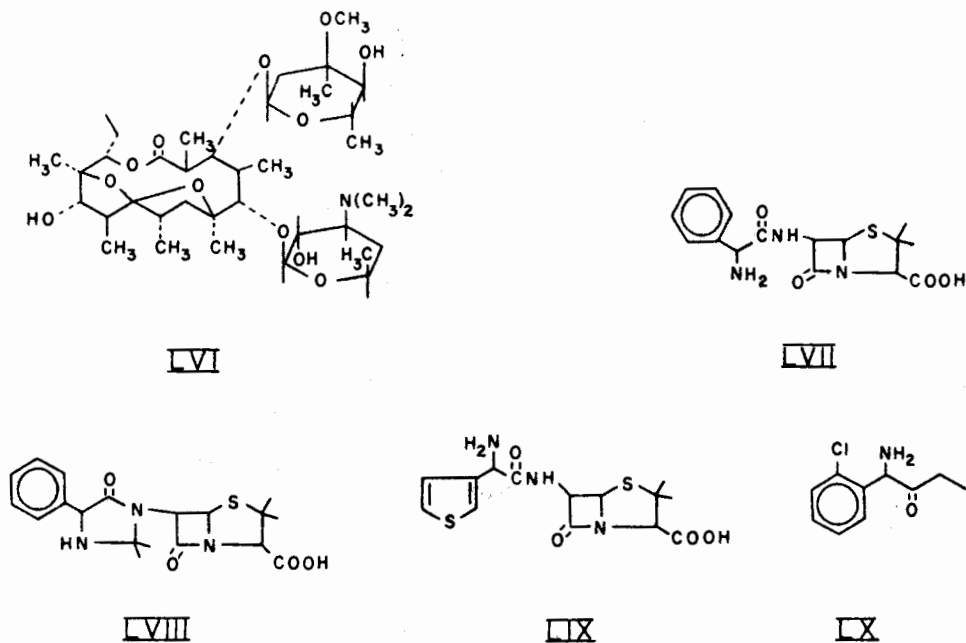
However the trimethoxybenzoic acid was measured spectrofluorometrically rather than spectrophotometrically in this case.

It is also possible to measure a substance by the change in absorbance when a chromophore is destroyed. The measurement of cephalosporin C using β -lactamase to destroy the chromophore has already been mentioned in Section V.B.1).⁴⁹ Steroids with the 4-en-3-one chromophore in the A ring have also been analyzed by using absorbance loss.⁴⁶⁻⁹¹ Gorog⁹⁰ found that 4-en-3-one steroids could be reduced quantitatively in 15 min at room temperature using a solution of sodium borohydride in methanol. The 1,4-dien-3-ones are much harder to reduce and require heating with alkaline sodium borohydride for 1 hr. Chafetz et al.⁹¹ found that lithium borohydride in tetrahydrofuran reduced 4-en-3-one, 1,4-dien-3-one, and 4,6-dien-3-one steroids in just 10 min at room temperature. Chafetz et al.⁹¹ tested their procedure on cortisone(XXXXVIII), cortisone acetate(XXXXIX), hydrocortisone(L), progesterone(LI), chlormadinone acetate(LII), betamethasone(LIII), and triamcinolone(LIV) with good results.



Many special techniques for developing chromophores in specific molecules are also available. For example, Kuzel et al.⁹² found that a chromophore ($\lambda_{MAX} = 236$ nm) can be developed quantitatively in erythromycin(LV) by warming it with aqueous base. Anhydroerythromycin(LVI), a hydrolysis product of erythromycin, does not interfere in the analysis. A correction can be made for other UV absorbing species in the sample by dividing it in two parts and gently hydrolyzing one part in the presence of sulfuric

acid to convert the erythromycin to anhydroerythromycin before developing the chromophore with alkali. The difference in absorbance of the hydrolyzed and unhydrolyzed portions at 236 nm is then a measure of the erythromycin originally present in the sample. Monteleone et al.⁹³ measured ampicillin (LVII) in the presence of hetacillin (LVIII) by forming an ampicillin-Ni(II) complex in dimethylsulfoxide and measuring its absorbance at 330 nm. Hetacillin, seven other penicillins, and eight related compounds were tested and only two of them (LIX and LX) caused interference. Another UV spectrophotometric procedure for ampicillin has been reported by Bundgaard⁹⁴ and Bundgaard and Ilver.⁹⁵ Bundgaard acetylated ampicillin with acetic anhydride and then formed the mercuric mercaptide which is measured at 325 nm. The reaction scheme is shown in Figure 12. An alternate procedure was also described in which ampicillin is measured in the presence of its degradation and polymerization products.⁹⁴



Other UV spectrophotometric methods which are specific for simple organic functional groups but which will not be discussed here include formation of oximes, semicarbazones, and hydrazones from aldehydes, ketones, semicarbazines, and hydrazines; alkoxymercuration of alkenes and alkynes; and formation of borate complexes with sugars and sugar derivatives.

C. Colorimetric Methods of Analysis

1. Direct Methods

A few compounds of pharmaceutical interest absorb visible light strongly enough that direct colorimetric measurements can be used to quantitate them. Several examples from this group are presented in Table 16. The principal problem encountered in devising a direct colorimetric analysis is separation of the analyte from potential interferences. As with direct UV methods, the separations are usually accomplished by extraction, thin layer chromatography, or column chromatography. However, the potential for interference is less in colorimetric than in UV measurements, since relatively

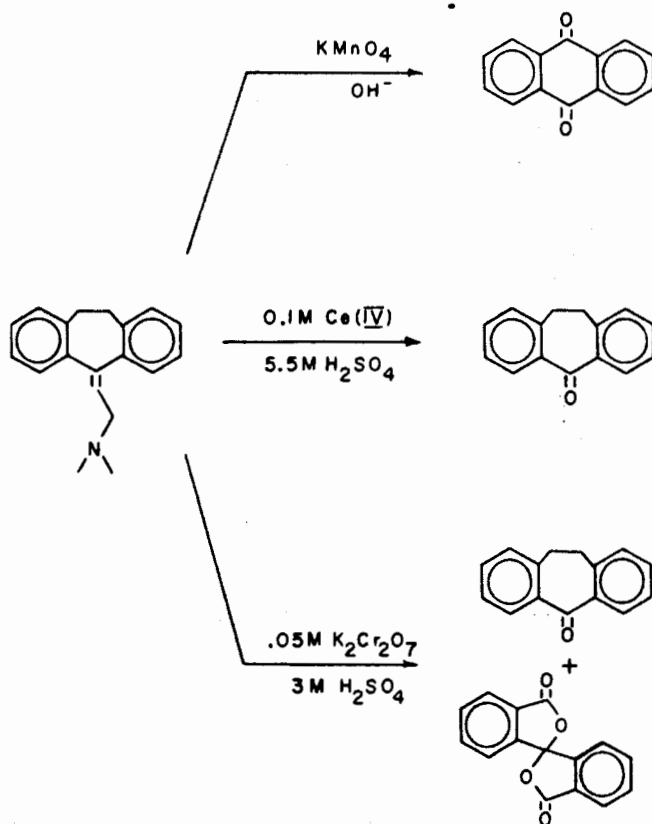


FIGURE 11. Oxidation of amitriptyline. Amitriptyline: λ_{max} (ethanol) = 238 nm, ϵ_{238} = 13,800 l mol⁻¹cm⁻¹. 9,10-Anthraquinone: λ_{max} (ethanol) = 252 nm, ϵ_{252} = 42,658 l mol⁻¹cm⁻¹. Dibenzosuberone: λ_{max} (hexane) = 251 nm, ϵ_{251} = 56,234 l mol⁻¹cm⁻¹.

few substances absorb at visible wavelengths, and therefore measurements can be made without separation in many cases.

2. Indirect Colorimetric Methods: Chemical Production or Modification of a Chromophore

A chromophore can be introduced into or prepared from a substance of almost any chemical class using relatively simple chemical procedures. The availability of procedures for most classes of compounds combined with the sensitivity and precision of spectrophotometric methods has led to widespread use of colorimetry in pharmaceutical analysis. The discussions in the following sections will be limited to colorimetric techniques for relatively complex structures, such as substituted aromatic molecules or steroids, where a large part of the molecule participates in the chromophore.

a. Aromatic Substitution Reactions

i. Electrophilic Aromatic Substitution

a. Orientation and Reactivity

Electrophilic aromatic substitution reactions are widely used in the analysis of substituted aromatic compounds and include many of the most familiar colorimetric methods. A brief summary of the mechanism of this class of reactions will be helpful in

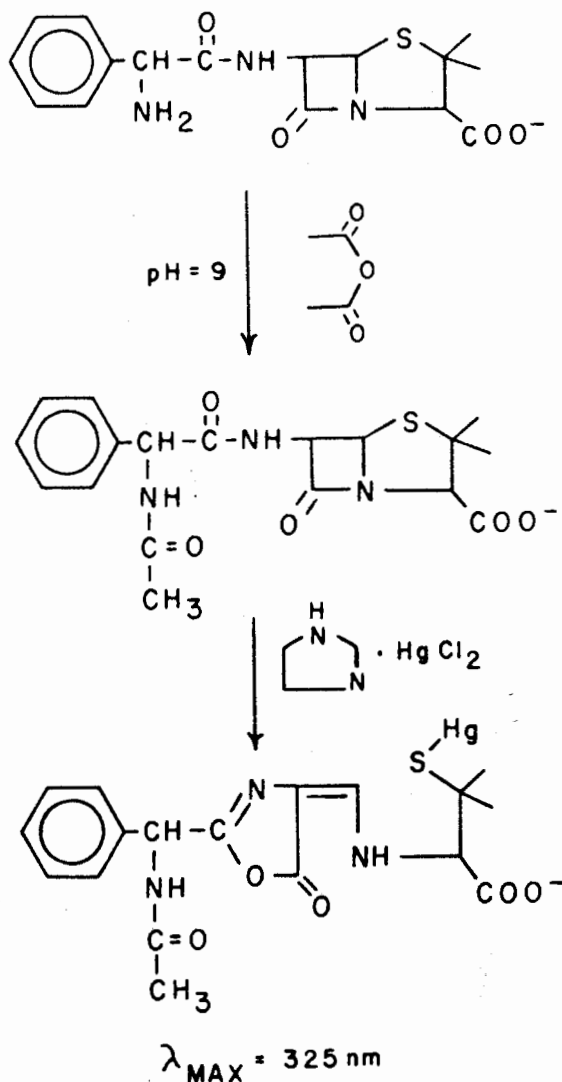
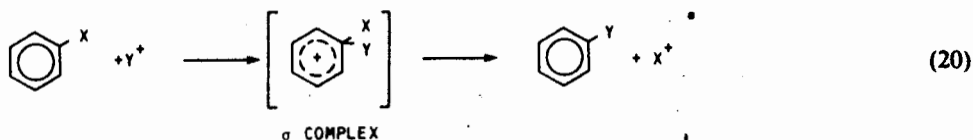


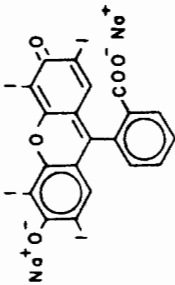
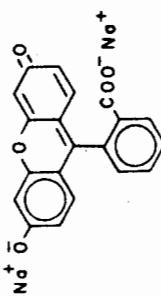
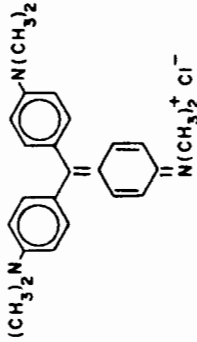
FIGURE 12. Reaction scheme for spectrophotometric measurement of ampicillin.⁶⁴

developing new colorimetric methods of analysis for drugs containing substituted aromatic groups. The reaction mechanism may be represented as:



The electrophile, Y^+ , is usually a cation as indicated in the equation, but may also be the positive end of a dipolar species.^{96,97} Typical electrophiles are I^+ , Cl^+ , Br^+ , SO_3^+ , NO_2^+ , $\text{R}^+\text{C}\equiv\text{O}$, and $\text{Ar}-\text{N}\equiv\text{N}^+$. The substituted benzene shown in the equation may be replaced by a variety of substituted aromatic hydrocarbons or aromatic heterocyclic

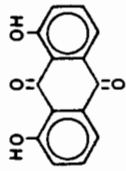
Table 16
PHARMACEUTICALS ABSORBING VISIBLE LIGHT

Compound	λ_{MAX} , nm	ϵ , l moles ⁻¹ cm ⁻¹	Solvent	Ref.
 <p>Erythrosine sodium</p>	531		Ethanol	134
 <p>Fluorescein sodium</p>	494		Water	134
 <p>Gentian violet (Crystal violet)</p>				134

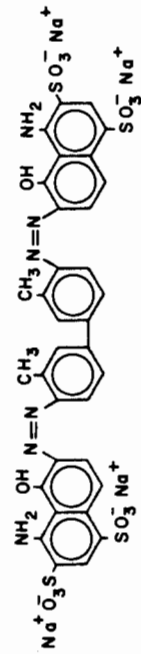
44 Chloroform

4,350
4,600

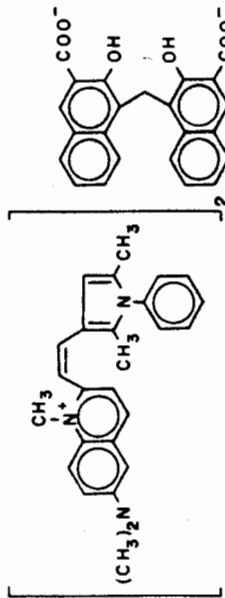
430
250



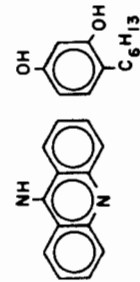
Danthron



Evan's blue



Pyrvinium pamoate



Acrisorcin

134

134

44

134

236
356
503

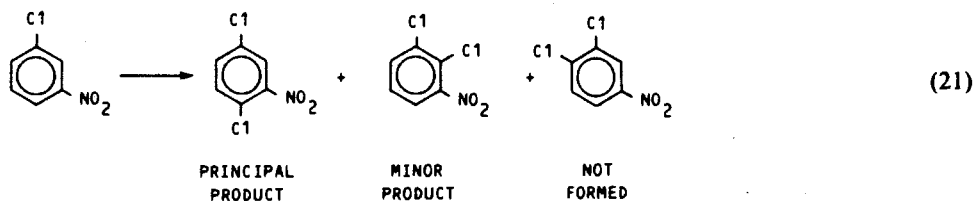
Table 16 (continued)
 PHARMACEUTICALS ABSORBING VISIBLE LIGHT

Compound	λ_{MAX} , nm	ϵ , l moles ⁻¹ cm ⁻¹	Solvent	Ref.
<p>Methylene blue</p>				134
<p>Sulfobromophthalein sodium</p>				134
<p>Phenolsulfonphthalein</p>				44

compounds. The leaving group, X, is a hydrogen atom in most analytical applications of this reaction. A discussion of reactions involving other leaving groups may be found in March,⁹⁷ and in other standard texts.

Attack of the electrophile on the ring is much slower than the subsequent displacement of the leaving group. Since the electrophile is a positive species, it will react most rapidly at the ring position with the greatest electron density, assuming steric factors are the same at all ring positions. Ring substituents affect both the magnitude and distribution of electron density and therefore control the overall rate and orientation of electrophilic substitution reactions. An electron releasing group such as $-\text{NH}_2$ or $-\text{OH}$ on a benzene ring increases the electron density at all ring positions, but increases the density at the *ortho* and *para* carbons by the greatest amount. Therefore $-\text{OH}$ and $-\text{NH}_2$ direct electrophilic substitution to the positions *ortho* and *para* to their own position on the ring. Electron withdrawing groups such as $-\text{NO}_2$ or $-\text{COOH}$ on a benzene ring decrease the electron density at all ring positions but again affect the *ortho* and *para* positions most, thus leaving the greatest electron density at the *meta* position. Therefore $-\text{NO}_2$ and $-\text{COOH}$ groups direct electrophilic substitution to the carbons *meta* to their own position on the ring. The effects of a number of common substituents on electrophilic substitution in the benzene ring are summarized in Table 17. An alternate explanation of orientation and reactivity in electrophilic substitution on the benzene ring has been given by March⁹⁷ in terms of the stability of the intermediate σ complex. March's approach leads to the same conclusion in the case of common substituents, but provides a better understanding of results obtained with less common substituents and heterocyclic systems.


The effects of multiple substituents on the reactivity of a benzene ring can also be predicted with reasonable confidence since the effects of substituents are approximately additive. Thus addition of an activating group to any benzene derivative will result in increased reactivity in electrophilic substitutions, while addition of a deactivating group will result in lowered reactivity. A strong activating group combined with a deactivating group results in a moderately activated ring system. A strong activating group will also dominate other groups and control the orientation of substitution. Thus substitution will occur *ortho* and *para* to a strong activating group when it is present on a ring containing weaker activating groups or deactivating groups. Steric factors also have a strong effect on the orientation of reaction in multiply substituted rings. Thus the position between two substituents which are oriented *meta* to one another is the least likely position for attack in an electrophilic substitution. When an *ortho*, *para* directing group and a *meta* directing group are *meta* to one another on the ring, substitution almost always occurs *ortho* to the *meta* directing group.^{97,99} Thus⁹⁷



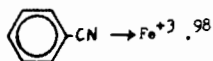
Quantitative treatment of reactivity and orientation in electrophilic aromatic substitution can also be made using Hammett equations,^{97,100,101} but quantitative estimates of rates are not usually required in the design of analytical procedures.

The carbons in fused ring and heterocyclic systems are not all equivalent and certain orientations are preferred in electrophilic substitution in these systems even in the ab-

Table 17
 SUBSTITUENT EFFECTS ON ELECTROPHILIC SUBSTITUTION
 ON THE BENZENE RING


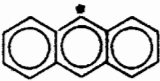

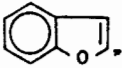



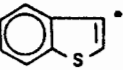


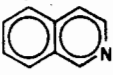
Activating, and <i>ortho, para</i> directing		Deactivating and <i>meta</i> directing		Weakly deactivating, <i>ortho, para</i> directing
O^- $-NR_2$ $-NHR$ $-NH_2$ $-OH$	} Strong	NR_3^+ $-NO_2$ $-CN^a$ $-SO_3H$	} Strong	$-Cl$ $-Br$ $-I$ $-F$ (Very little effect)
$-OR$ $-NHCR$ $-OCR$ $-NO$ $-SR$	} Moderate	$-CHO$ $-CR$ $-COOH$ $-CNH-$		
 $-R$ $-COO^-$	} Weak	$-CCl_3$ $-CF_3$ $-NH_3^+$		
		Most positively charged groups		

^a The $-CN$ group becomes *o,p* directing and activating when complexed to iron.



sence of substituents on the rings. Furan, thiophene, and pyrrole are more reactive than benzene and substitutions in the 2-position are preferred. However, the rings are reactive enough that substitution in the 3-position will occur if the 2-position is blocked. Pyridine is much less reactive than benzene and substitution occurs preferentially at the 3-position. Naphthalene is more reactive than benzene and is attacked primarily at the 1-position. Anthracene is more reactive than either benzene or naphthalene and is substituted primarily at the 9-position. The preferred orientations for reaction for several ring systems are summarized in Table 18. As with benzene derivatives, the heterocyclic and fused ring molecules will be more reactive when substituted with an activating group and less reactive when substituted with a deactivating group (Table 17). However prediction of the orientation of the reaction is difficult and will not be

Table 18
 ORIENTATION OF ELECTROPHILIC AROMATIC SUBSTITUTION IN
 CONDENSED AND HETEROCYCLIC RING SYSTEMS

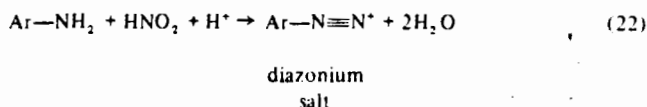
Compound	Structure	Principal position of substitution
Naphthalene		2
Anthracene		9
Furan		2
Benzofuran		2
Pyrrole		2
Indole		3
Thiophene		2
Benzothiophene		3
Pyridine		3
Quinoline		8
Isoquinoline		5

discussed here. Discussions of the reactivity and preferred orientation of reaction for a variety of ring systems can be found in References 102 to 105.

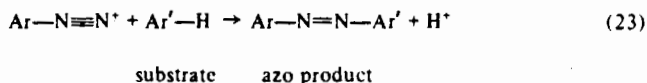
β. Diazotization and Coupling

Diazotization and coupling is used more widely in colorimetric analyses than any

other electrophilic aromatic substitution reaction. The reaction proceeds in two steps, diazotization:



and coupling:



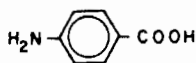
The azo compounds produced by the coupling reaction all absorb light in the visible region of the spectrum, but the wavelength and absorptivity depend on the structure of the aromatic groups, pH, and solvent. The absorptivity is usually large enough to allow concentrations of a few micrograms per milliliter to be measured. The sensitivity usually cannot be increased by employing fluorescence rather than absorbance measurements, because the azo group tends to quench the fluorescence of molecules that would otherwise be expected to fluoresce. The substrate, ArH, must contain a strongly activated aromatic ring if the reaction is to proceed rapidly and quantitatively. The usual substrates are therefore phenols, phenolate ions, or anilines, although phenyl ethers will couple satisfactorily with the more reactive diazonium salts. Satisfactory coupling also requires that an activated *ortho* or *para* ring position be available which is neither directly blocked by a substituent nor sterically hindered by a bulky substituent on an adjacent ring position.

Analyses based upon coupling with a diazonium salt may be divided into three groups: direct coupling of the analyte with a diazonium reagent, conversion of the analyte to a diazonium salt and coupling with an appropriate substrate, and conversion of the organic analyte to nitrous acid which is then determined by the coupling reaction.

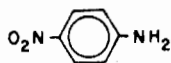
a.i. Direct Coupling of the Analyte with a Diazonium Salt

The diazonium salts derived from *p*-aminobenzoic acid(LXI),¹⁰⁶ *p*-nitroaniline(LXII),¹⁰⁷ *p*-sulfanilic acid (Pauly Reagent, LXIII),¹⁰⁸⁻¹¹² 2-aminobenzothiazole(LXIX),^{113,114} 3-phenyl-5-nitrosamine-1,2,4-thiadiazole(LXX),^{115,116} and 4-amino-6-chloro-1,3-benzene-disulfonamide(LXXIa)¹¹⁷ are commonly used reagents for direct coupling procedures. The arylamine coupling reagents are converted to their diazonium salts by reacting them in 1 to 2 M HCl with excess sodium nitrite. The reaction is usually carried out in an ice bath, the excess nitrite is removed by reaction with sulfamic acid or ammonium sulfamate, and the pH is adjusted for the coupling reaction. The reagent is used immediately since most diazonium salts are not stable. The diazotization proceeds quite rapidly with the substituted anilines in the preceding reagent list, being complete in 5 to 10 min in most cases. Usually 30 to 40 min are required for diazotization of 2-aminobenzothiazole, but the resulting 2-diazobenzothiazole is a very reactive diazonium salt and can be used to analyze a broader range of compounds than can the diazobenzenes derived from the aniline-type coupling reagents. 2-Diazobenzothiazole can also be produced by treating Compound LXX with 30 to 35% perchloric acid in ethanol,¹¹⁵ which offers the advantages of both rapid reaction and high reactivity. Several aryldiazonium fluorborates, including *p*-nitrobenzenediazonium

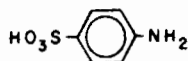
fluoroborate(LXXIb) are stable solids¹¹⁸⁻¹²⁰ and do not need to be prepared fresh for each analysis.^{121,122} The aryldiazonium fluoborates react more slowly than other diazonium salts and are only useful for the analysis of the more reactive substrates. The relative reactivities of ring substituted aryldiazonium salts can be predicted from the fact that groups that activate the ring toward electrophilic substitution (see Table 17) deactivate the corresponding diazonium ion toward coupling. The effects of several substituents on the rate of coupling of *para* substituted phenyldiazonium ions are given in Table 19.¹²³



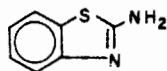
LXI



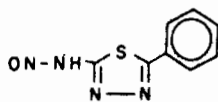
LXII



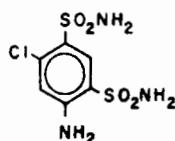
LXIII



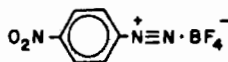
LXIX



LXX



LXXIa



LXXIb

The most common source of interference in analyses using the direct coupling procedure is impurities in the sample which also couple with the diazonium salt and exhibit some absorbance at the wavelength chosen for the analysis. Interferences of this type can only be avoided by employing a separation step prior to color development. Although diazonium salts undergo a wide variety of reactions, most side reactions will not lead to colored products and therefore will not affect the assay results provided a sufficient excess of reagent is used to assure complete conversion of the analyte to the coupled product.

Several examples of the analysis of drugs by direct coupling with a diazonium salt are listed in Table 20.

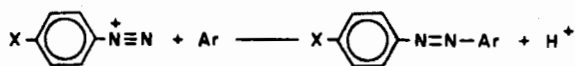
αii. Diazotization of the Analyte and Coupling

The second group of diazo coupling reactions are those in which the analyte is converted to a diazonium salt and then coupled to a substrate before measurement. Procedures of this type are encountered much more frequently than direct coupling procedures in pharmaceutical analysis. The most common substrates are (1-naphthyl)ethylenediamine (Bratton-Marshall Reagent, LXXII)¹³¹ and 2-naphthol(LXXIII). LXXII is preferred for quantitative work because the products are usually soluble and have high molar absorptivities. LXXIII often forms insoluble cou-

Table 19
RELATIVE COUPLING RATES FOR *PARA*
SUBSTITUTED PHENYLDIAZONIUM IONS¹²³

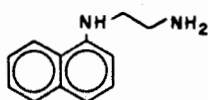
X	Relative rate
-OCH ₃	0.1
-CH ₃	0.4
-H	1
-Br	13
-SO ₃ ⁻	13
-NO ₂	1300

Reaction:

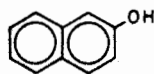


Note: The substrates, Ar, tested included 1-naphthol-4-sulfonate, 1-naphthol-3,8-disulfonate, 2-naphthol-6,8-disulfonate and 2-naphthol-3,6-disulfonate.³⁷⁴

pling products and is more frequently used for qualitative identification tests. When the substance being analyzed is diazotized, optimization of the reaction conditions and times becomes especially important because the diazonium salts are generally unstable and any loss through decomposition or side reactions will decrease the sensitivity and precision of the analysis.



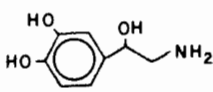
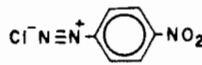
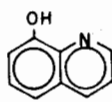
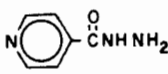
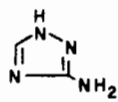
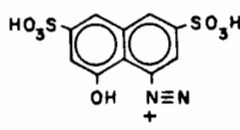
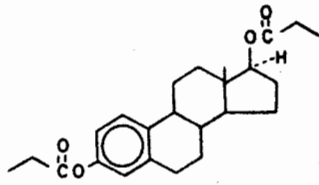
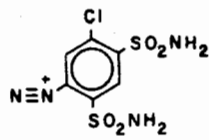
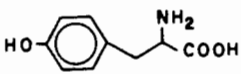
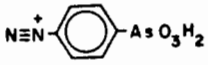
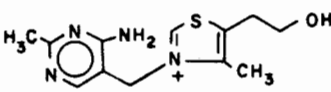
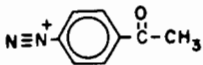
LXXII



LXXIII

The reaction of aromatic amines with nitrous acid to form diazonium salts is very general and can be carried out regardless of other ring substituents. The mechanism of the reaction^{132,133} is outlined in Figure 13. The effective nitrosating agents under normal reaction conditions are the nitrous acidium ion, nitrosyl salt, and dinitrogen trioxide. The reaction must be carried out in an acidic medium in order to have appreciable concentrations of these species present. Even though an acidic medium is used, the nitrosating agent couples with the small amount of free amine present rather than with the amine salt. Consequently the reaction may be slowed in an excessively acidic solution due to the reduction in free amine concentration. The effect of pH on the reaction rate can be used to achieve diazotization of an arylamine in the presence of an aliphatic amine without reaction of the latter. Below pH 3.0 the more basic aliphatic amine will be protonated to such an extent that it will not react with the nitrosation

Table 20
ANALYTICAL APPLICATIONS OF DIRECT COUPLING OF THE DRUG
WITH A DIAZONIUM SALT

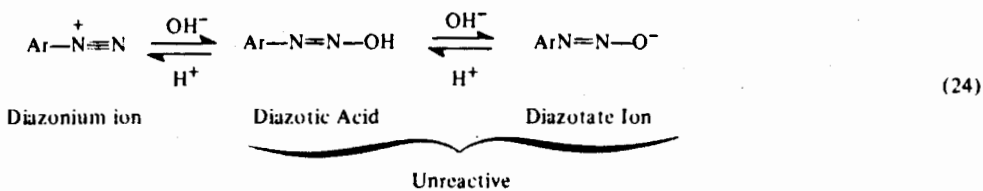
Drug substance	Coupling agent	λ_{MAX}	Ref.
 Levarterenol	 <i>p</i> -Nitrophenyldiazonium chloride	640 nm	124
 8-Hydroxyquinoline	<i>p</i> -Nitrophenyldiazonium chloride	~600 nm	125
 Isoniazid	<i>p</i> -Nitrophenyldiazonium Fluoroborate		126
 3-Amino-1H-1,2,4-triazole	 1-Naphthol-8-diazo-3,6-disulfonic acid	530 nm	127
 Estradiol dipropionate	 4-Diazo-6-chloro-1,3-benzenedisulfonamide		117, 128
 Tyrosine	 <i>p</i> -Diazobenzenearsenic acid	480 nm	129
 Thiamine	 <i>p</i> -Acetylphenyldiazonium ion		130

reagent while there will still be a sufficient concentration of the less basic, free arylamine for the diazotization to proceed. Note that the reaction of a primary aliphatic amine with nitrous acid does not yield a stable diazonium salt, but rather a diazo compound which rapidly decomposes to a variety of products: secondary amines react to give

relatively stable N-nitroso compounds, but these do not couple with the aromatic substrate.

Formation of the diazonium salt is usually fast enough that any convenient pH between 0 and 3 can be used. In cases where the reaction is unusually slow or where the diazonium salt is unusually labile, it might be well to optimize the pH in order to increase the reaction rate and minimize the effects of decomposition. The reaction rate is also increased if the pH is adjusted with hydrochloric rather than sulfuric acid, since NOCl is a better nitrosating agent than NOHSO₄. The rate can be increased even further by adding NaBr or KBr, presumably due to the formation of NOBr which is an even better nitrosating agent than NOCl.¹³³

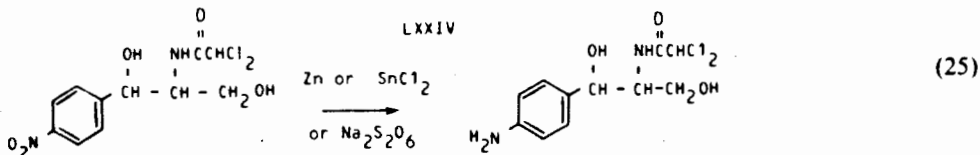
The coupling reaction requires a polar solvent to accommodate the ionic intermediates, and water and ethanol are most frequently used. Careful control of solvent pH is very important in achieving rapid, quantitative reaction. Since only the free arylamine and phenolate anion are active in coupling, too low a pH inhibits the reaction by converting arylamines to anilinium ions and phenolate ions to neutral phenols. Thus the arylamines will react most rapidly above pH 5 and phenols require as high a pH as possible. However, at pH 9 and above the diazonium ion is converted to unreactive species according to the following equilibria:

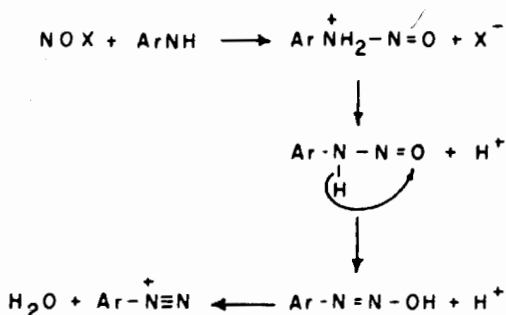
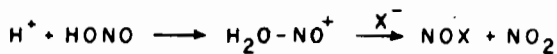


Therefore coupling to amine substrates should be carried out between pH 5 and pH 9, and phenols between pH 9 and pH 10. The exact pH must be determined experimentally in each case. Since the diazonium salt is formed at low pH, a pH adjustment must be made between the diazotization and coupling steps.

The spectrophotometric measurement of the coupling products of diazotized sulfonamides with LXXII (Bratton-Marshall Reagent) has been studied in detail.^{145,146} The absorption maximum is near 545 nm for coupled sulfonamides and maximum absorption intensity is achieved in the pH range 1 to 2. The maximum molar absorptivity is on the order of $5 \times 10^4 \text{ l mol}^{-1}\text{cm}^{-1}$. The absorptivity decreases in solutions with high salt concentration. Beer's Law is not followed over wide concentration ranges so special care must be taken to prepare a calibration curve using a matrix similar to the sample matrix and covering the full range of concentrations expected for the samples.

A few examples of the use of diazotization and coupling for the analysis of drugs containing an aromatic amine group are given in Tables 21 and 22. Specificity is achieved in most applications by including extraction or chromatographic separation prior to the coupling reaction, although separation after coupling should also be possible in many cases. A number of drugs have also been determined by first converting them to a diazotizable species which is then coupled in the usual manner. Chloramphenicol (LXXIV) contains a nitro group which can be reduced to an amine with metallic zinc, stannous chloride, or sodium dithionite. Reduction to the amine followed by the Bratton-Marshall:



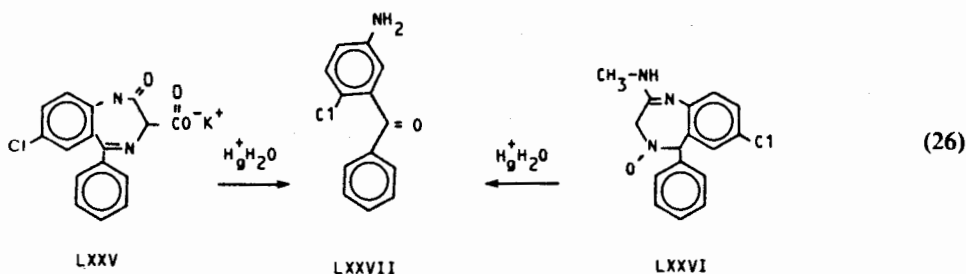


Nitrosating Agents

↓ Decreasing Reactivity ↓	NO^+	nitrosonium ion
	H_2O-NO^+	nitrous acidium ion
	$Br-NO$	nitrosyl bromide
	$Cl-NO$	nitrosyl chloride
	NO_2-NO	dinitrogen trioxide
	$HONO$	nitrous acid

FIGURE 13. Mechanism for diazotization of aromatic amines.^{132,133}

reaction has been used for the analysis of this drug.¹⁴⁷⁻¹⁴⁹ Chlorazepate potassium (LXXV)¹⁵⁰ and chlordiazepoxide(LXXVI) can be analyzed by hydrolyzing them in strong acid to 2-amino-4-chlorobenzophenone(LXXVII) which is then quantitated by the Bratton-Marshall reaction.



α.iii. Determination through Conversion of the Analyte to Nitrous Acid

Nitrous acid can be determined by using it to diazotize an aromatic amine, coupling the diazotized amine with a suitable substrate, and measuring the product spectrophotometrically. The Griess method for nitrite employs sulfanilic acid (LXIII) as the source of coupling agent and α -naphthylamine(LXXVIII) as the substrate.¹⁵¹ This reaction has been used for analysis of nitrite in pharmaceutical preparations including gel-

Table 21
APPLICATIONS OF DIAZOTIZATION OF A DRUG AND SUBSEQUENT COUPLING:
THE BRATTON-MARSHALL REACTION^a

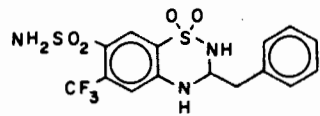
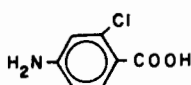
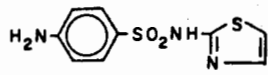
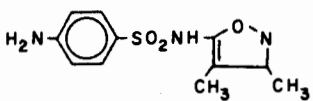
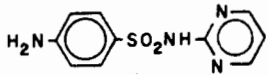
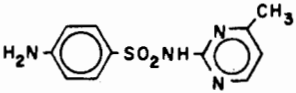
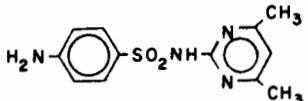
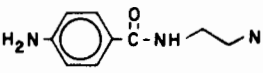
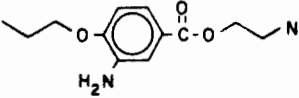
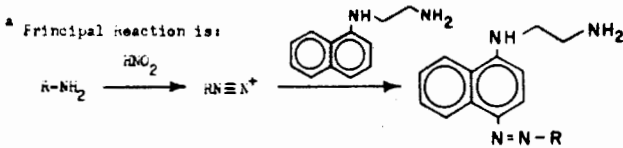
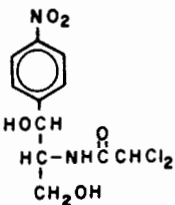
Compound	λ_{MAX} , nm	Ref.
 Bendroflumethiazide	515	128
 2-Chloro-4-aminobenzoic acid		128
 Sulfathiazole	550	128
 Sulfisoxazole	540	128
 Sulfadiazine	~545	134
 Sulfamerazine	~545	134, 137-140
 Sulfamethazine	~545	134
 Procainamide	550	135, 136

Table 21 (continued)
 APPLICATIONS OF DIAZOTIZATION OF A DRUG AND SUBSEQUENT COUPLING:
 THE BRATTON-MARSHALL REACTION^a

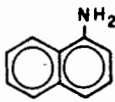
Compound	λ_{MAX} , nm	Ref.
	550	141



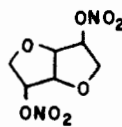
atin capsules, and can also be used for nitrate after a reduction step. Quantitative reduction of low levels of nitrate to nitrite is most easily accomplished over a coppered cadmium catalyst.¹⁵² However, the most important applications of this group of reactions are in the analysis of pharmaceutically important nitrate esters. Aliphatic nitrate esters hydrolyze quantitatively in alkaline solution at room temperature to nitrite ions, and dinitro and polynitro aromatic compounds hydrolyze in a similar fashion at elevated temperatures. Mononitro aromatic compounds do not hydrolyze appreciably under these conditions. A typical procedure will therefore consist of an alkaline hydrolysis, followed by acidification, diazotization of a coupling agent, and finally coupling with the substrate.¹⁵³



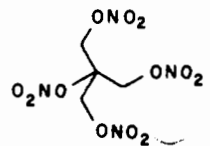
LXXIV



LXXVIII



LXXIX



LXXX

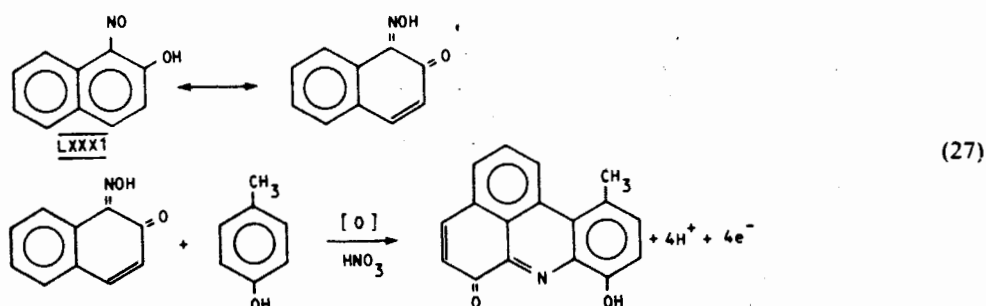
Isosorbide dinitrate(LXXIX) has been determined using sulfanilic acid and naphthylamine (Griess method), and also using *p*-nitroaniline as the coupling agent and azulene as the substrate.¹⁵⁵ *p*-Nitroaniline and azulene have also been used to analyze pentaerythritol tetranitrate(LXXX).¹⁵⁶

γ. Oxidative Coupling Reactions

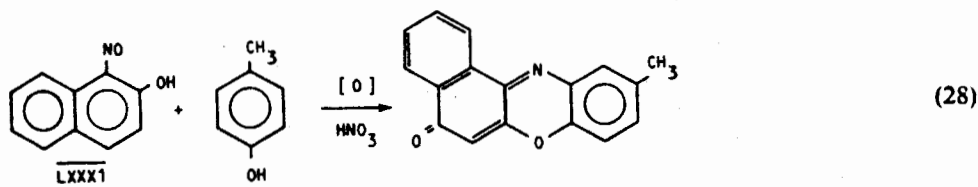
α.i. Coupling Phenols with 1-Nitroso-2-Naphthol

Phenols carrying groups activating them to electrophilic substitution can be coupled with 1-nitroso-2-naphthol(LXXXI) in nitric acid solution to form highly colored products. The reaction proceeds most rapidly with *p*-substituted phenols, but it is not specific for them as originally reported.¹⁵⁷ The mechanism of this reaction has not been

established. Anger and Ofri¹⁵⁸ proposed the following reaction:

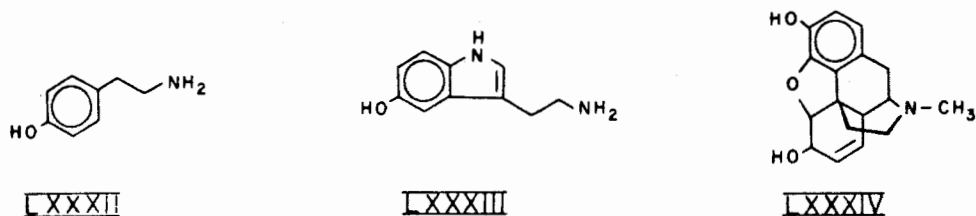


However, Umeda¹⁵⁹ isolated a phenoxazine rather than an acridine derivative from the reaction mixture and the net reaction therefore appears to be the following:



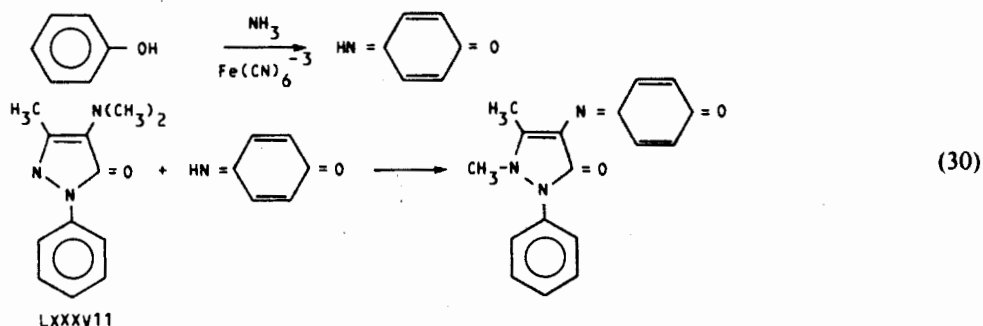
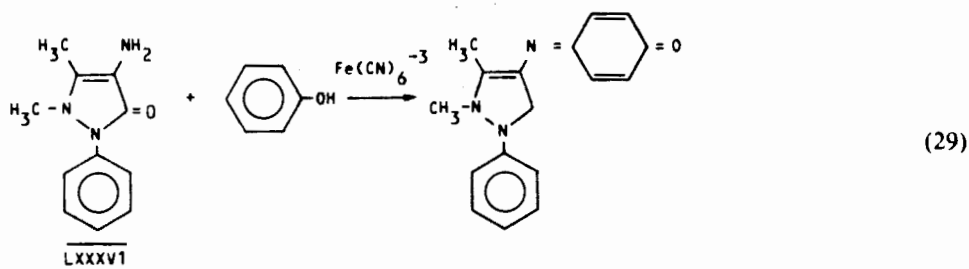
A typical analytical procedure based upon this reaction involves treatment of the sample with an excess of LXXXI prepared as a 2 to 3m *M* solution in ethanol or another relatively polar solvent. An equal volume of 0.01 *M* sodium nitrite in 3.5 *M* nitric acid is added and the solution is warmed (50 to 60°C) for 1 hr to effect the oxidation and coupling. The absorbance of the sample is measured after cooling. λ_{MAX} is usually between 450 and 500 nm for the products formed from simple phenols. Typical limits of detection are in the range of 1 to 20 $\mu\text{g/ml}$.

Coupling with LXXXI was originally used for the analysis of tyrosine in biological samples.^{157,160} Since then the reaction has been successfully employed with a large number of substituted phenols including tyramine(LXXXII),¹⁶¹ serotonin(LXXXIII),¹⁶² and morphine (LXXXIV),^{163,164} and hexestrol(LXXXV).¹⁶⁵ Bhansali¹⁶⁴ studied the reaction with 18 phenols and found only 3,4-dihydroxyphenyl compounds to be unreactive, but 3-hydroxyphenyl compounds gave products which did not absorb above 350 nm. Coupling with LXXXI is the official method of analysis for resorcinol in *U.S.P. XIX*.¹⁶⁶



α .ii. Coupling Phenols and Other Aromatics with 4-Aminoantipyrene or Aminopyrene

A variety of phenols can be oxidatively coupled with 4-aminoantipyrene (LXXXVI)¹⁶⁷⁻¹⁷² or aminopyrene(LXXXVII)¹⁷³ to give highly colored quinone imines as shown in Equations 29 and 30, respectively.



The reaction is usually carried out in an aqueous buffer at pH 8 to 9 with a tenfold or greater excess of LXXXVI or LXXXVII and potassium ferricyanide. The reaction mixture is allowed to sit at room temperature until color formation is complete and the absorbance of the product is measured (450 to 470 nm for LXXXVI, 490 to 510 nm for LXXXVII) directly on the aqueous solution or following extraction into chloroform. The limits of detection are usually in the range of 10 to 100 μg with aminopyrrole giving greater sensitivity than 4-aminoantipyrine.

The coupling reaction can be carried out with a wide variety of phenols,^{174,175} but is inhibited by the presence of deactivating groups on the ring. The presence of electron-withdrawing substituents also decreases the stability of the color formed.^{172,176} The position *para* to the hydroxyl must be free or contain a group such as a halogen, hydroxyl, or ether group.^{167,179} Thus compounds like *m*-cresol and α -naphthol react, while *p*-cresol, β -naphthol, salicylic acid and *p*-hydroxybenzoic acid do not react.

As suggested by Equation 30, interference in the analysis may arise from quinones or quinone-imines in the sample as well as from other phenols unsubstituted in the *para* position. Coupling to form a colored product may also occur with α,β -unsaturated ketones. LXXXVI is, in fact, frequently used for quantitative analysis of steroids^{176,177} having the 1-ene-3-one or 1,4-diene-3-one structures in the A ring: coupling with these steroids is carried out in acid solution at room temperature without an oxidizing agent. Interference in the analysis of phenols could also arise if the sample contained large quantities of a reducing agent.

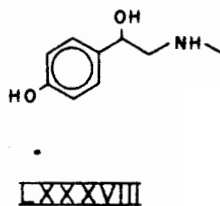
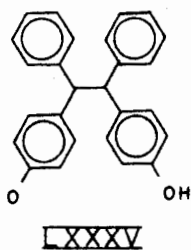
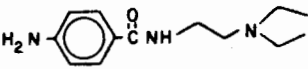
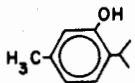
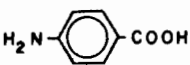
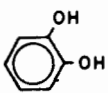
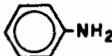
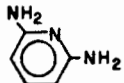
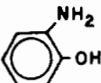
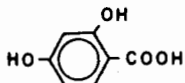
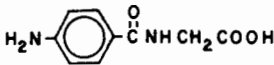
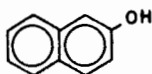
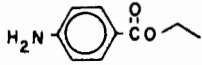
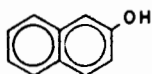
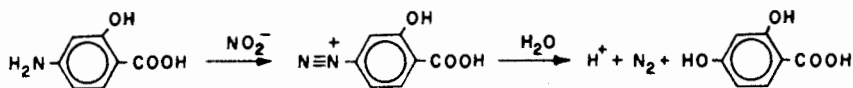


Table 22
 APPLICATIONS OF DIAZOTIZATION OF A DRUG AND SUBSEQUENT COUPLING:
 MISCELLANEOUS COUPLING AGENTS

Compound	Coupling agent	λ_{MAX} , nm	Ref.
 Procainamide	 Thymol		142
 <i>p</i> -Aminobenzoic acid	 Resorcinol	385	143
 Aniline	 2,6-Diaminopyridine	360	144
 <i>m</i> -Aminophenol	 2,4-Dihydroxybenzoic acid ^a	~430	128
 <i>p</i> -Aminohippuric acid	 β -Naphthol		134
 Benzocaine	 β -Naphthol		128

^a In the procedure reported in Reference 128, the 2,4-dihydroxybenzoic acid is generated *in situ* from *p*-aminosalicylic acid by the reactions:



Coupling with LXXXVI or LXXXVII has not been widely used in pharmaceutical analysis, except in the analysis of phenylephrine(LXXXVIII),^{181, 182} because most drugs having a phenolic hydroxyl group also have substituents *para* to the hydroxyl and therefore will not react. The usefulness of the reaction can, however, be extended to a variety of other compounds if they are converted to suitable phenols using the Hamilton hydroxylating^{183, 185} reagent prior to coupling.^{178, 186} The analyte is treated at pH 3.5 to 4.2 in an acetate buffer containing about 10^{-4} M catechol, 2×10^{-4} M ferric

perchlorate, $4 \times 10^{-3} M$ hydrogen peroxide, and 2 to $5 \times 10^{-1} M$ glucose at $70^\circ C$ for 10 min. The reaction mixture should be less than $10^{-3} M$ in the analyte. An aliquot is withdrawn at 10 min and added to an alkaline borate buffer to quench the reaction, and coupling is accomplished by adding aminoantipyrine and potassium ferricyanide to the borate buffer solution.¹⁷⁸ Glucose is used as a color stabilizing agent and can be replaced by a cyclodextrin, but reaction times are longer in the presence of cyclodextrins. The compounds tested by Albert and Connors¹⁷⁸ using this procedure are listed in Table 23. The modified Hamilton hydroxylation system can be used equally well to prepare phenols for coupling with other colorimetric reagents.

α .iii. Coupling Phenols and Arylamines with *p*-Diaminobenzenes (*p*-Phenylenediamines)

Phenols and arylamines couple under alkaline oxidizing conditions with *p*-diaminobenzenes to form indophenol (also called indaniline) and indamine dyes, respectively.^{186,187} This reaction has been used to analyze phenols,¹⁸⁶⁻¹⁹¹ diaminobenzenes,^{192,193} and arylamines.^{189,199} The absorption maxima for indophenols formed from simple phenols are generally between 580 and 650 nm, while the indamines absorb between 680 and 750 nm. Concentrations on the order of $10^{-5} M$ can usually be measured.

A typical procedure¹⁹¹ for analyzing phenols consists of mixing equal volumes of $10^{-3} M$ N,N-dimethyl-*p*-diaminobenzene in 0.01 *M* hydrochloric acid, 0.1 *M* pH 9 sodium carbonate buffer, $6 \times 10^{-3} M$ potassium ferricyanide, and a sample which is approximately $10^{-4} M$ in the unknown phenol. The reagents are added in the order given, allowed to sit for 1 min for color development, and then diluted 1:2 with ethanol prior to measurement. A solution which is $5 \times 10^{-3} M$ in both potassium ferricyanide and sodium dichromate should be substituted for the $6 \times 10^{-3} M$ potassium ferricyanide when analyzing arylamines, the pH reduced to 6, and the reaction time extended to 30 min.¹⁸⁹

The reaction involves oxidation of the *p*-diaminobenzene to a diiminium ion which then attacks the reactive arylamine or phenolate anion as shown in Figure 14.^{189,194,195} Potassium ferricyanide is frequently used as the oxidant since it oxidizes *p*-diaminobenzenes and N,N-dialkyl-*p*-diaminobenzenes rapidly and quantitatively to the corresponding *p*-benzoquinonediimines.^{196,197} However Kramer and Talentino¹⁸⁹ found ferricyanide unsatisfactory in the analysis of arylamines and recommended the use of dichromate or a mixture of dichromate and ferricyanide. The dichromate/ferricyanide mixture is also satisfactory for the analysis of phenols. Permanganate, hypochlorite, manganese dioxide, silver chloride, lead dioxide,¹⁹⁴ and persulfate¹⁹² have also been used as oxidants in the preparation of indophenol dyes but, with the exception of persulfate, have not been specifically evaluated for use in analytical applications of this reaction.

pH control is very important in this reaction. The pH must be high enough to assure a high concentration of the phenolate anion or free arylamine, but not so high that hydrolysis of the benzoquinone diimine to the monoimine^{196,198} becomes competitive with the coupling reaction. Corbett¹⁹¹ has shown that a pH between 9 and 10 will be best for most phenols. A pH of 6 is recommended for analysis of arylamines primarily to reduce hydrolysis of the indamine and thereby increase the stability of the color formed.¹⁸⁹

There are a number of potential sources of interference in analyses based upon this reaction. Since the reaction proceeds with a broad range of aromatic amines and phenols, the most likely source of interference is the presence of phenol and aniline-derived impurities in the sample which will cause a high bias in the analysis. If oxidation of the diaminobenzene is not complete, unoxidized reagent may react with aldehydes in

Table 23
 AROMATIC COMPOUNDS ANALYZED BY
 HYDROXYLATION AND COUPLING WITH 4-
 AMINOANTIPYRINE^{a, b}

Compound	Reaction time, min	Analytical wavelength, nm	$10^{-3}\epsilon_{\text{app}}$ ^c
Using the Glucose-Modified Hydroxylation Procedure			
Benzene	10	505	2.9
Anisole	10	505	4.4
Acetanilid	10	510	3.4
Phenacetin	10	490	3.1
Benzoic acid	10	495	1.7
<i>o</i> -Methoxybenzoic acid	10	493	3.7
<i>m</i> -Methoxybenzoic acid	10	493	2.8
<i>p</i> -Methoxybenzoic acid	10	485	5.5
Mandelic acid	10	500	1.3
Atropine	10	500	2.1
Ephedrine	10	505	2.2
Phenobarbital	10	515	1.3
Chloramphenicol	10	390	0.8
Chlorpheniramine	10	500	2.7
Using the Cyclodextrin-Modified Hydroxylation Procedure			
Acetanilid	120	510	3.9
Phenacetin	78	490	2.7
Aspirin	120	490	1.7
Benzenesulfonamide	120	505	1.1
Sulfanilamide	120	435	1.5
Chloramphenicol	120	390	0.7
Mandelic acid	120	500	2.1
L-Phenylalanine	60	495	2.6
Tropic acid	120	500	1.4
Atropine	120	500	2.6
Ephedrine	60	505	2.7
Phenobarbital	120	515	1.1
Antipyrine	120	500	0.9
2-Naphthalenesulfonic acid	120	510	0.6
L-Tryptophan	120	485	0.5
Quinine	120	490	0.5
Physostigmine	120	495	0.2

^a From Albert, K. S. and Connors, K. A., *J. Pharm. Sci.*, 62, 625, 1973. With permission.

^b Reaction conditions:

Glucose procedure — 1.17×10^{-4} M catechol, 5.94×10^{-5} M Fe(III), $3-4 \times 10^{-3}$ M H_2O_2 , 3.7×10^{-3} M glucose, pH 3.6—4.0, 10 min reaction time, 75°.

Cyclodextrin procedure — 1.17×10^{-4} M catechol, 5.94×10^{-5} M Fe(III), 3.4×10^{-3} M H_2O_2 , $1.0-1.3 \times 10^{-3}$ M β -cyclodextrin, pH 3.6—4.0, 25°.

^c Apparent molar absorptivity based upon total sample concentration regardless of fate, in the final colorimetric solution.

the sample to form Schiff bases, which in some cases will absorb light in the same wavelength region as the indophenol and indamine dyes. However, coupling with aldehydes usually proceeds most rapidly under slightly acidic conditions and will not be

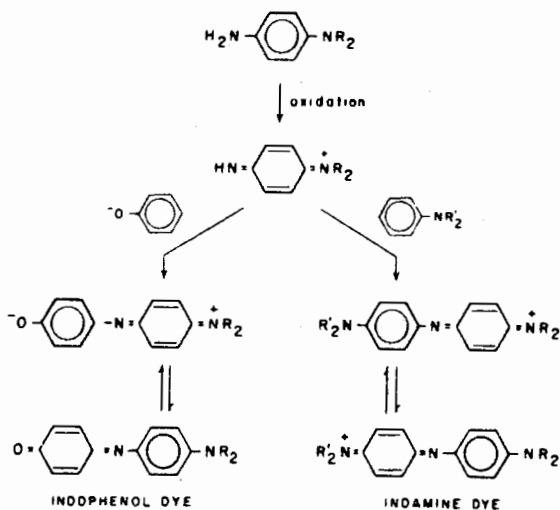
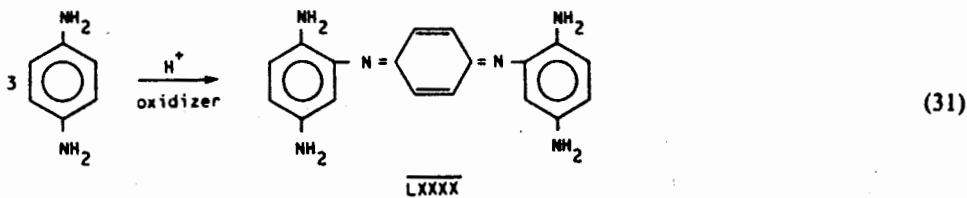


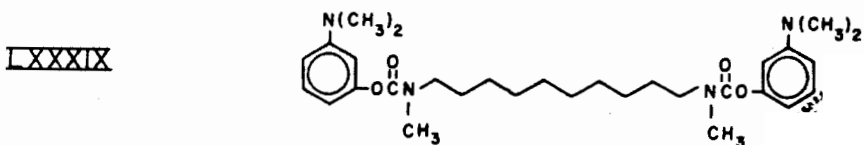
FIGURE 14. Oxidative coupling of phenols and arylamines with *p*-diaminobenzenes.

significant at the high pH values used in the indophenol reaction. If the oxidation is carried out under acidic conditions, it is also possible for the *p*-diaminobenzene to oxidatively couple with itself to produce Bandrowski's base (LXXXX)²⁰³ which absorbs at relatively long wavelengths

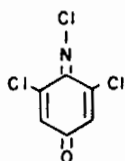


The presence of strong reducing agents in the sample can lead to low bias by reducing the quinone diimine before it couples or by reducing the product dye.

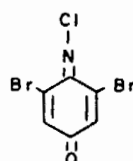
A variety of 2- and 3-substituted phenols and arylamines have been analyzed using this method. Specific procedures for phenol,^{188,189,191,192} thymol,^{190,191} *p*-aminophenol,¹⁹¹ 2,6-dimethylphenol,^{189,191} 2,6-dimethoxyphenol,¹⁸⁹ aniline,¹⁸⁹ *N*-methylaniline,¹⁸⁹ and *N,N*-dimethylaniline have been reported in the literature. The reaction is known to proceed with catechol, *o*-iodophenol, 3-cyanophenol, resorcinol, 2-aminopyridine, 3-hydroxypyridine, indole, and barbituric acid, but has not been used for quantitative analysis of these compounds. The color produced by coupling demarcium bromide(LXXXIX) with *N,N*-dimethyl-*p*-diaminobenzene after oxidizing it with hypochlorite is the basis of one of the identity tests for this substance in *N.F. XIV*. Coupling with thymol has been used for analysis of aryl amines,²⁰¹ and sulfonamides have been analyzed by coupling with phenol.²⁰³



Although analytical applications of this reaction have been restricted to *ortho* and *meta* substituted phenols and anilines, the reaction will proceed with certain *p*-substituted phenols. In particular, phenols substituted in the *para* position with $-Cl$, $-Br$, $-COOH$, $-SO_3H$, $-OR$, $-RCHOH$, or $RC=O$ will couple with displacement of the substituent group.²⁰⁰



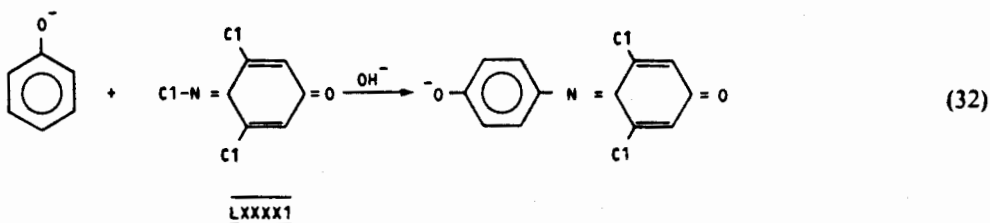
LXXXXI



LXXXXII

α .iv. Coupling Phenols with 2,6-Dichloroquinone Chlorimine and Related Reactions

One of the most sensitive colorimetric methods for the estimation of phenols is based upon coupling with 2,6-dichloroquinone chlorimine (LXXXXI), which is also known as Gibb's reagent.²⁰⁴⁻²⁰⁷ 2,6-Dibromoquinone chlorimine (LXXXXII) can also be used.²⁰⁸



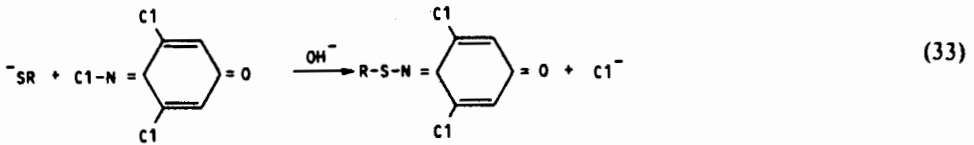
(32)

This reaction is not an oxidative coupling, but is discussed here because it is very closely related to the coupling reactions of aminopyrine, *p*-diaminobenzene, and other reagents in which species similar to LXXXXI are formed by *in situ* oxidation prior to coupling. The indophenol dyes formed by the coupling reactions of LXXXXI are acid-base indicators, and are typically yellow or brown in their neutral form and blue to red in their anionic form.

A typical analytical procedure involves preparing a solution of the phenol in a pH 8 to 9 aqueous buffer, reacting it with a 0.01 *M* solution of LXXXXI or LXXXXII in ethanol, and measuring the absorbance of the product directly in the aqueous solution or after extraction into butanol, chloroform, or another polar solvent. The reaction is usually complete within 10 to 30 min at room temperature.

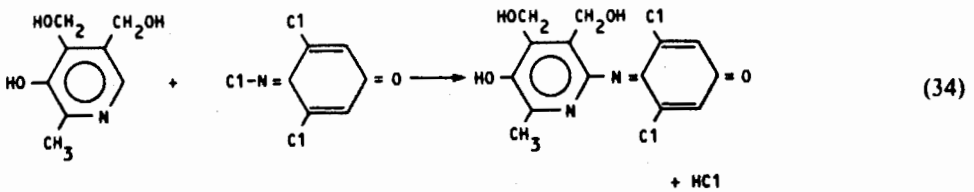
The specificity of the Gibb's reaction has been reviewed by Dacre.²⁰⁹ The reaction will proceed with a wide variety of phenols unsubstituted in the *para* position. However, highly substituted phenols may not react in spite of an unsubstituted *para* position.^{210,211} Certain *para* substituted phenols also react. All phenols with *p*-alkoxy groups probably react,²⁰⁹ including those with bulky groups such as *t*-butyl *ortho* to either the hydroxyl or alkoxy group.^{209,212,213} Many phenols with *para* alcohol, aldehyde, or carboxylic acid groups also react.^{209,211,214-218} However, the presence of an aldehyde, carboxylic acid, nitro, or nitroso group *ortho* to the phenolic hydroxyl will prevent coupling.²¹⁹ Some *p*-halophenols react with Gibb's reagent while others do not.^{209,215,220-222} In particular, phenols with two or more halogen substituents usually fail to react, presumably because of ring deactivation by the substituents. Amines,²²³

amino derivatives,²²⁴ and thiols²²⁵ yield colored products upon reaction with LIX or LX and are potential sources of interference in analyses by the Gibb's reaction. However, thiols substitute on the sulfur atom yielding products that usually absorb light at or below 400 nm and therefore would not interfere in the analysis of phenols which would be measured at much longer wavelengths. Strong reducing agents are potential interferences in this

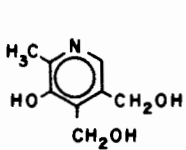


analysis since they will reduce the Gibb's reagent and prevent coupling.

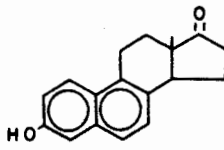
LXXXXI is used in the *U.S.P. XIX* assays for pyridoxine(LXXXXIII) raw material, tablets, and as a component in Decavitamin Tablets and capsules. After appropriate separations from other tablet constituents, color is developed in an alkaline ammonium buffer for 70 sec and the absorbance measured at 650 nm. The reaction is ²²⁶



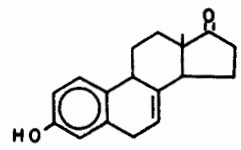
Banes²²⁷ used LXXXXII for the colorimetric analysis of equilenin(LXXXXIV) and equilin(LXXXXV) in the presence of estrone(LXXXXVI), since estrone does not react with this reagent. Reaction with LXXXXI has also been used to detect *nor*-mycophenolic acid(LXXXXVII),²¹¹ uric acid(LXXXXVIII),²²⁴ theophylline(LXXXXIX),²²⁸ dihydroerodin(C) and dihydrogeodin(Cl),^{229,230} propylthiouracil(CII),²³¹ and thiouracil(CIII).²³²⁴ However, the thiouracils react according to Equation 33 rather than 32 since their absorption maxima are at much shorter wavelengths. LXXXXII has been used to quantitate resorcinol in the presence of phenol in a procedure that is reported to be stability indicating.^{232B}



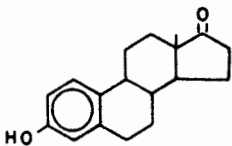
LXXXXIII



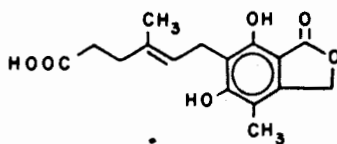
LXXXXIV



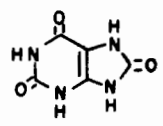
LXXXXV



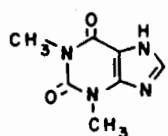
LXXXXVI



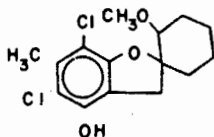
LXXXXVII



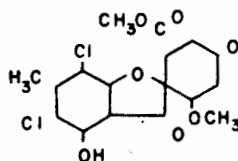
LXXXXVIII



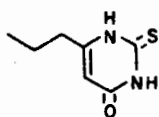
LXXXXIX



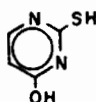
C



CI

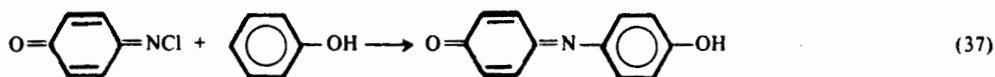
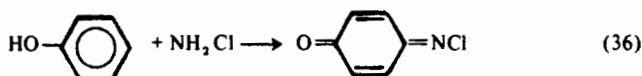


CII



CIII

A number of other analytical procedures for pharmaceutical products are based upon reactions closely analogous to Equation 32, i.e., they also involve coupling a phenol with a quinoneimine to produce an indophenol dye which is measured spectrophotometrically. Acetaminophen(XV) has been analyzed by hydrolyzing it to *p*-aminophenol in acid, then oxidizing to the quinoneimine with hypobromite, and finally coupling with phenol, to obtain an indophenol with maximum absorption at 625 nm.²³³ This procedure has been used to measure acetaminophen in blood,^{233,234} and to determine free *p*-aminophenol in acetaminophen. Murfin and Wragg^{235,236} improved the procedure by using sodium hypochlorite and then removing the excess oxidizer with sodium arsenite prior to raising the pH to 9.9 for the coupling step. The oxidation and coupling can both be carried out under alkaline conditions if potassium ferricyanide is used in place of sodium hypochlorite as the oxidizing agent.²³⁷ Aniline itself will couple with phenol in the presence of hypochlorite to yield an indophenol dye with absorption maximum at 600 nm. Another variation of this procedure provides a very sensitive analytical procedure for ammonia.²³⁸ Ammonia is first converted to chloramine by reaction with hypochlorite, and subsequent addition of an alkaline phenol solution (0.8% phenol in 0.3 M sodium hydroxide) results in formation of the indophenol dye as shown in Equations 35 to 37:



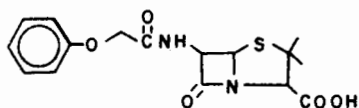
This procedure offers about ten times greater sensitivity than methods using Nessler's Reagent.

d. Nitration, Halogenation, and Friedel-Crafts Reactions

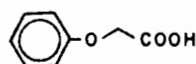
Reactions in this group are of limited use in quantitative analysis since they often fail to go to completion and because they often yield a mixture of products rather than a single product. Nonetheless these reactions are encountered often enough in pharmaceutical analysis to merit a brief discussion here.

Nitration is usually carried out with a mixture of concentrated nitric and sulfuric acids. The product may be a mono, di-, or trinitro derivative or mixture of them, depending upon the reaction conditions and how strongly activated the analyte is. A calibration curve prepared by nitration of a reference standard under the same conditions as the sample is required for quantitative work. Acetaminophen(XV) can be nitrated in a mixture of nitric and sulfuric acids at -5°C to obtain 2-nitro-4-acetaminophenol which can be measured at 370 nm in neutral or acid solution and at 430 nm in alkaline solution, as shown in Figure 15.^{239,240} Le Pedriel et al.²⁴¹ later found that the same reaction could be carried out under mild conditions with nitrous acid instead of nitric acid, and that *p*-aminophenol, phenacetin, and a number of other related compounds do not interfere in this procedure as they do when nitric acid is used for nitration. A careful evaluation of this method and an improved procedure have been reported by Chafetz et al.²⁴² Under the conditions he recommends for the analysis, Chafetz found approximately 90% of XV was converted to the nitro product. Nitration with 5 N nitric acid for 20 min at room temperature has also been used for the analysis of phenacetin(IV).²⁴³

Both phenoxymethylpenicillin(Penicillin V, CIVa) and phenoxyacetic acid (CIVb) and in CIVa have been analyzed by nitration with 10% nitric acid in concentrated sulfuric acid, and measurement of the absorbance in an ammonia solution. Specificity in the determination of CIVb in CIVa is achieved by extracting with benzene prior to color development²⁴⁴ or by thin layer chromatography.^{244B} Ida et al.²⁴⁵ determined CIVb in fermenter broth by extracting with chloroform, nitrating, and then quantitating the nitrated product polarographically. Nitration of xylene or phenodisulfonic acid is often used for quantitation or inorganic nitrate and nitrate esters.



CIVa



CIVb

Bromination and iodination have been used in the analysis of phenols and anilines. The reactions must be carried out under closely controlled conditions in order to obtain reproducible results, and the results must be evaluated using a calibration curve prepared under the same reaction conditions. Aniline, phenol and phenyl ethers react rapidly in bromine water to produce the corresponding 2,4,6-tribromo derivatives. The tribromo derivatives obtained in this manner are often crystalline materials suitable for use in qualitative characterization of the analyte. Identity Test B for glyco-biarsol(CV) is based upon bromination of the substituted aniline to yield 2,4,6-tribromoaniline. In this case, cleavage of the amide function and displacement of the arsenate group accompany bromination.

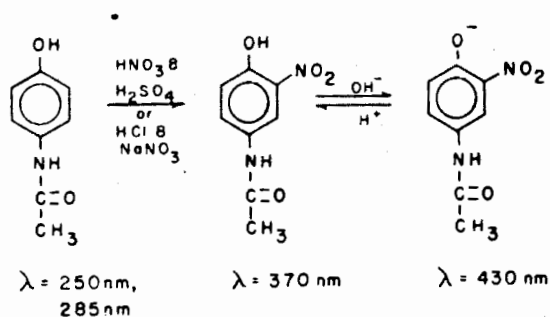
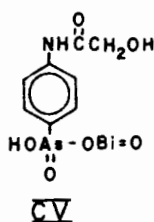
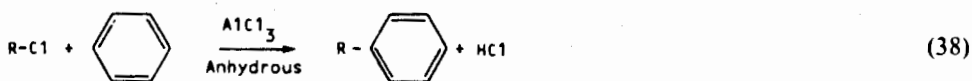


FIGURE 15. Spectrophotometric analysis of acetaminophen by nitration.

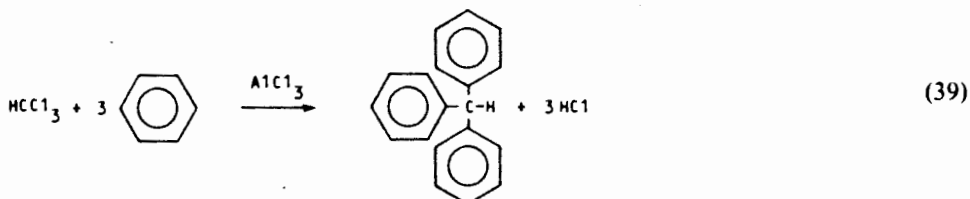


Phenols can be analyzed quantitatively by bromination either by measuring the absorbance of the brominated product²⁴⁶ or by determining the excess brominating agent titrimetrically.²⁴⁷ Iodination can also be used to determine phenols spectrophotometrically or by titration of the excess iodine with thiosulfate.²⁴⁸ Certain *o*-alkyl and *o*-aryl phenols also react with iodine in an alkaline medium to form coupled products which can be quantitated spectrophotometrically.^{249, 250} The structure of the products formed is not known.

Friedel-Crafts reactions may be used to couple acyl halides or alkyl halides to aromatic substrates to yield phenones or alkyl substituted aromatics, respectively, as shown in Equation 38



The reaction of chloroform with aromatic compounds in the presence of aluminum chloride for example produces triaryl methane derivatives which are colored in solution and can be used as a qualitative test for the presence of aromatic compounds²⁵¹ which are suitably activated toward

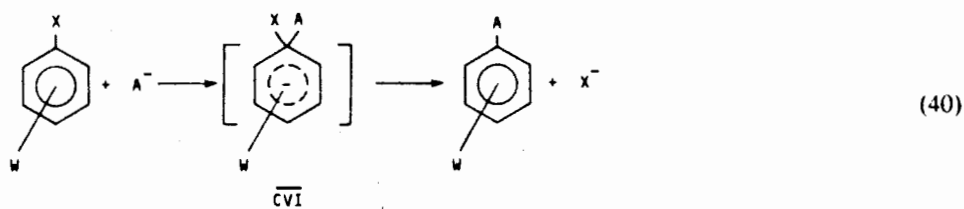


electrophilic substitution. Friedel-Crafts reactions have not been used for quantitative analysis, but as Connors²⁵² has pointed out, they offer a high degree of selectivity and good yields, and therefore offer an interesting possibility for future development.

ii. Nucleophilic Aromatic Substitutions

a. Orientation and Reactivity

A number of important reactions in organic analysis are of the nucleophilic substitution type. The general form of these reactions is given in Equation 40.



This reaction is similar to that shown in Equation 20 for electrophilic substitution, but the attacking species is now a nucleophile (usually an anion) and the aromatic substrate must be substituted with electron withdrawing groups. The requirement for electron-withdrawing groups is responsible in part for the importance of nucleophilic substitution reactions in pharmaceutical analysis since the presence of such groups prevents the use of the popular colorimetric procedures based on electrophilic aromatic substitution.

Nucleophilic substitutions on aromatic systems may proceed through several different mechanisms,²⁵³⁻²⁵⁷ but the reactions of analytical interest all involve intermediates like CVI. In some cases, such as substitutions on *m*-dinitrobenzene derivatives, the complexes are stable, highly colored species and are used directly for quantitation of the reactants. Only reactions in which CVI is a product or intermediate will be discussed in this chapter.

The rate limiting step in the nucleophilic substitution reaction is the formation of the intermediate complex. As a result, the reaction rate is relatively independent of the strength of the bond between the leaving group and substrate. The rates are accelerated by the presence of electron withdrawing groups, including the leaving group, on the substrate, and are inhibited by electron releasing groups. The effects on reaction rate are greatest when the substituents are *ortho* or *para* to the leaving group. A heterocyclic nitrogen atom also activates the ring and directs substitution to the *ortho* and *para* positions.^{258, 259}

The relative ease with which leaving groups are displaced from the substrate varies with the nucleophile and particular substrate molecule. For example, Bartoli and Todesco²⁶⁰ reported that the rate of nucleophilic substitution on 1-X-2,4-dinitrobenzene by the methoxide ion decreased in the order $F \sim NO_2 > -OC_6H_4NO_2 (p) > Cl > OC_6H_5 > SC_6H_4NO_2 (p)$ whereas the order with the thiophenoxide ion was $-NO_2 \gg -F > -Cl > -OC_6H_4NO_2 (p) \sim -SC_6H_4NO_2 (p) > -OC_6H_5$. Bartoli and Todesco²⁶⁰ developed an empirical relation to allow estimation of the relative rates of reaction for nucleophilic substitutions with different leaving groups. An approximate order that can be used as a guide in selecting suitable substrates for a nucleophilic substitution reaction is given in Table 24.^{253, 261, 262}

The ability of other ring substituents to increase the rate of a nucleophilic aromatic substitution follows the reverse of the order of their ability to enhance the rate of electrophilic substitutions (see Table 17). Relative reaction rates for substrates with

Table 24
 APPROXIMATE ABILITY OF
 SUBSTITUENTS AS LEAVING
 GROUPS IN NUCLEOPHILIC
 AROMATIC SUBSTITUTIONS^{a, b}

-F	decreasing ease of displacement
-NO ₂	
-OSO ₂ C ₆ H ₄ CH ₃	
-SOC ₆ H ₅	
-Cl	
-Br	
-I	
-N ₃	
-NR ₂ ⁺	
-OAr, -OR, -SR, -SO ₂ R, -NH ₂	

} close to one another

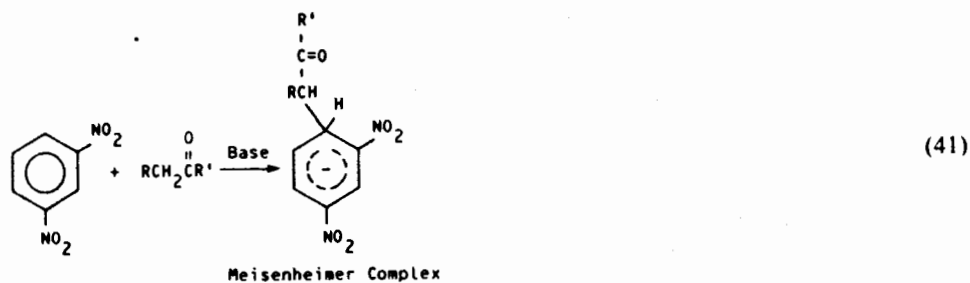
- ^a As discussed in the text, the exact order in a particular reaction depends upon the nucleophile and substrate as well as the leaving group.
- ^b After March, J., *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, McGraw-Hill, New York, 1968, 488; Bemporad, P., Illuminati, G., and Stegal, F., *J. Am. Chem. Soc.*, 91, 6742, 1969; Bartoli, G. and Todesco, P. E., *Acc. Chem. Res.*, 10, 125, 1977.

different ring substituents can be predicted quantitatively using a modified Hammett equation^{263, 264} employing σ^- values.²⁶⁵

The rate of reaction of a given substrate with a variety of nucleophiles correlates roughly with the basicity of the nucleophile, but the exact ranking of a series of nucleophiles depends on both the substrate and leaving group. Nonetheless, an approximate ranking is useful in the selection of reagents for the development of new analytical procedures. An approximate ranking of nucleophiles is given in Table 25.²⁵³

β. Coupling Active Methylene Compounds with m-Dinitrobenzene Derivatives

m-Dinitrobenzene derivatives react with active methylene compounds under basic conditions to produce colored complexes as shown in Equation 41.



The complexes are called Meisenheimer complexes,²⁶⁶ and the reaction is called the Janovsky reaction.^{267, 268} The reaction is often carried out in dimethylformamide or

Table 25
APPROXIMATE RELATIVE
NUCLEOPHILICITY OF SEVERAL
COMMON NUCLEOPHILES^{a, b}

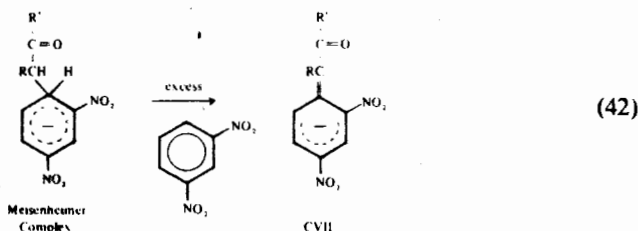
NH₂
(C₆H₅)₃C⁻
ArS⁻
RO⁻
R₂NH
ArO⁻
OH⁻
ArNH₂
NH₃
I⁻
Br⁻
Cl⁻
H₂O
ROH

- ^a As discussed in the text, the exact order in a particular reaction depends upon the substrate and leaving group as well as on the nucleophile.
- ^b From March, J., *Advanced Organic Chemistry: Reactions, Mechanisms, and Structure*, McGraw-Hill, New York, 1968, 488.

methanol-dimethylformamide mixtures with high concentrations (~10%) of a quaternary amine hydroxide as the base. An active methylene coupling agent such as acetone, butanone, or nitromethane must be present in excess and may serve as both solvent and reactant in the analysis of dinitrobenzene derivatives. Other relatively polar solvents may also be used. The reaction is rapid, usually being complete in a minute or less at room temperature. The absorbance is measured shortly after addition of the reagents since the colors often fade in time. Absorption maxima for many compounds are near 550 nm. The limit of detection for ketones is usually on the order of 0.01 $\mu\text{mol/ml}$.

Suitable substrates for the Janovsky reaction include *m*-dinitrobenzene,²⁶⁷⁻²⁷⁰ 1,3,5-trinitrobenzene,²⁷¹⁻²⁷³ picric acid,²⁷⁴ 2,4-dinitrophenylhydrazones,²⁷⁵ and various dinitrophenyl ethers. The dinitrobenzenes may be coupled with ketones bearing α hydrogens including acetone,²⁶⁷⁻²⁷⁰ butanone,²⁷⁷ and methyl ethyl ketone;²⁷³ alkyl nitrates²⁷⁸ including nitromethane;^{269, 270} dimethylsulfoxide;²⁷⁹ and alkyl nitriles.²⁷⁸ Coupling also occurs with alkoxides,^{258, 259} amines,^{280, 281} dialkylphosphites,²⁷² sulfite,²⁸² cyanide²⁷¹ and many other nucleophiles.

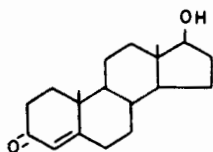
The Janovsky reaction has been used to quantitate *m*-dinitrobenzene derivatives^{283, 284} directly, and to quantitate a variety of other compounds after nitration, including benzene,²⁷⁷ ephedrine,²⁷⁶ phenobarbital,²⁷⁶ and atropine.²⁷⁶ The Janovsky reaction is not often used for the determination of active methylene compounds because an excess of the dinitrobenzene is required to assure complete reaction of the analyte, and in the presence of excess dinitrobenzene, the Meisenheimer complex is oxidized as shown in Equation 42.²⁸⁵⁻²⁸⁸



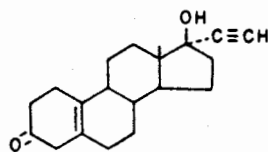
The reaction of an active methylene with an excess of a *m*-dinitrobenzene derivative to produce products such as CVII is called the Zimmerman reaction,^{289, 290} and has been widely applied to the analysis of ketosteroids. An excellent review of this reaction has been given by Pesez and Bartos.²⁹¹

The Zimmerman reaction is carried out under the same conditions as the Janovsky reaction, but color development is slower. The wavelength of the absorption maxima and the limits of detection are also similar to those of the Janovsky reaction.

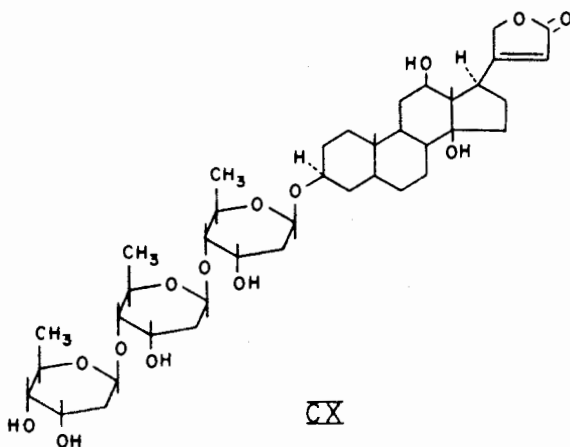
The Zimmerman reaction has been used to quantitate testosterone (CVIII), norethynodrel (CIX), hydrocortisone (L), cortisone (XXXXVIII), progesterone (LI), prednisolone (CXI), and other 3- and 17-ketosteroids by coupling with *m*-dinitrobenzene.^{291, 292} Coupling with *m*-dinitrobenzene is also used for identification and quantitation of digoxin (CX) in elixirs, injections, and tablets in *U.S.P. XIX*. Coupling with 1,3,5-trinitrobenzene has been used for the analysis of 3- and 17-ketosteroids, and offers the advantage of a more stable chromophore than dinitrobenzene.²⁹¹ 1,3,5-Trinitrobenzene has also been used for the analysis of cardiac glycosides.^{293A, 293B} The Meisenheimer complexes formed by the reaction of ouabain (CXII) or digitoxin with 1,3,5-trinitrobenzene and 2,4,6-trinitroanisole have been shown by Burns et al.^{293B} to be substituted on carbon 1 of the aromatic ring as shown in Figure 16. However, Kimura^{293C} reported that the picric acid reacts at an unsubstituted carbon. Picric acid is widely used for analysis of 3- and 17-ketosteroids,²⁹¹ cardiac glycosides,^{291, 294, 295, 296} and other active methylene compounds. Picric acid is used for the analysis of ouabain (CXII), methylphenidate (CXIII) and phentolamine mesylate (CXIV) in *U.S.P. XIX*, and for acetyl digoxin (CXV) and ophenadrine citrate in *N.F. XIV*.



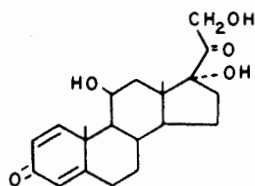
CVIII



CIX



CX

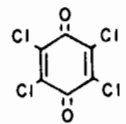
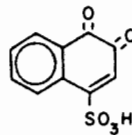
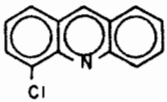
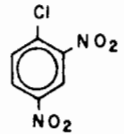
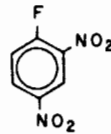
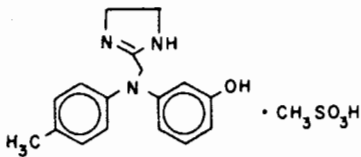
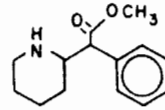
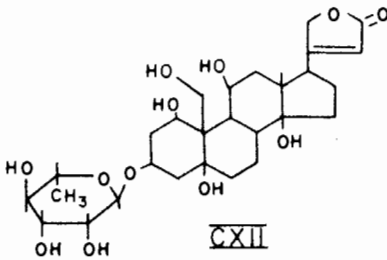


CXI

γ. Other Nucleophilic Substitutions

Several other nucleophilic substitution reactions are used in pharmaceutical analysis for determination of specific functional groups. Coupling of amines, phenols, and

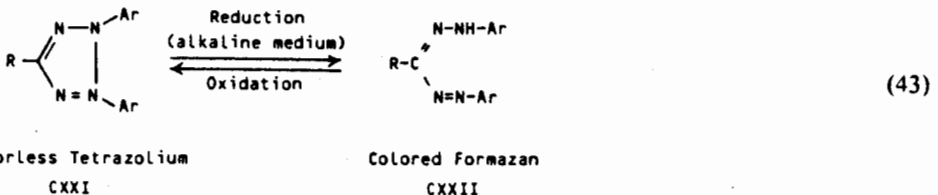
thiols with 1-fluoro-2,4-dinitrobenzene(CXVI, Sanger's reagent) or 1-chloro-2,4-dinitrobenzene(CXVII); primary amines with 9-chloroacridine(CXVIII) or 1,2-naphthoquinone-4-sulfonic acid(CXIX); and coupling of tertiary amines with chloranil(CXX) are examples of these reactions.



b. Colorimetric Determinations Based on Oxidation-Reduction Reactions

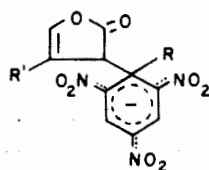
i. Determination of Steroids and Other Substances by Reduction of Tetrazolium Salts

Tetrazolium salts(CXXI) are easily reduced in alkaline solutions to highly colored formazans(CXXII) as shown in Equation 43:



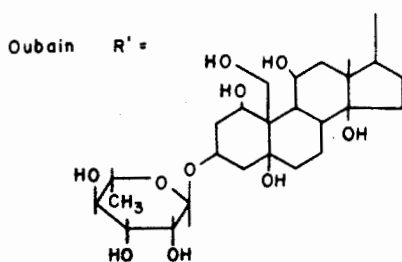
The reduction potential for CXXI is approximately -0.08 volts, allowing the reaction to be used for spectrophotometric determination of a wide range of reducing agents. The reaction has been widely used for the analysis of 20-one-21-ol steroids including the corticosteroids. Limits of detection of 1 to 2 μg can usually be achieved.

The formazans produced in the reaction may exist in the four configurations shown.



1,3,5-trinitrobenzene R = H

1,3,5-trinitroanisole R = OCH₃



Digitoxin R' =

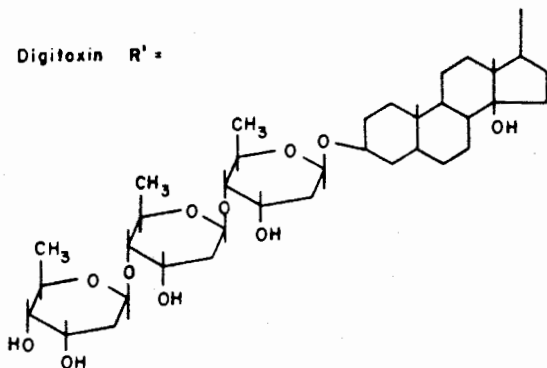


FIGURE 16. Structures of Mesenheimer complexes formed by reaction of ouabain and digitoxin with 1,3,5-trinitrobenzene derivatives.

in Figure 17, and the four isomers have quite different physical properties. For example, the *trans-syn* isomer of the diformazan (CXXIII) formed from blue tetrazolium(CXXIV) absorbs at a longer wavelength (blue, $\lambda_{MAX} = 625$ nm in dimethylformamide) and is less polar than the *trans-anti* isomer (red, $\lambda_{MAX} = 517$ nm in dimethylformamide).²⁹⁷ Since both the rate of reaction and the distribution of reaction products between the isomeric forms are strongly dependent on solvent,^{297,298} careful control of solvent composition is important in analytical applications of this reaction.

Polar solvents,²⁹⁷ and in particular water,^{298,299} reduce the rate of color formation. In solvent mixtures of equal dielectric constant, the reaction proceeds most rapidly in the mixture with the greatest capacity for hydrogen bond formation.²⁹⁷ In spite of the shorter retention times possible in non-polar media, 95% ethanol has been the most widely used solvent in applications of the tetrazolium reaction in pharmaceutical analysis.¹⁰⁰⁻¹⁰⁸ However, Graham et al.^{297,309,310} have used dichloromethane to good advantage in the analysis of corticosteroids with CXXIV, and Ascione and Fogelin³¹¹ have reported the use of chloroform-ethanol mixtures.

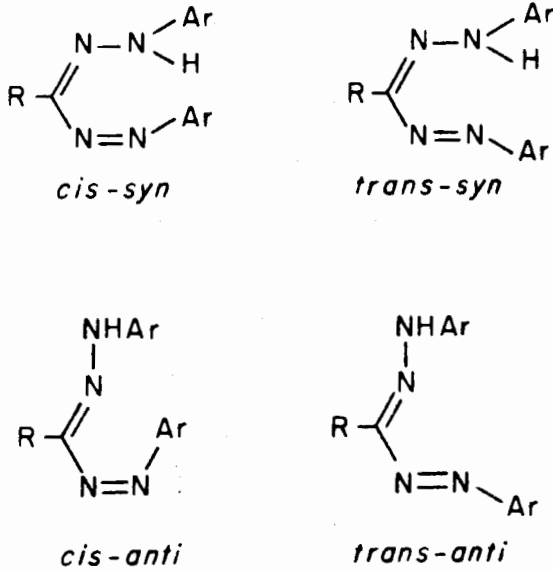
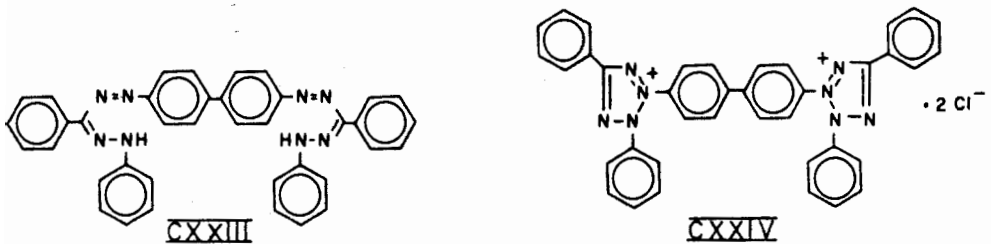
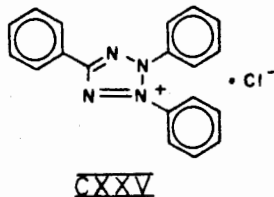


FIGURE 17. Possible configurations of formazans.



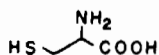
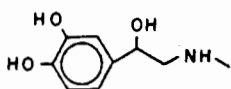
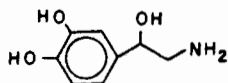
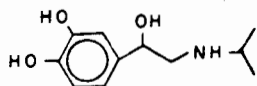
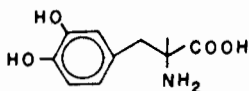
Triphenyltetrazolium(CXXV),³¹³⁻³¹⁷ CXXIV,³¹¹ and their formazans^{301,312-318} are light sensitive, although at least two studies concluded that light did not affect the reaction of CXXIV with corticosteroids.²⁹⁹ Graham et al.³¹³ have shown that light induced degradation of the formazans of CXXIV and CXXV is negligible when 95% ethanol is used as the solvent, but that the solution must be protected from light when other solvents such as cyclohexane, chloroform, or dichloromethane are used.³¹³ The use of actinic glassware in the analysis of corticosteroids with CXXV has been recommended.³¹⁵



A typical analytical procedure involves dissolving the sample in 0.01 M solution of tetramethylammonium hydroxide or other quaternary amine hydroxide in ethanol. An analyte concentration near 25 μM is usually satisfactory. Sufficient tetrazolium is

added, usually in an ethanol solution, to yield a final concentration of 500 μ M. The solution is protected from light during color development. The time and temperature for color development must be optimized for each substance, but times between 5 and 60 min at temperatures between 25°C and 60°C are common. The conditions chosen for the reaction also determine the specificity of the method since higher temperatures and longer reaction times allow reaction with a wider variety of reducing agents. A reagent blank must also be prepared in an identical manner. Dichloromethane can be substituted for ethanol provided the quaternary amine and tetrazolium stock solutions are prepared in anhydrous ethanol or methanol so that turbidity does not develop after the reagents are mixed. The compendia^{307,308} require that the absorbance of the samples and standards be measured with the reagent blank in the reference cell. However, Graham et al.^{309,310,313} find that the absorbance of the blank continues to rise even after the absorbance of the samples has stabilized. The increase in absorbance is not as rapid in the dark as in the light, but is still significant. Therefore, they recommend that samples, standards and blanks be measured against the solvent a precise time after the reagents are mixed rather than measuring the samples and standards directly against the blank.

Several tetrazolium salts have been used for chemical analysis. CXXV has been used for analysis of corticosteroids,^{320,321} reducing sugars³²²⁻³²⁶ including ascorbic acid (XX),^{324,327,328} cysteine (CXXVI),³²⁴ and catecholamines including epinephrine (CXXVII), levarterenol (CXXVIII), isoproterenol (CXXIX), and methyl dopa (CXXX).³⁰² However, the analysis of corticosteroids using CXXV often gives poor reproducibility³¹⁹ due to the effects of moisture^{298,299} and temperature^{299,329,330} on reaction rate, and the ease with which the formazan is oxidized back to the tetrazolium by oxygen.³⁰¹

CXXVICXXVIICXXVIIICXXIXCXXX

Better analytical precision can often be achieved by using CXXIV which is not as sensitive to atmospheric oxygen as CXXV. CXXIV is the most widely used tetrazolium salt in pharmaceutical analysis, and some of its applications are summarized in Table 26. The 21-hydroxy-20-one group of the corticosteroids is responsible for the reduction of CXXIV under the usual reaction conditions, and structural changes in other portions of the molecule have only a limited effect on the rate of the reaction.^{297,317} Therefore degradation products must be removed by column or thin layer chromatography prior to color development if an analysis is to be stability indicating.³³² Corticosteroids esterified at the 21-hydroxy position react as rapidly as the parent substance.²⁹⁷ Δ^1 - and Δ^4 -3-ketosteroids can also be quantitated by reaction with CXXIV although higher temperatures and longer reaction times are required.^{333-335,338} Meyer and Lindberg³³⁸

Table 26
U.S.P. and N.F. APPLICATIONS OF BLUE TETRAZOLIUM

Prednisolone: $R = H$ $R' = -H$

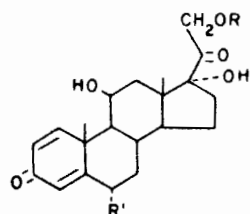
Methyl prednisolone: $R = H$ $R' = CH_3 -$

Methyl prednisolone acetate: $R' = CH_3 -$ $R = CH_3COO^-$

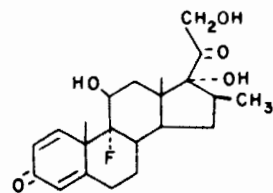
Methyl prednisolone sodium succinate:

$R' = CH_3 -$

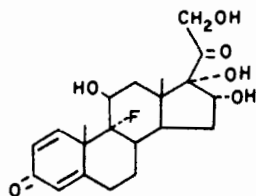
$R = Na^+ ^-OOCCH_2CH_2COO^-$



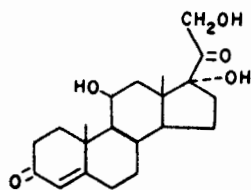
Betamethasone



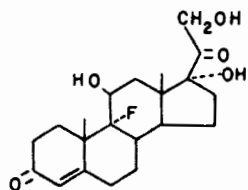
Triamcinolone



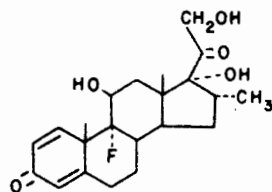
Hydrocortisone



Fludrocortisone



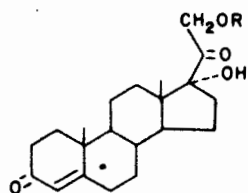
Dexamethasone



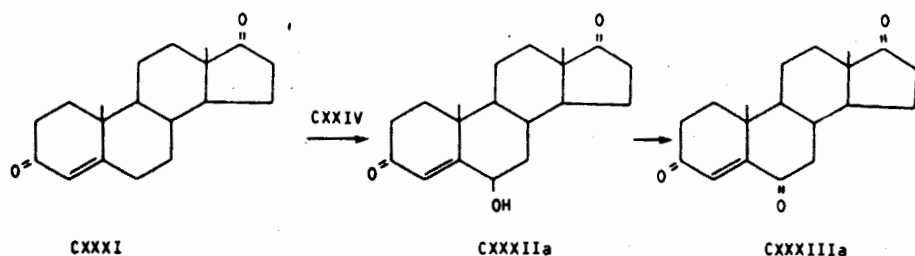
Desoxycortisone: $R = H$

Desoxycortisone acetate: $R = CH_3COO^-$

Desoxycortisone pivalate: $R = (CH_3)_3CCOO^-$



have shown that the products of the oxidation of 4-androstene-3,17-dione(CXXXI) by CXXIV include the 6-hydroxy(CXXXIIa) and 6-keto(CXXXIIIa) derivatives.



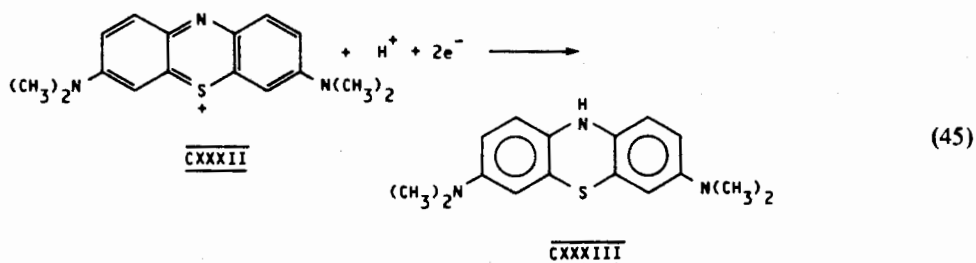
(44)

Aromatization of the A ring apparently occurs with other substrates,³³⁸ but these reactions have not been characterized. No reaction occurs with the 17-keto group or with the 3-keto group when the A ring is saturated.

Many nonsteroidal compounds reduce CXXIV. Manni and Sinsheimer³⁰³ have studied the reduction of CXXIV by non-steroidal α -hydroxyketones and Sinsheimer and Salim³⁰⁴ found that it was reduced by dihydroxybenzenes, diphenylacetonitrile, and malonitrile. Thiols are oxidized to disulfides by CXXIV.^{304,336} Dithiocarbamates,³³⁹ reducing sugars,³²⁰ and methyl ketones³⁰⁴ also react. Substances in each of these classes are potential interferences in analyses based upon the reduction of CXXIV and other tetrazolium salts. Several drugs and excipients that have been reported to be specific interferences in the analysis of corticosteroids with CXXIV are listed in Table 27.

ii. Redox Reactions with Methylene Blue

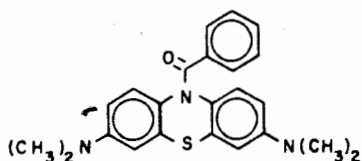
Methylene blue(CXXXII) can be reversibly reduced to the corresponding leuco (colorless) base as shown in Equation 45:



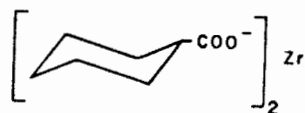
The reduction potential for CXXXII is 0.5 volts at pH 3,³⁴¹ and 0.36 volts at pH 0.³⁴² The color changes that occur upon reduction of CXXXII and oxidation of CXXXIII can be used for the colorimetric determination of reducing and oxidizing agents, respectively, but few applications of this reaction in pharmaceutical analysis have been reported.

The reduction of CXXXII by ascorbic acid(XX) is used as an identification test for XX in Ascorbic Acid, Ascorbic Acid Injection, and Ascorbic Acid Tablets in *U.S.P. XIX*.¹⁴³ Methylene Blue itself is used medicinally as an antidote to cyanide poisoning and methemoglobinemia. It has been determined directly by measuring its absorbance at 663 nm in ethanol, and by titrating with 0.1 *N* titanous chloride to a pinkish grey endpoint which marks complete reduction of CXXXII to CXXXIII.³³⁴

A derivative of CXXXIII, benzoyl leuco methylene blue(CXXXIV), has also been used for the determination of very low levels of peroxide.³⁴⁵ The reaction is carried out in 0.5% trichloroacetic acid in benzene with zirconium naphenate(CXXXV, $\sim 7mM$) added to accelerate the decomposition of the peroxide. The rate of reaction varies with the particular peroxide being analyzed,³⁴⁶ but reaction times of a few hours are usually sufficient. Less than 0.1 μg of peroxide can be determined by measuring the absorbance of the oxidized dye at 645 nm.³⁴⁶⁻³⁴⁹



CXXXIV



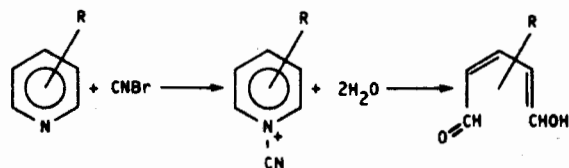
CXXXV

Many dyes undergo reversible oxidation and reduction reactions with concomitant changes in their absorption spectra, and all of these dyes are potentially useful in the analysis of oxidizing and reducing agents. Lists of several dyes and their reduction potentials can be found in References 341 and 342.

c. Colorimetric Methods for Nitrogen Heterocycles

i. Reaction of Pyridine Derivatives with Cyanogen Bromide

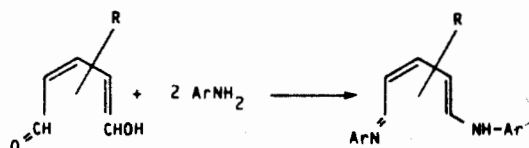
Pyridine derivatives with a hydrogen in at least one of the positions adjacent to the ring nitrogen react with cyanogen bromide to produce a derivative of glutaconic aldehyde(CXXXVI).³⁵⁰



CXXXVI

(46)

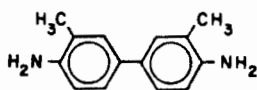
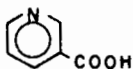
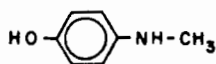
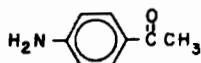
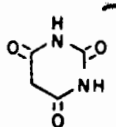
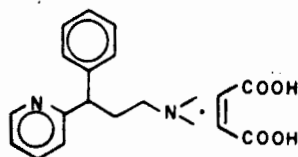
CXXXVI often absorbs at long enough wavelengths to be measured colorimetrically, but it is a reactive molecule and color stability is frequently poor. Therefore CXXXVI is often converted to the Schiff base of an aryl amine (Equation 46), and the absorbance of the Schiff base is measured. Aniline was originally used as the coupling agent,³⁵¹⁻³⁵⁴



(47)

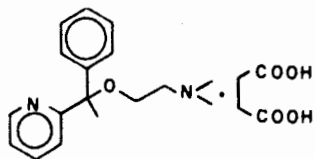
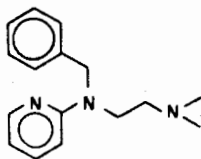
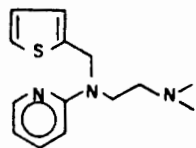
but *o*-tolidine(CXXXVII),³⁵⁰ nicotinic acid(CXXXVIII),^{355,356} *p*-methylaminophenol (CXXXIX),³⁵⁷ *p*-aminoacetophenone(CXXXX),^{358,359} barbituric acid (CXXXXIa, with cyanogen chloride rather than cyanogen bromide),³⁶⁰ sulfanilic acid(LXIII),³⁶¹ and others have been used.

The analytical procedure usually requires preparing a solution of the analyte in a pH 5.5 to 7.5 buffer at a concentration of 10^{-4} to 10^{-2} M. Sufficient cyanogen bromide is added to achieve a final concentration of approximately 4×10^{-2} M and the solution is allowed to sit from 10 to 90 min at room temperature. The color may be read directly at the end of this time, or a solution of the amine may be added to produce the Schiff base.

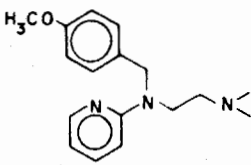
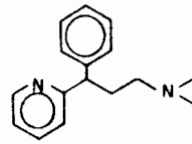
CXXXVIICXXXVIIICXXXIXCXXXXCXXXIXaCXXXIXb

Direct measurement of the absorbance of a glutamic aldehyde derivative at 480nm has been used for the analysis of pheniramine maleate (CXXXIXb).^{362,363} Perlman³⁵⁹ reported that colors developed upon treatment of doxylamine succinate (CXXXIXII), nicotinic acid (CXXXVIII), pyridine, and 2-aminopyridine with cyanogen bromide. Perlman³⁵⁹ also noted that several of the pyridine derived antihistamines developed fluorescence rather than color on treatment with cyanogen bromide. Development of fluorescence is associated with a 2-aminopyridine group in the molecule.

Cyanogen bromide treatment with subsequent condensation with aniline has been used in the analysis of CXXXVIII,^{355,356} and is specified in the *N.F.* for analysis of niacin and niacinamide³⁶¹ in pharmaceutical preparations. Jones and Brady³⁵³ also used condensation with aniline in the analysis of tripeleminamine (CXXXIXIII), thenylpyramine (CXXXIXIV), pyranisamine (CXXXIXVI), prophenpyridamine (CXXXIXVII), and doxylamine (CXXXIXII): these substances were analyzed in urine as well as in a variety of pharmaceutical preparations. Perlman³⁵⁹ used p-aminacetophenone (CXXXX) to develop colors with doxylamine (CXXXIXII), pyranisamine (CXXXIXVIII), pheniramine (CXXXIXb), nicotinic acid (CXXXVIII), pyridine and 2-aminopyridine.

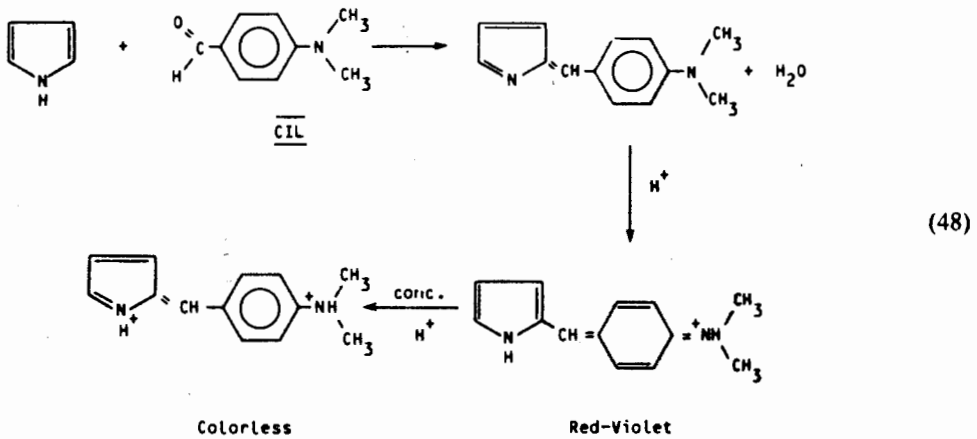
CXXXIXIICXXXIXIIICXXXIXIV

Interferences in this procedure do not appear to have been studied systematically. However, aldehydes in the sample matrix would form Schiff bases with the added alkyamine and therefore are a potential source of interference.

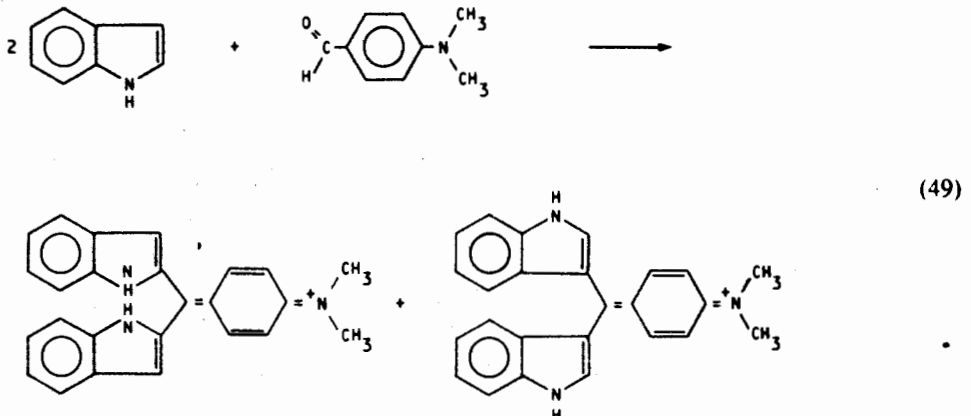
CXXXXVICXXXXVII

ii. Determination of Pyrroles and Indoles with *p*-Dimethylaminobenzaldehyde (Ehrlich Reaction)

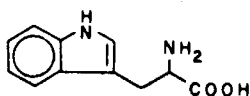
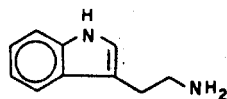
Ehrlich³⁶⁴ found the *p*-dimethylaminobenzaldehyde (CIL) condensed with pyrrole in an acid medium to produce a red-violet color. The colored product is a quinoneimine formed according to Equation 48.



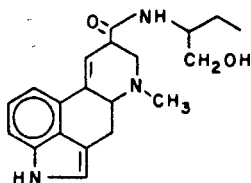
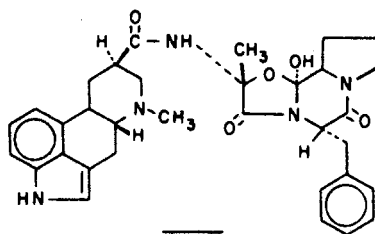
This is a special case of the general hydroxyalkylation reaction in which an aldehyde or ketone condenses with an aromatic ring. Hydroxyalkylation reactions have been reviewed by Hofmann and Schriesheim.³⁶⁵ The first step in the reaction is an electrophilic attack on the heterocyclic ring to give an alcohol. Dehydration of the alcohol gives the quinoneimine product shown in Equation 48. In many cases the intermediate alcohol is sufficiently reactive that it condenses with a second mole of the heterocycle. Condensation with 2 mol of nitrogen heterocycle is common with indole derivatives and produces the product shown in Equation 49.



Pyrroles and indoles with a hydrogen α or β to the ring nitrogen react rapidly with CIL.³⁶⁶⁻³⁶⁸ For example, CIL reacts with pyrrole, 2,4- and 2,5-dimethyl pyrrole, indole, and 2- and 3-methylindole to form quinoneimines with λ_{MAX} values between 520 and 565 nm, and molar absorptivities on the order of 60,000 to 90,000 $l\ mol^{-1}\ cm^{-1}$.³⁶⁷ Carbazole and substituted carbazoles do not react because the α and β ring positions are blocked. Substances such as tryptophan(CL) and tryptamine(CLI) react slowly if at all because the aminomethyl group is protonated under the acidic conditions used in this reaction, and the positively charged substituent group deactivates the ring to electrophilic substitution. Other ring substituents that are strongly deactivating to electrophilic aromatic substitution will also inhibit color formation (see Table 17).

CLCLI

A variety of solvents and conditions have been used for this reaction. A typical analysis employs a final analyte concentration between 10^{-6} and 10^{-2} M in approximately 1 M aqueous acetic, trifluoroacetic, sulfuric, or phosphoric acid. Normal reaction times range from 10 to 60 min at temperatures from 25°C to 50°C. The *N.F.*, for example, specifies 60 min at room temperature in 6 M sulfuric acid for analysis of methylergonovine(CLII) and dihydroergotamine(CLIII). The acid concentration in the *N.F.* procedure is higher than usually recommended for this reaction.

CLIICLIII

Aldehyde and ketone impurities in a sample can interfere in analyses based upon Equation 48 and 49 by formation of colored Schiff bases with CIL. Indoles may also couple with quinones under acid conditions to produce colored products. The analyte must be separated from these substances as well as extraneous pyrroles and indoles prior to color development. French³⁶⁹ found that benzhydrol(CLIV) also interfered in the analysis of skatole(CLIV) because it reacts with skatole (and presumably other indoles) under the conditions used with Ehrlich's reagent.

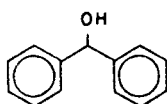
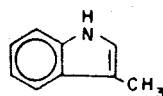
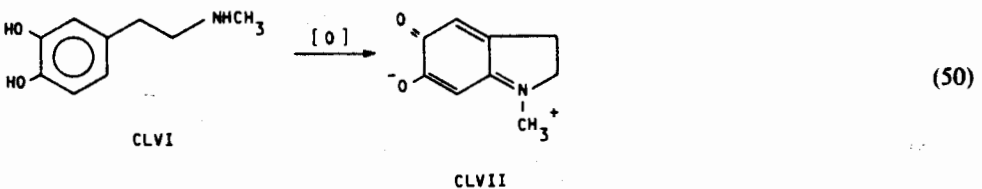
CLIVCLV

Table 27
INTERFERENCES IN THE
ANALYSIS OF
CORTICOSTEROIDS WITH
BLUE TETRAZOLIUM

Substance	Ref.
Acetone	331
Allantoin	331
Alloxantin dihydrate	303
Amphotericin B	331
Bacitracin	331
Dyclonin hydrochloride	331
Erythromycin	303
Erythromycin stearate	331
Lanolin	331
Novobiocin sodium	331
Nystatin	331
Peroxide, diethyl-	331
Polysorbate 60	331
Sorbitan monostearate	331
Sorbitan monooleate	331
Sulfur	331
Sulfide	331
Tetracyclines	303
Xylocaine	331

d. Oxidation of Catecholamines to Adrenochromes

Derivatives of methyl 2-(3,4-dihydroxyphenyl)ethylamine (CLVI) can be oxidized to adrenochrome derivatives (CLVII) as shown in Equation 50.

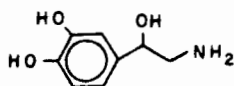
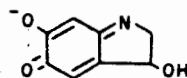


The adrenochromes are highly colored and can be used for spectrophotometric determination of the corresponding catecholamine. However, the adrenochromes are susceptible to further reaction to form adrenolutins which do not absorb in the visible but are fluorescent. Fluorimetric determination of catecholamines as adrenolutins is discussed in Chapter 4, Section V.D. of this chapter, and is much more frequently employed in pharmaceutical analysis than is their measurement as adrenochromes.

The oxidation is usually carried out in an aqueous buffer at a pH between 6 and 7. Highly alkaline solutions cause conversion of the adrenochromes to adrenolutins, and therefore must be avoided. The most frequently used oxidizing agents are iodine and potassium ferricyanide, but a variety of other oxidizing agents can be used. Arterenol (CLVIII), for example, has been analyzed by measuring the absorbance of the noradrenochrome (CLIX) formed by oxidation with iodine in a pH 6.0 acetate buffer.¹⁷⁰ The absorbance was measured at 529 nm.

Table 28
U.S.P. AND N.F. COLORIMETRIC ANALYSES OF STEROIDS USING
ALCOHOLIC SULFURIC ACID

Item	Reagent	Time and temperature	Wavelength
Ethinyl estradiol U.S.P. and Ethinyl estradiol tablets U.S.P.	70% Sulfuric acid in methanol	Room temperature, very short time	538 nm
Mestranol U.S.P.	70% Sulfuric acid in methanol	25°C for 6 min	545 nm
Mestranol tablets N.F.	70% Sulfuric acid in methanol	25°C for 6 min	545 nm
Prednisolone tebutate U.S.P.	125 ml Concentrate sulfuric acid + 350 ml methanol	100°C for 1 hr	550 nm

CLVIIICLIX

e. Development of Color in Steroids with Concentrated Alcoholic Sulfuric Acid

Many steroids undergo complex rearrangements in concentrated solutions of sulfuric acid in alcohol leading to products which can be determined colorimetrically or fluorometrically. Since the majority of the applications of this reaction are in fluorometric analysis, the discussion of reaction products and conditions is presented in Chapter 4 rather than here. Colorimetric applications of the reaction specified in the compendia are summarized in Table 28.

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