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REVIEW ARTICLE

Stability-Indicating Assay Methods for Drugs and Their Dosage Forms

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Several years ago, pharmaceutical scientists used to distinguish between *control* and *stability assay* methods. Batches of pharmaceuticals were released for testing or marketing on the basis of the control assay, whose primary attribute was that it provided a precise and accurate measure of the amount of drug incorporated in a formulation. In some cases, the same method could be used for stability studies, but often a special method, the stability assay, was developed for use in determining the integrity of the drug substance during accelerated shelflife studies. Advances in analytical technology and the legal requirements these have fostered have made the stability-indicating assay the criterion for product acceptance. The dichotomy between assays that measure the amount of drug added and those that measure the amount remaining persists in the use of content uniformity testing.

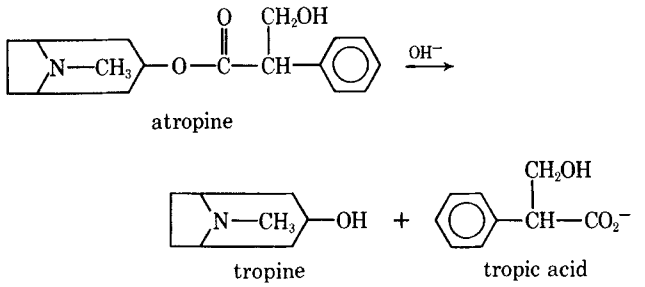
A stability-indicating assay method may be defined as a procedure that affords the selective determination of a drug substance in the presence of its decomposition and reaction products. Drug substances, the majority of which are highly refined organic chemicals, have intrinsic stability under their usual storage conditions. This stability property can often be predicted from inspection of chemical structure and physical properties. Moreover, the environment of a drug substance, *per se*, is easily controllable; there is relatively little limitation on the amount of sample that can be taken for testing; and there is no constraint on the variety of testing techniques and apparatus that can be applied. Most compendial assays for drug substances are relatively nonselective functional group assays chosen for their accuracy; however, the assay is invariably supported by other monograph requirements which provide information on drug quality.

A drug dosage form could be considered as a deliberate contamination of a therapeutic principle, a sophistication which is perpetrated to achieve uniformity and convenience of dosage, product identity, and the physical properties which enable it to be mass produced and shipped in commerce. Depending on its dosage, the active principle in a dosage form may be the major component on a weight basis or a very minor fraction. For example, meprobamate tablets may be 75% drug, while ethinyl estradiol may constitute only 0.007% of the total weight of a tablet. Because a drug dosage form represents a dilution or dispersion of a drug with other substances, the analytical problems with it are more complex and the stability less easily predictable than with the drug substance by itself. A procedure which is stability indicating for a drug in one instance is not necessarily valid for the drug in another environment where reaction possibilities differ. As used here, *stability indicating* is a relative, not an absolute, descriptor in assessing the validity of a method; one method may be more nearly valid than another without either of them being absolutely conclusive. This review provides a critical examination of assay methods for representative drugs with respect to their usefulness in establishing and monitoring chemical stability.

With few exceptions (1, 2), assay methods are combinations of separation and measurement operations. Separation may be as simple as dissolution of a drug from a solid dosage form matrix with filtration of insoluble excipients or as complex as differential migration in a chromatographic system. Measurement could be as simple as weighing a precipitate, or it might require elaborate and expensive instrumentation. The selectivity required may be derived primarily from the separation operation or from the measurement technique used, but it is a function of the method considered as a whole. Obviously, there can be several appropriate approaches to devising stability-indicating assay methods.

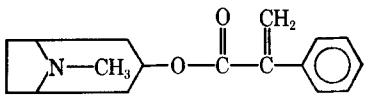
One could determine drug stability or instability, *i.e.*, measure intact drug by a highly selective method, measure total drug content nonselectively and estimate the degradation products, or selectively measure both drug and decomposition products. *A priori*, the third alternative is the most desirable; however, it may not be either possible or practical.

Selective Determination of Active Drug—Determination of intact drug in the presence of decomposition products requires knowledge of degradation routes in most cases. Some investigators (3, 4) considered that the most likely degradation route for atropine would be ester hydrolysis with formation of tropine and tropic acid (Scheme I). They measured hydrolysis rates using a

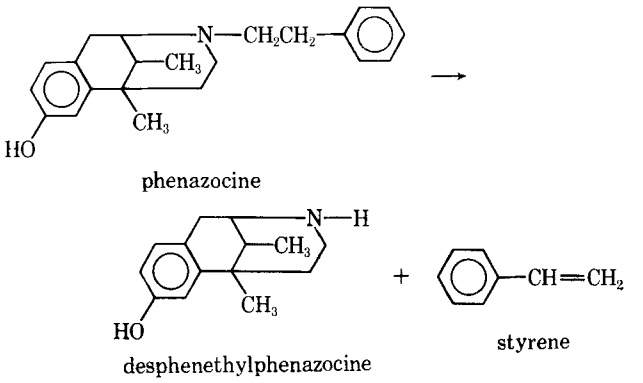


Scheme I

modification of the compendial Salts of Organic Nitrogenous Bases method, where the amine is separated by extraction from alkali into an organic solvent, extracted into dilute aqueous acid, and determined by UV spectrophotometry. Since the UV absorption of atropine is conferred by the benzene ring in the tropic acid moiety, and tropic acid present as a result of hydrolysis would be retained as the anion in the aqueous alkali, the procedure would appear to be valid as a measure of ester hydrolysis. Recently, however, other investigators (5, 6) criticized these studies on the basis that they did not recognize the concomitant formation of apoatropine, which has a much more intense UV spectrum than atropine.



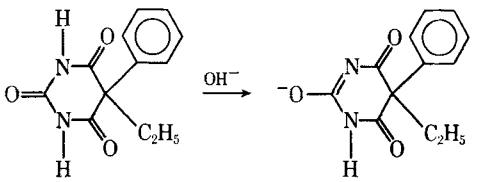
Eisdorfer *et al.* (7) found that phenazocine HBr was unaltered by thermal stress either as the salt or in acid solutions, but the base or neutral or alkaline solutions showed evidence of degradation. They determined that decomposition produced styrene and a secondary amine (Scheme II). They recommended two methods for



Scheme II

determining the drug in the presence of its decomposition products. In one, phenazocine was separated by cyclohexane extraction from alkaline solution and then determined by differential UV spectrophotometry in an extract of the organic solvent. In the other, phenazocine was determined by nonaqueous titration after chloroform extraction of a 0.1 *N* hydrochloric acid solution.

Tishler *et al.* (8) studied assay methods for phenobarbital in relationship to its degradation products. They determined that the UV spectrum of the drug at pH 10, due to the monoanion of the ureide ring, was greatly diminished with cleavage of the ring to decomposition products (Scheme III). Only the intact drug



Scheme III

showed a difference in the UV spectrum with a change in pH from acid to alkaline. They recommended differential UV spectrometry, measurement of an aliquot at pH 10 *versus* an acidified aliquot, as a stability-indicating assay method for phenobarbital. Since the spectra of other 5,5-disubstituted barbiturates are similar to phenobarbital, the method is generally applicable.

In the foregoing examples, the decomposition products were well characterized; however, there are many instances where this situation does not obtain. Khoury and Cali (9) demonstrated the specificity of the fluorometric determination of ethinyl estradiol by TLC of estrogen samples that had been partially degraded by heating or by exposure to light. The chromatograms exhibited several spots when sprayed with phosphomolybdic acid, a general oxidizing agent, but only one fluorescent spot, corresponding in R_f to the intact drug, when the fluorogenic[1] reagent, 90% sulfuric acid, was

[1] As used in this paper, a fluorogenic or chromogenic reagent is a reactant used to produce fluorescence or an absorption band. A fluorophore or chromophore is a molecule that fluoresces or has an absorption band.

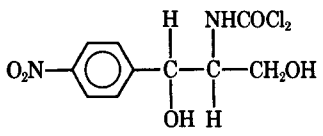
used for detection. Although it might be more satisfying intellectually to know the mechanism of the fluorogenic reaction and the decomposition pathways, this example illustrates the validation of an empirical method where the chemistry of neither has been elucidated. The use of chromatography on paper or thin layers in conjunction with the fluorogenic or chromogenic reagent for spot detection is generally applicable.

Chafetz *et al.* (10) used gas chromatography as the reference method for the methanol–sulfuric acid colorimetric assay for quinestrol, ethinyl estradiol 3-cyclopentyl ether, after intentional degradation of the drug with hydrogen peroxide, dilute acid, and base. They obtained excellent correlation of gas chromatographic and colorimetric assay values for intact drug. Templeton *et al.* (11) used a similar scheme to validate mestranol assays; however, they used intentionally degraded tablets. Use of the dosage form as the model may have advantages in providing additional opportunities for drug decomposition, but excipient decomposition under unrealistic stress conditions may complicate the interpretation of results. It is often possible to correlate actual drug decomposition in dosage forms with stress-induced decomposition of the pure drug substance by comparison of the gas chromatographic or thin-layer patterns produced by the degradation products.

Solubility Analysis as a Reference Method—Comer and Howell (12) evaluated the use of solubility analysis in judging the suitability of routine stability-testing methods for a number of drugs. They pointed out that it is only possible to use this tedious technique on the drug substance free from formulation excipients. In one instance, with propoxyphene, they found no decomposition after thermal stress either by IR spectroscopy, the stability test method, or the reference method. The suitability of the IR method was not established.

Biological Assays as Reference Methods—Since drug assays by physicochemical means are intended to assure the biological activity characteristic of the intact drug, the direct measurement of biological activity would appear to be ideal. In many cases, however, the endpoint of a biological test cannot be measured precisely enough to distinguish between a product that would pass and one that would fail by physicochemical assay. Where the drug is antimicrobial, microbiological assay is often both relatively accurate and convenient.

Higuchi and his students (13–16) performed a classical study on the stability of the antibiotic chloramphenicol. *A priori*, they considered that the most likely



chloramphenicol

degradation routes would be hydrolysis of the amide function or of covalently bound chlorine, and they measured the rates of both reactions. The rate of amide hydrolysis, determined by a column partition chromatography procedure selective for intact drug, was significantly faster than formation of ionic chloride, indicating that the first route was the primary degradative pathway. They inferred from the excellent correlation obtained between the chromatographic assay and a microbiological assay that racemization could not be an important decomposition route.

Garrett (17) showed that a microbiological assay for tetracycline yielded an overestimate of its potency when applied to partially degraded samples, because the decomposition product, although much lower in antibacterial potency, diffused more readily in the culture medium than did the intact antibiotic drug. Here, as is often the case with physicochemical measurements, the sensitivity of the analytical measurement differed markedly for the intact drug and its decomposition products. As with any other method, the specificity of a biological assay should not be taken for granted.

Estimation of Decomposition Products—Using an assay method selective for the intact drug molecule usually entails accepting a relative standard deviation of 1% or more. In stability studies on new drugs or new formulations, it is often difficult to discriminate between normal assay variation and incipient degradation without several data points or an independent measure of degradation products. Accurate measurement of degradation products requires their characterization; however, estimation of their amounts often suffices when they are present in relatively small amounts, *i.e.*, about 1%. In those relatively rare instances where drug degradation leads to a toxic product, as in the formation of nephrotoxic 4-epianhydrotetracycline from tetracycline, the pharmaceutical chemist is obligated to control and limit the amount formed (18–21).

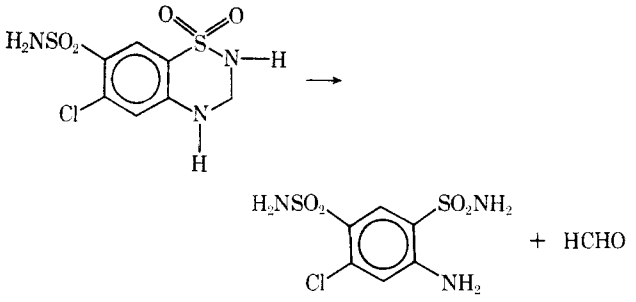
Paper, thin-layer, and gas chromatography techniques have made monitoring decomposition products relatively simple; however, their effective use requires some sophistication. A number of systems of differing polarity should be employed in searching for unknown decomposition products, and the system chosen for routine use should have been demonstrated capable of separating known decomposition products. It is sometimes difficult to discriminate between drug decomposition products and trace impurities present in the drug substance as synthesis contaminants and precursors.

Drug impurities are usually controlled to a level of 1–2%, a concentration below that in which one is generally interested in drug degradation. Since the detectability of spots or peaks on a chromatogram is a function of the loading of the system, the detection technique used, and other factors, the parameters often can be adjusted so that trace impurities are not evident but decomposition above the 1% level is seen. Artifacts from formulation excipients may further confound interpretation of chromatograms, but these can be identified by examination of a blank formulation such as a placebo used in clinical studies.

Quantitation by Means of Decomposition Products—With many drugs, the analytical features of the decomposition product may be more attractive than the intact drug. For example, salicylic acid has a UV absorption band at a long wavelength and more intense absorption than aspirin, and salicylic acid forms an intensely colored chelate with ferric ion. One could measure intact aspirin in a dosage form by the dif-

ference in values of determinations of free salicylic acid and total salicylic acid determined after intentional hydrolysis. (This is rarely done, since relatively simple means for separation of intact aspirin are available.)

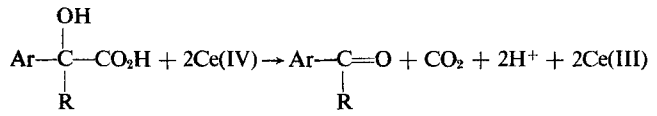
Rehm and Smith (22) studied the stability of hydrochlorothiazide. They found that the drug undergoes a reversible hydrolytic reaction to form 4-amino-6-chloro-1,3-disulfonamide and formaldehyde (Scheme IV). The



Scheme IV

disulfonamide hydrolysis product can be diazotized with nitrous acid and coupled with chromotropic acid or other phenolic compounds to give intensely colored azo dyes. They determined total hydrochlorothiazide by alkaline hydrolysis prior to diazotization and coupling.

Chafetz (23) suggested an approach to the stability analysis of arylglycolate ester drugs, of which eucatropine, glycopyrrolate, homatropine, mepenzolate, oxyphencyclimine, and penthienate are examples recognized in the USP and the NF. The method is based on the selective oxidation of free arylglycolic acids with ceric ion according to Scheme V, where Ar is aromatic

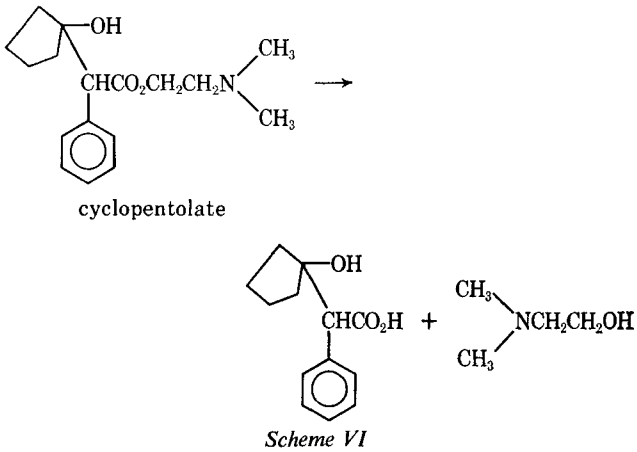


Scheme V

or heterocyclic and R is aromatic, aliphatic, alicyclic, or hydrogen. The formation of a carbonyl group in conjugation with an aromatic ring affords a tremendous increase in UV absorptivity over that of the parent compound, and the chromophore is easily isolated by extraction with a nonpolar solvent. Since intact esters are not oxidized in the reaction, one can: (*a*) determine free arylglycolic acid present as the result of drug hydrolysis; (*b*) determine the total amount of arylglycolate ester by oxidation after saponification; and (*c*) estimate the amount of intact ester by the difference in values. Chafetz and Daly (24) described a variation of this technique for homatropine HBr ophthalmic solutions, determining by titration the amount of ceric ion consumed by saponified and untreated aliquots. Although the titration method eliminates the need for a reference standard, it is less selective than the UV assay.

Solvent Extraction Methods—Extracting the unionized form of an acid or basic drug into an organic solvent and then returning it to aqueous medium for measurement is perhaps the most widely used separation procedure because of its simplicity. Because of its extensive use for compendial drugs, a general method for Salts of Organic Nitrogenous Bases is provided in the USP and the NF. This method uses UV spectrophotometry as the measurement after isolation of the drug by transfers between aqueous base and acid. The method affords separation from acidic and neutral chromophores which might be present in a formulation.

Solvent extraction is often used as a preliminary to titration. The USP XVII assay for Homatropine Hydrobromide Ophthalmic Solution required basification of a measured volume of the preparation, extraction of the base with several portions of chloroform, and titration of it with standard acetous perchloric acid. Chafetz and Daly (24) critized the method, pointing out that the aminoalcohol formed by hydrolysis, tropine, would be extracted and measured as homatropine by this procedure. The USP assay for Cyclopentolate Hydrochloride Ophthalmic Solution is similar, except that prescribed volumes of hexane are used for extracting the base. If one considers hydrolysis as the most probable degradation route, 2-dimethylaminoethanol would be the basic decomposition product formed (Scheme VI).



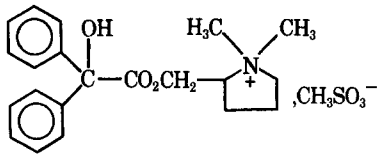
Scheme VI

The partition coefficients for this aminoalcohol between chloroform–water, ether–water, and isooctane–water are 0.22, 0.044, and 0.0043, respectively (25). Hexane should extract very little of the decomposition product; thus the method is stability indicating with respect to hydrolysis. In general, choice of an extraction solvent with the least possible polarity that accomplishes the desired separation provides the greatest selectivity.

Partition chromatography can be considered a special form of solvent extraction; however, the ability to control polarity often affords an extremely high degree of selectivity. Moody (26) described a simple and elegant partition chromatographic method for isolating ephedrine, amphetamine, and other water-miscible amine bases from dosage forms. A portion of the drug dosage form is incorporated with dilute sulfuric acid on chromatographic siliceous earth in a glass column, the column is washed with water-saturated chloroform, and then the internal phase is made alkaline by adding ammonium hydroxide adsorbed on the support. The drug is eluted with wet chloroform, extracted into dilute sulfuric acid, and determined by UV spectrophotometry. Essentially, the method is a highly efficient version of the Salts of Organic Nitrogenous Bases procedure, and it provides the same information.

Dye-Complex and Ion-Pair Formation—Separations by ion-pair formation have been used for many years, although recognition of the phenomenon has been relatively recent. Auerbach (27) used the formation of a solvent-extractable dye complex to measure benzalkonium chloride. In general, ion-pair formation may be considered as an equilibrium process in which oppositely charged ions (neither ion by itself extractable into an organic solvent) form a solvated species in which the hydrophilic ionic moieties are hidden within a hydrophobic envelope. Most of the dye-complex methods use sulfonated dyes as the anion in the pair, and they measure the color of the extracted (ion-paired) dye to provide an indirect measurement of the drug. Since these methods depend on a substituted ammonium function and measure the reagent consumed, they are no more selective than acidimetric titration.

Singleton and Wells (28) reported that dye-complex methods for poldine mesylate did not distinguish between the drug and its hydrolysis product; however, they found that measurement of the chloroform-extracted color of the complex formed by reaction of poldine with ammonium cobaltothiocyanate is selective



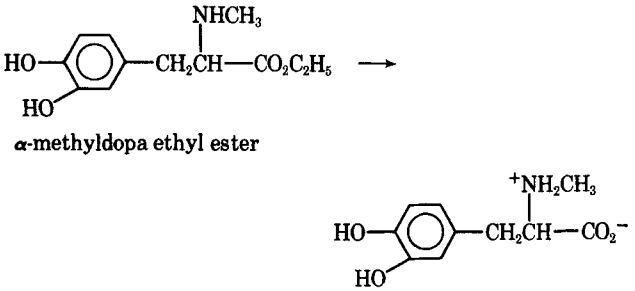
poldine mesylate

for the intact ester. It is likely that the pyrrolidinium alcohol formed by hydrolysis complexes with the cobaltothiocyanate also, but the charged species are not sufficiently well shielded to permit solvent extraction. Higuchi and coworkers (29, 30), Levine and coworkers (31–33), and Temple (34) are among a number of investigators who studied ion-pair techniques for separation of drugs from each other. Relatively little work has been directed toward application of these techniques to separating drugs from their degradation products, although this would seem to be a fruitful direction for exploration. Eisdorfer *et al.* (7) demonstrated that phenazocine could be separated from its desphenethyl decomposition product by extraction with chloroform from dilute hydrochloric acid, undoubtedly an ion-pairing phenomenon. Extraction of lipophilic amines from hydrochloric acid with chloroform is frequently observed. Other anions, such as *p*-toluenesulfonate, trichloroacetate, and cyclamate, can be used; variations in pH, concentration, and solvents can provide very efficient separations.

INCOMPATIBILITIES IN DOSAGE FORMS

Where a pure drug substance may be subject to decomposition by the action of light, heat, adsorbed moisture, and oxygen, incorporation of it in a dosage form extends the possibilities for degradation. Lach and his students (35–39) demonstrated a number of drug–drug and drug–excipient interactions in solid dosage forms which affect drug stability and bioavailability. Solutions of drugs afford much more opportunity for chemical reaction than solid dosage forms; for example, no one has yet provided a stable aspirin elixir, although the preparation of one is a classical problem in pharmaceutics. Solubility and pH requirements for one component of a formulation may provide a hostile environment for another. Talmage *et al.* (40) discovered that cyclamate hydrolyzed to a significant extent in a hydroalcoholic solution used as a vehicle for an antibacterial amine. Although the sweetener is very stable in water, its hydrolysis rate was found to be proportional to the alcohol content of a vehicle and inversely proportional to pH. Considerations of isotonicity and tolerance impose limits on formulation variables for injections and eye solutions, often affording suboptimal conditions for drug stability.

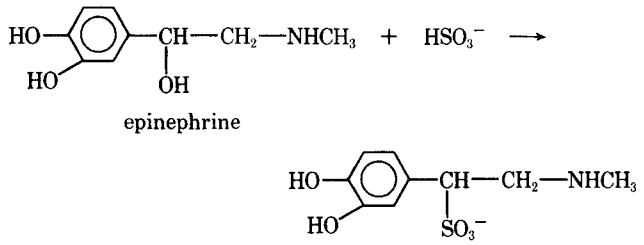
Catecholamines–Bisulfite Reaction—Catecholamines such as epinephrine, isoproterenol, and levarterenol are easily oxidized by air and other oxidants to adrenochromes and products of its further oxidation. Doty (41) showed that catechols form intensely colored derivatives with a ferrocitrate reagent. Since oxidation of epinephrine and its analogs destroys the catechol function on which the Doty reaction depends, the colorimetric method is stability indicating with respect to oxidation. Higuchi *et al.* (42) assayed epinephrine by UV spectrophotometry of its triacetyl derivative following chromatographic isolation of the derivative. The results obtained by this method and the Doty reaction were comparable on solutions degraded by oxidation. Marcus and DeMarco (43) determined α-methyldopa ethyl ester by the Doty reaction after partition chromatography on buffered cellulose. The chromatographic procedure separated the ester from its hydrolysis product (Scheme VII); however, oxidized ester was eluted with the intact drug. Because the measurement technique was selective for the unoxidized compound and interference by the hydrolysis product was eliminated in the procedure; the overall procedure was stability indicating with respect to both routes of degradation.



α-methyldopa ethyl ester

Scheme VII

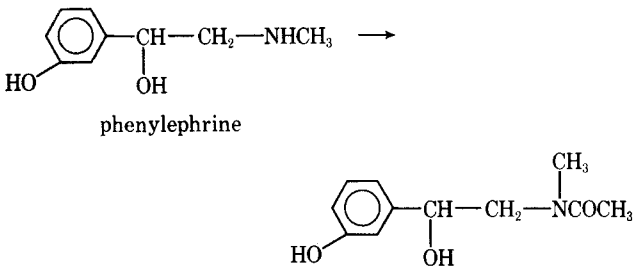
Schroeter *et al.* (44) reported that epinephrine reacts with sodium bisulfite, added to aqueous formulations to inhibit oxidation, to form a biologically inactive sulfonic acid derivative (Scheme VIII). Because the sulfonic acid product retains the catechol group, the Doty reaction does not distinguish between the drug and its reaction product; however, the triacetylation method is applicable. Several years later, Welsh and Sammul (45) reported that isoproterenol, the *N*-isopropyl homolog of epinephrine, reacts with bisulfite in the same way. They found that extraction of the ion pair formed by the catecholamine with bis(2-ethylhexyl)phosphoric acid



Scheme VIII

provides a means for separating isoproterenol from its reaction product, and they used UV spectrophotometry for measurement. These authors cited unpublished work, by E. L. Pratt and M. E. Auerbach of the Sterling-Winthrop Research Institute, which showed that oxidation to the fluorescent adrenolutin also distinguished the drug from its reaction product. Recently, Kaistha (46) proposed two methods for assay of isoproterenol in the presence of decomposition products: a modification of the ion-pairing technique and determination as 3,4-dihydroxybenzaldehyde after periodate oxidation. The latter requires an 18-hr. reaction time in acid solution. The author proposed use of these techniques for tablets as well as solutions, although the rationale for its use with tablets is not clear.

Aspirin Acetylations in Tablets—Troup and Mitchner (47) discovered that phenylephrine reacts with aspirin in tablet formulations, with *N*-acetylation occurring at room temperature and progressive acetylation of the phenolic and secondary alcohol —OH functions taking place during storage at elevated temperatures (Scheme IX). They used a combination of two



Scheme IX

assay methods: (*a*) the 4-aminoantipyrine–ferricyanide reaction (48), selective for a phenol with H or an easily displaceable *para*-substituent, and (*b*) a colorimetric method selective for the secondary amine function, measurement of the cupric-ion complex of the dithiocarbamic acid formed by reaction of the drug with carbon disulfide. One would expect that the Kelly and Auerbach (49) assay method for phenylephrine, colorimetric determination of the azo dye formed by coupling with *p*-nitrobenzenediazonium chloride following separation by ion-exchange chromatography, would be applicable in determining phenylephrine in the presence of *N*-acetylphenylephrine. The Levine and Doyle (50), procedure, partition chromatography of the bis(2-ethylhexyl)phosphoric acid ion pair followed by UV spectrophotometry, should also be stability indicating for this incompatibility. Determination of phenylephrine as 3-hydroxybenzaldehyde as described by Chafetz (51) will distinguish between the drug and its reaction products.

Jacobs *et al.* (52) showed that aspirin can acetylate the alcohol function of codeine at elevated temperature in the presence of moisture; however, the reaction proceeds slowly. They detected the incompatibility by TLC, and they recommended a partition chromatographic method for its separation and determination.

Koshy *et al.* (53) and Boggiano *et al.* (54) found by chromatography that acetaminophen is acetylated at the phenolic function by aspirin in some commercial formulations (Scheme X). Many of the colorimetric methods (55–57) described for acetaminophen require preliminary hydrolysis of it to *p*-aminophenol; none of these would be stability indicating with respect to this incompatibility. The colorimetric method described by Le Perdriel *et al.* (58), measurement of 2-nitro-4-acetamidophenol, formed by nitration of the drug with nitrous acid, would appear to be selective for acetaminophen in the presence of its reaction product, because this reaction is peculiar to phenols.

Some Further Incompatibilities—One may generalize from the aspirin interactions already noted. Of these, the incompatibility with phenylephrine appears the most serious, probably because amides are more stable than esters of alcohols or phenols. One would expect that transacylation reactions in dosage forms may occur with mixtures of esters or other acyl compounds with primary or secondary amines, alcohols, and phenols. Relatively labile esters such as diethylaminoethyl esters of aromatic acids, *e.g.*, tetracaine and procaine, would serve as good sources of acyl groups in such reactions. The ubiquitous use of chromatographic techniques will doubtless yield further examples of transacylation reactions.

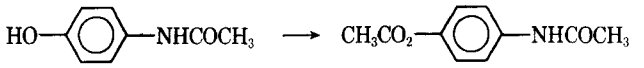
Metal ions have been found to catalyze the decomposition of many drugs. El-Shibini *et al.* (59) found that cupric ion, and lead and iron to a lesser extent, catalyze the decomposition of alkaline phenylephrine solutions. Lewbart and Mattox (60) and Monder (61) reported oxidation of the ketol function of hydrocortisone to a glyoxal by traces of copper ion, and several others (62–64) reported trace metal catalysis of the oxidative degradation of corticosteroids.

Duvall *et al.* (65) attributed the browning of spray-dried lactose to formation of 5-hydroxymethylfurfural. This and other aldehydes in pharmaceutical formulations may react with primary amines to form Schiff bases. Kabasakalian *et al.* (66) showed that benzocaine reacted with citric acid, corn syrup, and natural cherry flavor in a throat lozenge formulation. In each case, the ester group was shown to be intact in the product, and the incompatibility involved the aniline moiety.

MEASUREMENT OF LABILE FUNCTIONAL GROUP

A functional group assay[2] is one that is selective for a particular structural feature in a molecule. Provided that the structural feature determined does not persist in any of the decomposition products, such a method will be stability indicating. Inspection of the structure of chloramphenicol (page 337) shows that its UV

[2] The term is often applied to group-selective reactions. As used here, it applies both to these and to physical methods that are dependent on specific structural features.

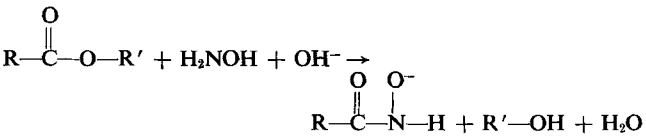


acetaminophen

Scheme X

spectrum derives from its *p*-nitrobenzyl alcohol component. However, several years after publication of the definitive studies by Higuchi *et al.* (13–16), direct UV spectrophotometry was a legally acceptable alternative to microbiological assay for chloramphenicol dosage forms. Schwarm *et al.* (67) criticized this, demonstrating its invalidity by quantitative TLC. Polarographic assay (68) suffers from the same limitation; it measures the nitrobenzene moiety common to the antibiotic and its hydrolysis product. Although neither spectrophotometry nor polarography is a stability-indicating measurement, either could be used in a stability-indicating procedure if provision is made to separate the amine degradation product from the neutral amide intact compound.

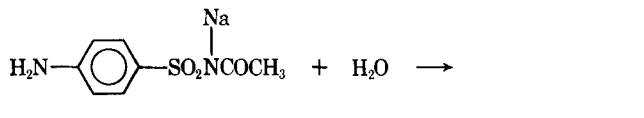
Ferric–Hydroxamate Colorimetry—Alkaline solutions of hydroxylamine react with esters, amides, acyl halides, and other acyl derivatives to form hydroxamic acids (Scheme XI). The red-colored complex formed



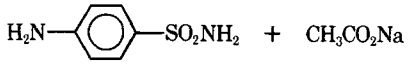
Scheme XI

by treatment of the hydroxamic acid with ferric salts in acid solution serves as the basis of a useful colorimetric method. Carboxylic acids do not react with hydroxylamine under these conditions; thus the method is stability indicating with respect to hydrolysis.

Colorimetric analysis of chloramphenicol has been reported using this procedure (69), and it would appear to be valid. Siegel *et al.* (70) used the method as a means for following the hydrolysis rates of methylphenidate. Schleider *et al.* (71) recommended the method for determining sodium sulfacetamide in the presence of its hydrolysis products, pointing out that the official nitrite titration assay will not distinguish between the drug and its sulfanilamide hydrolysis product, because both bear the primary amine function on which the titration is based (Scheme XII).



sodium sulfacetamide

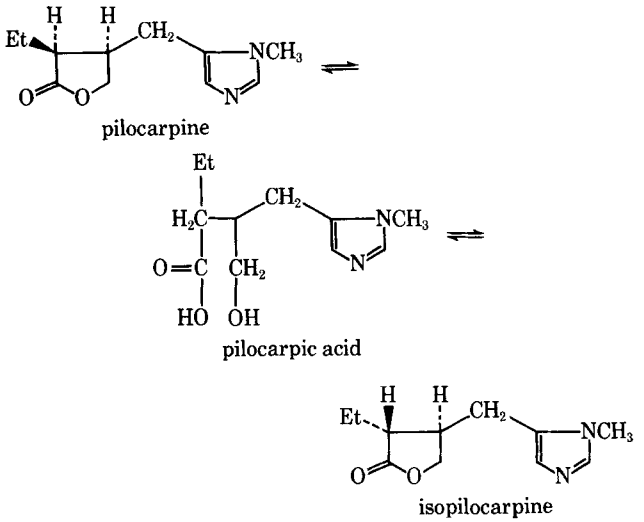


sulfanilamide

Scheme XII

Vincent and Schwal (72) investigated ferric–hydroxamate colorimetry for a number of alkaloids, including pilocarpine, cocaine, physostigmine, atropine, and scopolamine. Of the alkaloids tested, the tropine esters were found to give very weak colors, perhaps because the hydrolysis reaction rate predominates over the rate of hydroxylaminolysis. A number of workers, including Brochmann-Hanssen *et al.* (73), Gibbs and Tuckerman (74), and Baeschlin *et al.* (75–77), recommended this method for analysis of pilocarpine eye solutions.

Baeschlin *et al.* (75–77) used the method in conjunction with TLC and NMR spectroscopy, reporting that it is useful in studying the position of the equilibrium between pilocarpine and pilocarpic acid. Anderson (78) and Anderson and FitzGerald (79), however, used optical rotation measurements to show that an additional equilibrium should be considered, inactivation of pilocarpine through conversion to isopilocarpine (Scheme XIII). Since both stereoisomers have the lac-

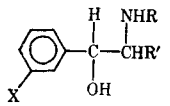


Scheme XIII

tone function measured by the colorimetric assay, it provides an overestimate of solution potency. It would appear that neither the TLC nor NMR method discriminated between the isomers.

Spectrophotometry after Oxidation of Phenalkanolamines—Nicolet and Shinn (80) showed that periodate oxidizes compounds with vicinyl hydroxyl and primary or secondary amine functions to aldehydes and ammonia or a primary amine. Pisano (81) used the reaction as the basis for an assay of metanephrine and normetanephrine in urine, determining the UV absorbance of the vanillin formed by oxidation. Heimlich *et al.* (82) used the reaction to determine phenylpropanolamine in urine as benzaldehyde. Chafetz (51) described dosage form assay methods for phenylpropanolamine, ephedrine, and phenyramidol as benzaldehyde and phenylephrine as 3-hydroxybenzaldehyde. It has been shown (83) that compounds determinable by this method must have the general structure shown in Table I, that the amine function must be basic and unhindered sterically, and that acylated compounds and oxidation products do not interfere. Catecholamines are oxidized to adrenochromes, which do not interfere in methods where the aromatic aldehyde reaction product is separated by solvent extraction before spectrophotometry. The method will not distinguish between optical isomers; however, racemization does not appear

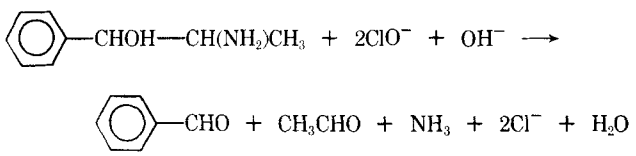
Table I—Structure of Some Phenalkanolamine Drugs



Compound	X	R	R′
Ephedrine	H—	CH_3—	CH_3—
Phenylpropanolamine	H—	H—	CH_3—
Phenylephrine	—OH	CH_3—	H—
Metaraminol	—OH	H—	CH_3—

to be an important degradation route for this class of drugs (84, 85).

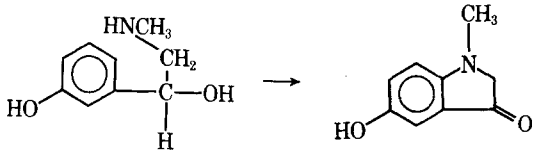
Chafetz *et al.* (86) recently described an alternative method for determination of ephedrine and related compounds; oxidation to benzaldehyde with alkaline sodium hypochlorite (Scheme XIV). Unlike periodate oxidation, this method can be applied directly to the drugs in syrups, and the reaction is significantly faster.



Scheme XIV

However, the selectivity of the method has not been studied as extensively.

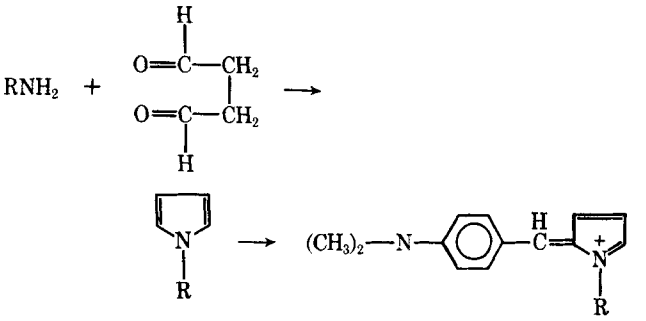
Some Further Methods for Amine Functions—Schriftman (87) used quantitative paper chromatography to study the degradation of phenylephrine HCl solutions in water, 50% glycerin, and 5% dextrose after heating at 75° for 1 week. He found that only intact phenylephrine could be detected with ninhydrin, a reagent applicable to the determination of primary and secondary amines. El-Shibini *et al.* (88) used the cupric dithiocarbamate method described by Troup and Mitchner (47) to follow the rates of phenylephrine decomposition in alkaline solution. The Egyptian workers suggested that phenylephrine cyclizes to form 5-hydroxy-*N*-methylindoxyl; however, they offered no proof of structure (Scheme XV).



phenylephrine

Scheme XV

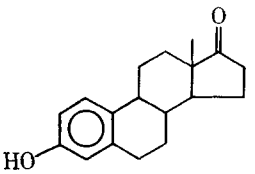
Whatever the structure of decomposition products, selective determination of the secondary amine function would appear to be informative. Sekhon *et al.* (89) and Fujimoto and Ose (90) described photometric methods for ephedrine using ninhydrin. Sawicki and Johnson (91) described a colorimetric method for primary amines, producing an indole by condensation with 2,5-dimethoxytetrahydrofuran, the diacetal of succindialdehyde, and condensing the indole with Ehrlich's reagent (Scheme XVI). This scheme has been used for automated colorimetry of phenylpropanolamine (92) and for the determination of amphetamine (93).



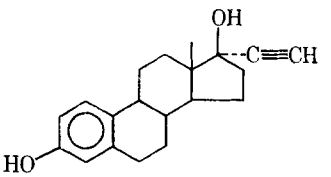
Scheme XVI

Feldmann (94) used the reaction of procaine with 1,2-naphthoquinone-4-sulfonate to determine this primary aromatic amine, reporting no interference from other anesthetics that had no primary aromatic amine function, *e.g.*, meprylcaine, lidocaine, and piperocaine. Auerbach and Angell (95) used this reagent for determining norepinephrine in epinephrine. Pesez and Bartos (96) described a fluorometric method for primary and secondary aralkylamines in which their reaction products with 1,2-naphthoquinone-4-sulfonate are extracted with methylene chloride, reduced with potassium borohydride, and measured after acidification.

Methods for Estrogenic Steroids—Estrogenic steroids have phenolic A-rings and an oxygen function at C-17, as typified by the structures of estrone and ethinyl estradiol. Intentional degradation experiments on



estrone



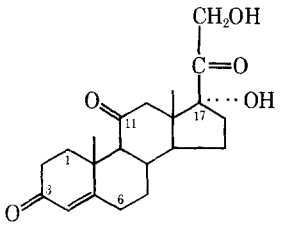
ethinyl estradiol

ethinyl estradiol (9) and two of its 3-ethers (10, 11) monitored by chromatography, showed only small amounts of identifiable products among the several products formed. Empirically, however, the sulfuric acid-induced fluorescence and color have been shown to provide methods selective for these estrogens in the presence of their degradation products and other estrogens. The Kober reaction (97, 98) and its many modifications, production of a pink color by reaction of estrogens with sulfuric acid–hydroquinone or sulfuric acid–phenol–iron, is remarkably sensitive to small changes in structure. Empirical methods based on these reactions have been described for the determination of the components of conjugated estrogens extracted from equine urine (99, 100). Jones and Hähnel (101) studied the specificity and mechanism of the Kober reaction, and they proposed that the Kober chromophores are resonance-stabilized carbonium ions.

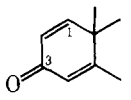
The phenolic moiety of the estrogens provides a UV chromophore, which is similar for most of them, and many of the compounds fluoresce in acid solution. This latter property is used by the British Pharmacopoeia (102) in the assay of ethinyl estradiol tablets. Because they are phenols, they may be coupled with diazonium salts to form azo dyes. Colorimetric methods based on this reaction were described by Urbanyi and Rehm (103)

and others. It has not been established whether, or to what extent, these methods are stability indicating. Most of the attention of investigators of estrogen stability has been directed to development of selective separation procedures for intact drug. Determination of the fate of various functions in the estrogen molecule in its dosage forms or characterization of the principal degradation products would provide valuable insight into the selectivity of these methods.

Corticosteroid Methods—The structure of cortisone and a partial structure of prednisolone, which may be considered prototypes for this important class of drugs, are represented here. The unsaturated ketone system



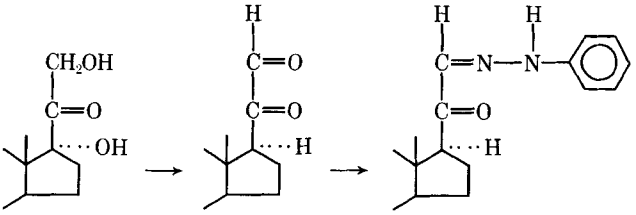
cortisone



prednisolone

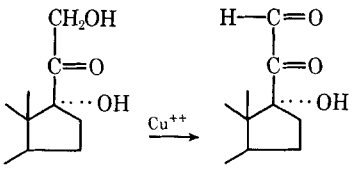
in the A-ring of these steroids is a very good UV chromophore. Görög (104) published a differential UV spectrophotometric method for conjugated ketosteroids. The Δ^4-3-keto chromophore is reduced by sodium borohydride at room temperature to a Δ^4-3-hydroxy structure, which has no spectrum in the near UV. The $\Delta^{1,4}$-3-one system of prednisolone is reduced to the alcohol, but this requires heating. Determination of an untreated aliquot *versus* a reduced aliquot affords a measure of the steroid in the presence of nonspecific UV chromophores. Umberger (105) described a colorimetric method for steroids with the unsaturated ketone structure, formation of an isonicotinic acid hydrazone by reaction with isoniazid. Avdovich *et al.* (106) reported an NMR method for the $\Delta^{1,4}$-3-one corticosteroids. The method is based on the signals derived from the A-ring protons, and it is applicable to their determination in the presence of the cortisone-type structures and dosage form excipients. Although all of these methods based on the properties of the A-ring are useful, and many of them are applicable to the determination of progestins and androgens, they are not stability indicating for corticosteroids.

The most labile group in the corticosteroid structure is the 17α-ketol, which is easily oxidized to a variety of inactive products (60–64), all of which have unaltered A-rings and which would be undetected by the methods described. Mader and Buck (107) exploited the reducing properties of the corticosteroids by measuring the color produced when tetrazolium salts are reduced to formazan dyes in alkaline solution. Tetrazolium methods have been extensively studied, and the blue tetrazolium method has been adopted for corticosteroid dosage form assay by the official compendia. Since the color measured is that of reduced reagent, the method is subject to interference from easily oxidized excipients. Porter and Silber (108) described a method using phenylhydrazine in alcohol and sulfuric acid to produce a yellow color with steroids containing the dihydroxyacetone group at the 17-position. The chromophore has been identified as the 21-phenylhydrazone formed after acid-catalyzed rearrangement (109, 110) (Scheme XVII).



Scheme XVII

One disadvantage of the Porter–Silber reaction for dosage form assay is that heating with the acid reagent often produces nonspecific color by reaction of excipients. Another is that the reaction is inapplicable to corticosteroids that lack a 17-hydroxy function. Lewbart and Mattox (111) described a modification of the Porter–Silber reaction which overcomes these difficulties. The steroid is oxidized to the glyoxal with methanolic cupric acetate; this product reacts immediately with Porter–Silber reagent (Scheme XVIII). Both 17-desoxy-



Scheme XVIII

α-ketols and steroids with the dihydroxyacetone side chain are chromogens in this procedure. Although traces of copper catalyze the oxidation of corticosteroids to glyoxals, these rearrange readily to α-hydroxyacids, which may be further oxidized. Thus this modification would appear to be stability indicating. This method does not appear to have been reported in drug dosage form assay.

Guttman (112) noted that hydrolysis of the ester was a prerequisite for determination of cortisone-21-acetate by the blue tetrazolium method. This consideration is doubtless true for esters in the Porter–Silber reaction as well. One may infer from this that neither procedure is stability indicating with respect to ester hydrolysis.

SUMMATION

It has become widely recognized within the past few years that such factors as crystal form, particle size, and complexation phenomena, undetectable by chemical analysis, may significantly affect drug performance. Nevertheless, a primary requirement for drugs and their dosage forms is that they contain the declared amount of the intact chemical substance within reasonable and predetermined tolerances. A critical review of some methods for determining this fundamental parameter of drug quality has been presented.

The selection of references cited to illustrate principles has been arbitrary, guided by the experience and predilections of the writer. It is conceded that many important drugs and drug classes have been ignored here, and mention of several elegant studies of drug stability has been omitted.

The introduction of the photoelectric UV spectrophotometer and the glass-electrode potentiometer, both of which became commercially available during the 1930's, provided a quantum jump in the capability and productivity of pharmaceutical analysis laboratories. There has since occurred an enormous proliferation of optical, electrochemical, and chromatographic instrumentation and techniques. Computer systems are now available not only for the rapid reduction of the data gushed out by this instrumentation but for controlling their operation as well. The number and compass of the tools available to the pharmaceutical analyst have provided many more alternative solutions to problems and many more means for corroboration. Notwithstanding this sophistication in hardware, the primary asset of the pharmaceutical analyst lies in the creativity of his approach to a problem.

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RESEARCH ARTICLES

Structural Approach to Partitioning: Estimation of Steroid Partition Coefficients Based upon Molecular Constitution

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Abstract □ Partition coefficients are directly related to the free energy of transfer of a substance between two immiscible phases and thus have a first principal character rarely ascribed to them. In addition, partition coefficients have been shown to be additive constituitive in character, allowing for their calculation from the individual contributions of the molecular components. These factors, taken together, make partitioning a meaningful and convenient physical phenomenon to match against biological activity. For these reasons, the structural relationships between a large group of steroids and their ether–water parition coefficients were explored. Hansch-like π_e values were estimated for a number of functional groups. These data allow the calculation of partition coefficients of highly substituted steroids from stripped steroid skeletons. The implications of these results from both physical–chemical and biological activity standpoints are discussed.

Keyphrases □ Corticosteroids—partition coefficients □ Partition coefficients—ether–water partitioning, steroids □ Structural group contribution—steroid partition coefficients □ Blue tetrazolium, isonicotinic acid procedures—analysis

The fundamental work of Meyer (1) and Overton (2) at the turn of the century that introduced the lipid-partitioning hypothesis brought a significant new dimension to biopharmaceutical research. Since then a myriad of investigators have sought correlation of biological activity with some measurable physical–chemical parameter of a drug family. It is obvious that no single property is capable of correlating all drug activities, because every type of bonding makes its contribution to the forces of action between the pharmacological agent and its environment and, in particular, its interaction with the "receptor." In the cases where one interaction factor predominates or is variant while all other factors are invariant, good correlations will be obtained with a closely related physical–chemical parameter. Good correlation is also possible with relatively meaningless parameters if the choice of compounds is limited to a homologous series or is restricted in some similar fashion.

Invariably, activities are compared with partition coefficients obtained in some seemingly arbitrary partitioning system. Hansch and coworkers (3–5) were extremely successful in correlating a spectrum of biological response data with octanol–water partition coefficients. Beckett and Moffat (6) found that *n*-heptane–water partitioning gives excellent rank correlation with buccal absorption for several series of compounds. Other investigators made similar contributions (7, 8).

From the practical standpoint, it is perhaps of equal importance that recent investigators (in particular, Hansch) have provided evidence that the partition co-