CHAPTER 8

Alkaloidal Assay and Crude Drug Analysis

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8.1 GENERAL CONSIDERATIONS

Alkaloids are of widespread occurrence in the plant kingdom and are to be found in a variety of structures and tissues. The term *alkaloid* is usually

restricted to the relatively complex bases of natural origin and to those synthetic compounds which are closely related to them. All possess one or more basic nitrogen atoms and may be classified by the nature of their ring systems.

Medicinally, the alkaloids form one of the most important groups of compounds derived from plants, and they may be administered in the form of the whole drug, dried exudate, tincture, liquid or solid extract, crude alkaloidal mixture, or as the purified isolated alkaloids. Any of these forms may be admixed with other medicaments or vehicles as in tablets, injections, mixtures or powders. It is not surprising, therefore, that much effort has been necessary to devise assays for these diverse potent preparations. An assay suitable for a particular alkaloid contained in one preparation may be, for reasons of concentration or presence of other bases, entirely unsatisfactory for another preparation containing the same alkaloid.

As a commercial standard for many crude drugs and galenicals, it is often sufficient to determine the content of total alkaloids. For other drugs it may be desirable to perform an assay for the most important alkaloid present, and in research work it often becomes necessary to evaluate quantitatively the individual components of a complex mixture. In the latter respect, methods are now available for most of the common drugs.

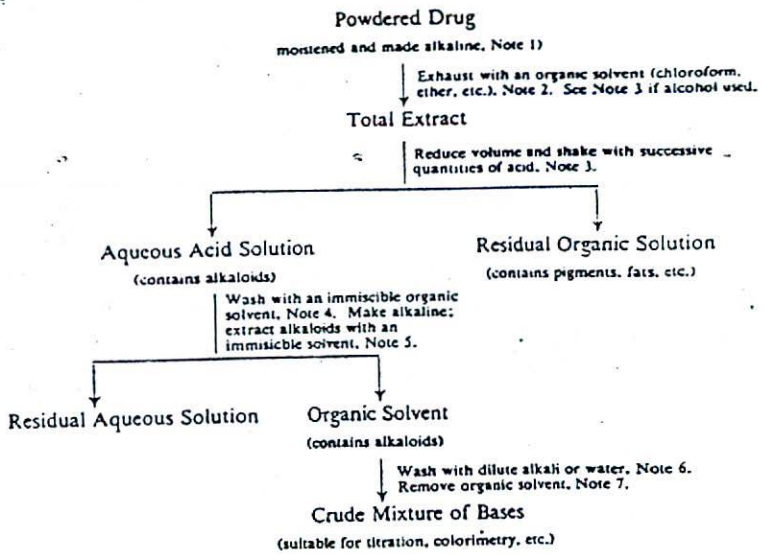
Quantitative alkaloidal analysis resolves itself into an extraction procedure by which the alkaloids are separated from other extraneous matter followed by the quantitative determination of the separated alkaloids, either as a mixture or after resolution of the mixture into individual components.

8.2 EXTRACTION OF ALKALOIDS

The simple salts of many alkaloids are water-soluble but much less soluble in organic solvents, whereas the reverse is true for free bases. This property affords a valuable method for the separation of many alkaloids from extraneous material, distribution of the bases being effected between acid or alkaline aqueous solutions and immiscible organic solvents. The principle appears to have been first adopted in 1856 by Otto, who employed ether in his modification of Stas' process for the detection of poisonous alkaloids. One scheme, based on this method, for the extraction of alkaloids is indicated in Scheme 8.1.

Note: 1. The powdered drug is moistened, often by the addition of dilute ammonia solution or by calcium hydroxide and water.

2. Percolation, repeated maceration, or continuous extraction may be employed. For continuous extraction the alkaloids must be stable for considerable periods at the boiling point of the solvent. Common solvents include ether, chloroform, alcohol, or mixtures of these.



SCHEME 8.1: Scheme for Extraction of Alkaloids Based on the Stas–Otto Principle

Complete extraction of alkaloids is conveniently tested for by micro-precipitation. A few milliliters of the percolate or extract are evaporated to dryness on a watch-glass, the residue dissolved as completely as possible in 1 to 2 ml of 1.0 *N* acid and, a suitable reagent (Mayers or iodine in potassium iodide solution) run in. No, or only a faint, turbidity is produced at the interface of the liquids. For the detection of many alkaloids, iodine solutions are more sensitive than Mayers reagent (potassium mercuric iodide) but will give precipitates with some nonalkaloidal substances. Alternatively, the sample of filtrate can be concentrated, spotted on to filter paper and the dried spot sprayed with a suitable alkaloidal reagent such as iodine in carbon tetrachloride or Dragendorff's reagent. Low concentrations of alkaloid can be detected in this way, provided there is little extraneous material present.

3. The nature of the evaporated extract may vary. If alcohol was used as a solvent and water added to moisten the drug initially, the evaporated solution is probably aqueous and contains considerable deposited material. Chloroform or ether should be added at this stage and used to transfer the extract to a separating funnel for the subsequent shaking with acid. Some alkaloidal salts (strychnine and lobeline hydrochlorides, nitrates of strychnine, and codeine) are soluble in chloroform so that attention should be paid to the mineral acid used; sulfuric acid is usually satisfactory.

4. The aqueous acid solution is washed with organic solvent to remove material entrained during the previous extraction. The organic washings are shaken with acidulated water which is returned to the main alkaloidal fraction.
5. Since some alkaloidal salts are soluble in chloroform (see above), they must be completely decomposed in the alkaline solution, because any salt would not be detected in subsequent titrations.
6. The alkaline washings are shaken with fresh solvent, which is returned to the main fraction.
7. Care is necessary as this stage, since mechanical loss of alkaloid may result by decrepitation (particularly strychnine). Alcohol is often added when the organic solution has nearly evaporated to dryness. All ammonia must be removed if the bases are to be titrated. Where necessary (assay of belladonna), volatile bases are eliminated by heating the residue at 105°.

Tinctures can be assayed similarly by first removing most of the alcohol by evaporation, and liquid extracts prepared by dilution, as necessary, with water. Ammonia solution is added and the alkaloids extracted with an organic solvent. Emulsions may make complete extraction of the alkaloids difficult at this stage, and some pharmacopoeias (USP—Ipecacuanha) direct that a measured volume of ether be added to the aqueous phase. After the shaking and standing, an aliquot of the supernatant ether is then taken for subsequent estimation. Powdered tragacanth may also be added (*Austrian Pharmacopoeia*) to facilitate the separation of the layers.

The Stas–Otto process can also be employed by first making an aqueous acid extract of the drug or preparation; this is washed with an immiscible organic solvent, the bases regenerated and recovered in chloroform. It is particularly applicable to the assay of extracts and solutions of alkaloids, but the inconvenience of concentrating large volumes of aqueous percolates or macerates makes it generally less suitable for crude drugs.

The tedious procedures of alkaloidal extraction and purification using separatory funnels can often be overcome by employing a column technique to utilize the immiscible solvent method. Thus, the evaporated total extract from a drug, in ether or chloroform is passed through a column of kieselguhr supporting half its weight of acid. Pigments, fats, and some other neutral substances are eluted from the column with more solvent, and the alkaloids can then be collected from the column in ammoniacal chloroform. Adsorption columns can also be employed for the same purpose, but here the Stas–Otto process is not involved. A tincture of ipecacuanha or nux vomica is filtered through an alumina column and the chromatogram developed with 70% alcohol; pigments and interfering impurities remain on the column while the alkaloids pass through into the filtrate where they can be titrated.[1] A similar method has been described for the assay of tincture of colchicum.[2]

Not all alkaloids can be manipulated by the Stas–Otto process; these include certain weak bases (reserpine, rescinnamine), which are extractable from acid solutions with chloroform, very water-soluble alkaloids (ergonovine), phenolic alkaloids (morphine, cephaeline) and alkaloids, the salts of which are appreciably soluble in chloroform. For the assay of drugs containing these types of alkaloids, special procedures are required.

A different approach to the extraction and purification of alkaloids for assay has been described by Brochmann-Hanssen.[3] In this, the alkaloids are extracted from the powdered drug by agitation with an aqueous suspension of a strongly acidic, cationic exchange resin. The crude drug is removed by a backwash technique, and the alkaloids are eluted from the resin with methanolic ammonia. With two drugs that present extraction problems, cinchona and nux vomica, an extraction of the alkaloids was completed in 30 and 15 min respectively, and was more efficient than conventional maceration procedures. This method has also been applied to ipecacuanha.

Ion exchange resins can also be employed for the purification of extracts and salts. An alcoholic solution of the alkaloid can be added to an anion exchange resin column and the bases eluted in ethanol; many impurities are retained by the column. The method has been employed for strychnine nitrate atropine sulfate, morphine hydrochloride, brucine hydrochloride, ephedrine sulfate, quinine, and cinchonidine[4] and adapted to the determination of the total alkaloids of cinchona, ipecacuanha, nux vomica, belladonna, hyoscyamus, and their preparations.[5] Weak cation exchangers have been suggested for the removal of alkaloids from colored extracts; nonbasic impurities are washed from the column with solvent, and the bases are then displaced from the column with acid or alkali according to their strength.[6]

Microsublimation has been used to separate some alkaloids from plant material. The method is readily applicable to the isolation of caffeine from tea and has been used for the main alkaloids of cinchona bark. In the latter case,[7] the dried cinchona powder, previously alkalinized, is heated at 140 to 175° and the sublimate collected quantitatively.

8.3 FRACTIONATION OF ALKALOIDAL MIXTURES

A. CHEMICAL METHODS

Phenolic groups give alkaloids possessing them, amphoteric properties which can be exploited in separtion processes. Ipecacuanha root contains the alkaloids emetine, cephaeline, psychotrine, psychotrine methyl ether, and emetamine. Emetine, psychotrine methyl ether, and emetamine are nonphenolic and in roots derived from *Cephaelis ipecacuanha* (Rio ipecacuanha) may constitute over two-thirds of the alkaloidal mixture. This species was, at one time, the only one recognized by some pharmacopeias and the standard

imposed for nonphenolic alkaloids eliminated varieties of the drug derived from *C. acuminata*; the latter, although often possessing a high total alkaloid content, contains proportionately less emetine. Erratic supplies of the Rio drug necessitated the use of both species; hence it is now more general to set a standard for total alkaloids only. For the determination of the nonphenolic alkaloids, the titration liquids of the total alkaloids are rendered alkaline with sodium hydroxide solution and the nonphenolic alkaloids extracted with ether and titrated. The phenolic alkaloids (mainly cephaeline) remain in the aqueous solution as sodium salts. Similarly, the phenolic properties of morphine can be utilized in the assay of its preparations and of opium. Morphine and atropine injection, BPC, and morphine and atropine sulfate tablets, NF, are treated in aqueous solution with sodium hydroxide solution and the liberated atropine collected in chloroform or ether. The morphine can then be recovered from the aqueous solution by adjusting the pH, adding ammonium sulfate, and extracting with a chloroform-alcohol mixture.

Nux vomica seeds contain 2 to 3.5% alkaloids, slightly less than half of which is usually strychnine and the remainder brucine. Pharmacologically, strychnine is the more active of the two alkaloids and is often determined separately. For this, advantage is taken of the relative ease of nitration of the two compounds. Nitration of the total alkaloidal mixture is conveniently effected by a mixture of nitric and sulfuric acids containing a trace of nitrite, the conditions being such that complete nitration of the brucine is obtained with virtually no change of the strychnine. The latter is extracted in the normal way from the reaction mixture and determined by titration. Over the years this method has been subjected to considerable investigation with the object of reducing the strychnine loss; the prescribed methods should be closely adhered to and the use of heat avoided.

B. PHYSICAL METHODS

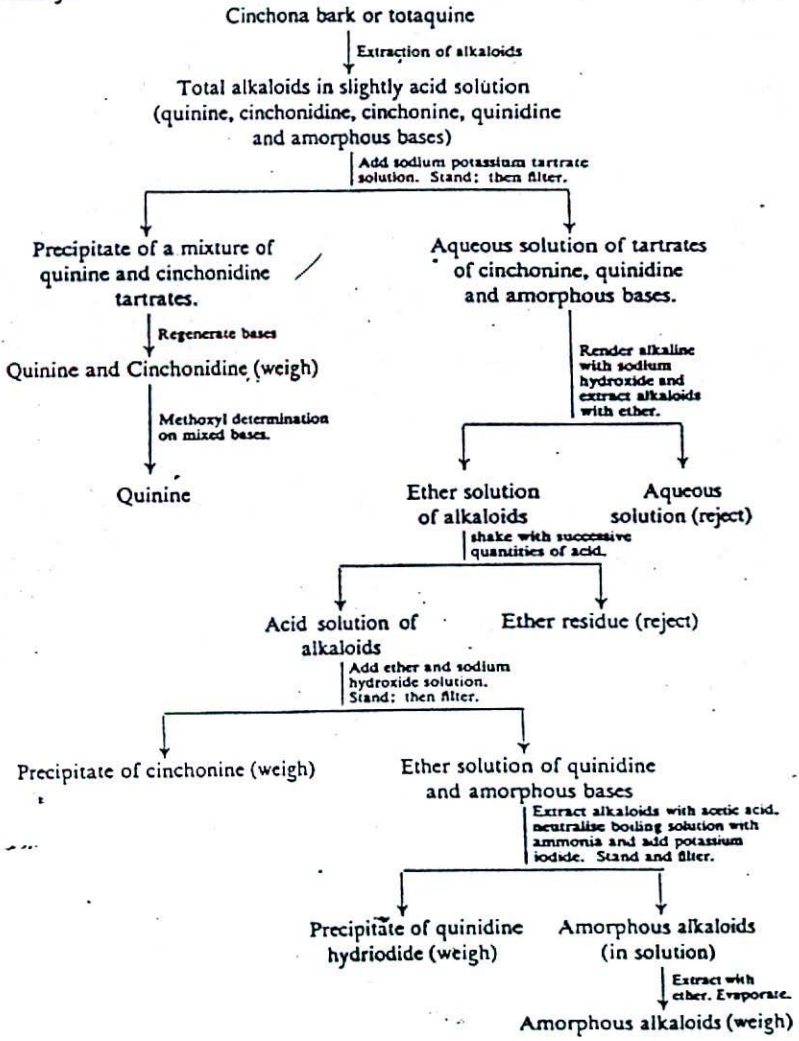
The majority of methods for the quantitative separation of alkaloids depend on exploiting physical differences between the components of a mixture.

1. Solubility

Differences in solubility of various alkaloids or their salts are sometimes large enough to afford quantitative separations. The determination of hydrastine, the effective therapeutic alkaloid of *Hydrastis canadensis*, in the presence of the other alkaloids of the root (berberine and small amounts of canadine and others) is a simple example of the method. Hydrastine is soluble in ether, whereas berberine is only slightly so; thus an ether extract of the alkaline powdered root will contain the desired alkaloid only. The extracted hydrastine can be purified by transferring it to acid and then again collecting the alkaloid in ether from the alkalinized solution. In another method for hydrastis extracts, based on relative solubilities, the berberine is

precipitated with potassium iodide and filtered off. The hydrastine is then extracted with ether from the ammoniacal filtrate.

A more complex assay utilizing solubility differences is that for the individual alkaloids of cinchona bark and totaquine. The latter is a mixture of the alkaloids from suitable species of *Cinchona* and was at one time extensively employed as a cheap source of quinine; it is included in USP XIII; BP, 1953 and *Pharm. Franc.*, 1949. Scheme 8.2 illustrates typical separations employed in the assay.



SCHEME 8.2

Preparations containing strychnine and quinine (compound syrup of hypophosphites, BPC) or strychnine and caffeine (compound syrup of glycerophosphates, BPC) may be assayed for strychnine by precipitation of the insoluble ferrocyanide in slightly acid solution. Caffeine and quinine do not give insoluble complexes. The strychnine base is regenerated from the salt and weighed. Mixtures of strychnine and brucine are quantitatively resolved by treatment of an alcoholic solution of the bases with Mayer's reagent (potassium mercuri-iodide solution) to give an insoluble strychnine complex suitable for gravimetric determination; brucine remains in solution and can then be determined by standard means.[8]

2. Fractional Liberation

The fractional liberation of bases from a mixture, although extremely useful in qualitative separations, rarely gives sufficiently clear-cut separations to be of value in quantitative analysis without considerable refinement.

In 1938, Kuhn and Schäfer[9] separated hyoscine from hyoscyamine and atropine by exploiting its lower basicity and greater solubility in ether. As a development of this,[10] hyoscine was liberated from a mixture of salts at pH 8.5 by the addition of an equivalent of sodium bicarbonate to the solution; by this means belladonna, stramonium, and hyoscyamus were evaluated. In 1941, King and Ware[11] demonstrated the use of buffer solutions to control the hydrogen ion concentration of the aqueous phase in a standard technique for the separation of mixtures of alkaloids by partition between immiscible solvents. The principle has been applied to countercurrent distribution and chromatography (Section 8.3A3, and following), and in a recent extension,[12] two buffer solutions of pH 2.5 and 7.5 are fed on to a column in such a way as to give a linear change of pH. The alkaloidal mixture, dissolved in chloroform, is held on the column and the buffer solution passed through; the latter passes to a larger column filled with Raschig rings and chloroform. In this way, as the pH of the buffer solution increases, a fractional liberation of bases takes place.

3. Countercurrent Distribution

Countercurrent distribution exploits differences in the equilibrium constants of different solutes between two immiscible liquids to achieve separations. Craig's "counter current extractor," described in 1944, made the procedure practical for the study of small amounts of material; modern machines are fully automatic. Although chiefly applicable to qualitative separations, countercurrent extraction has been applied to some quantitative alkaloidal fractionations. By using chloroform and selected phosphate buffer solutions, Banerjee and co-workers[13] separated a crude preparation of the total alkaloids of *Rauwolfia serpentina* into eight fractions each containing different components in a nearly pure form. They suggested the method as an assay

procedure for the drug. A mixture of ether-chloroform (3:1) and a citric acid-disodium hydrogen phosphate buffer solution, pH 3.1, has been used for the estimation of reserpine in reserpine-rich fractions obtained from *R. serpentina* and *R. comitoria*.[14] Seventeen transfers were stated to give reserpine, essentially free of interfering material; but presumably the fraction also contained rescinnamine, a related alkaloid having similar properties. Mixtures of berberine, canadine, and hydrastine have been quantitatively separated by countercurrent distribution and the method applied to the assay of tincture and extracts of hydrastis; the separated alkaloids can be determined spectrophotometrically.

4. Partition Chromatography

Partition chromatography is particularly effective for the separation of alkaloids since, like countercurrent distribution, differences in both ionization constants and partition coefficients of the individual bases may be utilized. As a column process, it has an efficiency which far exceeds anything, practically obtainable, with separatory funnels. Advantages compared with countercurrent extraction are that it requires less elaborate apparatus, is usually less time-consuming, and is not hampered by the formation of interfacial emulsions, so often a problem with crude plant extracts. The important factors in partition chromatography which can be varied, in addition to the column loading, are the pH of the buffer solutions used and the nature of the mobile phase. At high pH values, most alkaloids pass readily through a column with any mobile phase in which they are soluble. As the pH of the buffer solution decreases the R_F value for a particular alkaloid becomes less until, at about pH 4.0, most alkaloids are retained in the stationary phase. An intermediate pH value which gives the most satisfactory degree of separation for the alkaloids concerned is usually chosen. A series of immiscible solvents, of increasing eluting power, may be employed to give further scope to the method, but it should be borne in mind that the order of elution of the components of a mixture may not necessarily be the same with different solvents. Petroleum ether, ether, and chloroform are commonly used as the moving phase, and with many alkaloids the eluting power increases in that order.

The method was applied to the quantitative separation of the solanaceous alkaloids[15] in 1948 and was subsequently developed into a quantitative assay for the crude drugs.[16] An evaporated ether extract containing the total alkaloids of the drug is placed on a kieselguhr column loaded with phosphate buffer solution at pH 6.0. Carbon tetrachloride or petroleum ether, b.p. 40–60°, is used to elute pigments and other extraneous material from the column. Ether removes the hyoscine; then chloroform the hyoscyamine; these bases are estimated by titration. By a similar technique, the separation and determination of the *Duboisia* alkaloids[17] and of nicotine and nornicotine[18] has been effected. The roots of *Datura* species contain a complex

mixture of alkaloids which can be assayed with partition columns of the above type.[19]

Two celite columns in series have been used for the quantitative separation of the alkaloids of ipecacuanha.[20] The first column contains sodium hydroxide solution and the second a buffer solution, pH 6.4. The total root-alkaloids in ether are chromatographed and the phenolic and nonphenolic alkaloids retained in the upper and lower columns, respectively. From the upper column, cephaeline is eluted with chloroform and psychotrine with chloroform-ethanol (5:1). Ether, chloroform, and chloroform-ethanol (5:1) are used to recover emetine and other bases such as emetamine and *o*-methyl psychotrine from the lower column. The separated emetine, cephaeline, and psychotrine can be determined by nonaqueous titration.

The weakly basic alkaloidal mixture of *Rauwolfia serpentina* contains reserpine, rescinnamine, and deserpidine, and these alkaloids can be quantitatively separated from the more basic material of the root extract on a column loaded with citrate buffer solution; the upper phase of the mixture isooctane-chloroform-water-alcohol, (200:100:100:40), is a suitable eluting solvent.[21] These three alkaloids can themselves be fractionated on celite using the upper layer of a mixture of *n*-heptane, chloroform, morpholine, and formamide as the mobile phase and, the lower layer as the stationary phase.[22]

The water-soluble and water-insoluble alkaloids of ergot have been quantitatively separated on a silica gel column loaded with buffer, pH 7.0, by successive elution with chloroform-trichloroethylene (1:1).[23] For the same purpose Alexander and Banes[24] used celite loaded with 0.1 *m*-citric acid; the water-insoluble group was eluted with chloroform and the water-soluble group recovered from the extruded column. Aqueous solutions of the ergot-alkaloids form a mixture of the diastereoisomers and ergotamine and ergotaminine can be quantitatively separated on citric acid columns.[25] This method has been incorporated into the revised monograph for "Ergotamine Tartrate Injection," *First Supplement, USP XVI*, 1962.

A method for the determination of the most important secondary alkaloids of opium (codeine, thebaine, papaverine, and noscapine) involves chromatography at pH 4.8. Noscapine and papaverine are eluted together by ether-benzene (3:1) and are followed by thebaine; codeine is then removed with ammoniacal chloroform.[26]

Those alkaloidal salts which are to some extent soluble in chloroform can be separated by partition chromatography notwithstanding the fact that the partition coefficient is usually in favor of the aqueous phase. Thus, the hydrochlorides of hyoscine and hyoscyamine may be quantitatively separated by the elution of the latter in chloroform from a hydrochloric acid impregnated column.[27] Quinine can be separated from strychnine by a similar method, the latter being eluted from the column in chloroform as the hydrochloride[28]; this is applicable to the assay of such preparations as syrup of ferrous phosphate with quinine and strychnine, BPC. Many alkaloid toluene

sulfonates are chloroform-soluble and can be quantitatively separated in a similar manner.[29]

5. Paper Chromatography

Of all the processes used for alkaloidal fractionation, that of paper chromatography has been one of the most extensively studied. All the common variants of the technique are to be found in alkaloidal analysis and include ascending and descending, one- or two-dimensional, and circular chromatography. The paper used may be untreated or impregnated with buffer solution, acid, or formamide. A recent report[30] describes the subdivision of the paper into many strips each treated with a different buffer solution. The solvents vary from a single liquid of one component to complex mixtures.

Examples of paper chromatographic systems which have been employed for the quantitative analysis of some common alkaloidal mixtures are given in Table 8.1. For completeness the assay methods are also included; these are discussed in Section 8.4.

6. Paper Electrophoresis

Developed independently in the late 1940s and early 1950s by a number of workers for the separation of amino acids, electrophoresis on paper is the same in principle as conventional electrophoresis. Molecules possessing charged groups migrate under the influence of an electric field to the anode or cathode, depending on the nature of their charge. The migration velocity of a molecule depends on its size and shape as well as on the magnitude of the charge. Filter paper is used as the support for the electrolyte, and it commonly dips at either end into a buffer solution in which the electrode is immersed. The central area of paper is suitably supported and the whole enclosed in a container. A stable, direct current is applied through the electrodes across the paper for up to about 18 hr, the potential difference being usually of the order of 2 to 10 volts per cm of length of strip. A simple apparatus can be easily constructed for elementary laboratory use but more refined equipment is commercially available, particularly for high-voltage work. A recent modification is the use of a layer of starch gel on a glass plate to replace the paper.

Most reports deal only with the fractionation of alkaloids by this method but, once separated, individual alkaloids can be detected and quantitatively estimated by the same means employed in paper chromatography. The following examples illustrate the scope of the method: The quantitative determination of strychnine and brucine by planimetry after electrophoresis at pH 3.1[62] and strychnine, brucine, and quinine in tinctures after electrophoresis at pH 1.5 (2 *N* formic acid).[63] The separation at pH 9.9 of the tropane alkaloids followed by spectrometric estimation of the methyl orange complex.[64] UV spectrophotometry of reserpine in crude extracts after

TABLE 8.1: The Quantitative Evaluation of Alkaloids by Paper Chromatography

Sample	System for separation	Detection and estimation	Ref.
Aconite	Amyl alcohol-25% formic acid-benzene (24:26:30)	Location of alkaloids with Dragendorff's reagent; aconitine and benzoylaconine determined spectrophotometrically	31
Conium maculatum	*t*-Pentanol-*t*-butanol-*N*-HCl (9:3:2)	Nitroprusside reaction for coniceine; bromothymol blue reaction for coniine, N-methylconiine and conhydrine	32
Conium maculatum	*t*-Pentanol-*t*-butanol-*N*-HCl (9:3:2)	Location of spots with bismuth iodine reagent; measurement of spot areas by "4-point bioassay technique"	33
Ergot alkaloids	*n*-Butanol-glacial acetic acid-water (4:1:5) (upper phase)	Fluorescence of spots compared with reference compounds	34
	Papers impregnated with buffer solutions pH 2–7; ether saturated with water; separated zones eluted with ethanol	Paradimethylaminobenzaldehyde reagent	35
	Formamide-impregnated paper; chloroform; separated spots cut out	Paradimethylaminobenzaldehyde reagent	36
	Paper impregnated with formamide and benzoic acid; carbon tetrachloride-chloroform-benzene (7:2:1) or carbon tetrachloride-dibutyl ether (8:2)	Paradimethylaminobenzaldehyde reagent	37
Clavine alkaloids of Pennisetum ergot	Paper impregnated with formamide and benzoic acid; benzene-pyridine (6:1)	Paradimethylaminobenzaldehyde reagent	38
Lobeline in crude drugs and galenicals	Paper impregnated with formamide-ammonium formate-formic acid; chloroform-benzene (9:1)	Spectrophotometric absorption of lobeline at 245 mμ	39
Lupin alkaloids-sparteine, lupanine, hydroxylupanine, and others	Circular paper chromatography; butanol-toluene-hydrochloric acid; *n*-butanol saturated with hydrochloric acid, 0.1 *N*	Alkaloids detected with Dragendorff's reagent, eluted, determined colorimetrically	40,41
	Alkaloids separated from plant extract by ion exchange; then fractionated by descending paper chromatography	Elution and nephelometric determination	42

Morphine in opium	Ethylacetate-formic acid-water (10:1:3)	Eluted alkaloid determined at 450 mμ by nitrite colorimetric reaction	43
	Butanol-28% ammonia-water (50:9:15) (upper phase)	Absorption of eluted alkaloid measured at 286 mμ in benzene	44
Morphine in urine	Untreated paper and paper impregnated with buffer solution, pH 6.3; *n*-butanol-formic acid-water (12:1:7); amylene hydrate-di-*n*-butyl ether-water (80:7:13)	Polarography of eluted morphine	45
Morphine, codeine, and thebaine in pharmaceuticals	Paper treated with 2% ammonium sulfate solution and dried; iso-butanol-acetic acid-water (10:1:2.4)	Potassium iodoplatinate spray for detection; alkaloids evaluated with self-integrating densitometer	46
Morphine in poppy latex	Morphine first separated from codeine and thebaine; these two fractions chromatographed; *n*-butanol-acetic acid-water (5:1:2)	Modified Dragendorff's reagent for detection. Spot areas measured and "4-point bioassay" technique applied	47
Muscarine in *Inocybe spp.*	Paper impregnated with buffer solution pH 4.5; *n*-butanol-methanol-water (10:3:2)	Modified Dragendorff's reagent; visual comparison with standard	48
Reserpine in crude root and preparations	Stationary phase: propylene glycol-methanol-acetic acid; Mobile phase: benzene-cyclohexane (1:1)	UV absorption of eluted alkaloid	49
Reserpine in *Rauwolfia spp.*	Paper impregnated with a formamide methanolic solution; benzene-cyclohexane (1:1)	Visual comparison of fluorescent spots in UV light against standard reserpine spots	50
Strychnine and brucine in nux vomica	Mixed alkaloids treated with 4 *N* nitric acid; quantitative conversion of brucine into O-brucichinone; mixture chromatographed; butanol-acetic acid-water (4:1:5); strychnine, $R_f = 0.76$; O-brucichinone, $R_f = 0.1$	Elution of alkaloidal spots and estimation by standard procedures	51
	Butanol-propanol-0.05 *N*-hydrochloric acid (1:2:1)	Alkaloids detected by UV light and eluted with water; determined by spectrophotometry at 255 mμ	52

Table 8.1 (continued)

Sample	System for separation	Detection and estimation	Ref.
Strychnine and brucine in tinctures and extracts	Butanol saturated with water	Alkaloids determined by radiometry after detection with P^{32}-phosphomolybdic acid and excess acid washed off chromatogram	53
Tropane Alkaloids			54–56
Hyoscine and hyoscyamine in *Datura* and *Duboisia*	Paper impregnated with buffer solution, pH 7.4; water-saturated *n*-butanol	Alkaloids eluted with ethanol; Modified Vitali-Morin assay	57
Hyoscine and hyoscyamine in drugs and galenicals	Formamide-impregnated paper; chloroform or chloroform-benzene (4:6)	Alkaloids eluted; modified Vitali-Morin assay	58
	Paper impregnated with buffer solution pH 6.77; *n*-butanol saturated with water	Chromatogram sprayed with Dragendorff's reagent; comparison of alkaloid spots using dilution limits	59
	n-Butanol saturated with water-glacial acetic acid (100:5)	Densitometric measurement after spraying chromatogram with Dragendorff's reagent	60
Atropine and its hydrolytic products	*n*-Butanol-hydrochloric acid-water (5:1:3) for atropine and tropine; *n*-butanol-acetic acid-water (10:1:6) for atropine, tropine, and tropic acid.	Photoelectric densitometry after staining with Dragendorff's reagent or bromophenol blue	61
Hyoscine and hyoscyamine in herbs	*n*-Butanol saturated with water-glacial acetic acid (10:1)	Alkaloids eluted and determined using colour complex with tropeoline 00	

electrophoresis on 5 *N* acetic acid-impregnated paper.[65] A similar separation for the assay of rescinnamine and ajmalicine in solutions and tablets.[66]

7. Gas Chromatography

Gas-liquid chromatography has obvious applications in the fractionation of mixtures of volatile alkaloids. Qualitative separations of the tobacco alkaloids on columns having polyethylene glycol, polypropylene glycol, and polybutylene glycol as the stationary liquid phase have been studied.[67] The main difficulty in applying quantitative gas chromatography to alkaloids is to prepare a suitable concentrate of volatile bases which will correspond exactly to a known weight of plant material. Fairbairn and co-workers[68] have overcome this difficulty in assaying hemlock fruits for volatile alkaloids by extracting the bases as nonvolatile salts and then liberating the free base in situ on the column. A silicone-rubber column has proved satisfactory[69] for the detection and estimation of cocaine in illicit drug samples and for the determination of morphine in opium.[70] In the latter case the morphine was first separated from other opium alkaloids by ion exchange and then converted to the trimethylsilyl ether by treatment with hexamethyldisilazane before chromatography.

8. Adsorption Chromatography

Although adsorption chromatography with columns has proved valuable for the quantitative separation of total alkaloids from other nonbasic constituents of plant extracts, its application to the quantitative resolution of alkaloidal mixtures has been less extensively employed. For the estimation of the brucine and strychnine contents of nux vomica, the total basic mixture in trichloroethylene can be chromatographed on alumina and the solvent rejected. Strychnine is then quantitatively eluted with a mixture of carbon tetrachloride containing 9% acetone, and brucine is eluted with ethanol. The separated alkaloids are back titrated.[71] The behavior of opium alkaloids on alumina columns has also been studied and a method reported for the quantitative determination of morphine in opium.[72]

9. Thin-Layer Chromatography

The resolution of mixtures on thin films of alumina or silica-gel spread on glass plates has achieved remarkable success in all analytical fields. Often it has proved superior to paper chromatography in time, degree of resolution, and recovery of separated components; its use is being continuously extended for the separation of alkaloidal mixtures. With alumina plates, the process is analogous to column adsorption chromatography and, with silica-gel plates, buffer solutions can be used in the same way as with paper chromatography. Formamide-impregnated cellulose, kieselguhr, and ion exchangers have also been used as thin layers.

TABLE 8.2: The Quantitative Evaluation of Alkaloids by Thin-Layer Chromatography

Sample	System for separation	Detection and estimation	Ref.
Cinchona	Kieselgel G plates; isopropanol-benzene-diethylamine (2:4:1); benzene-ether-diethylamine (20:12:5)	Planimetry of separated spots	73
Ergot			
Clavine alkaloids	Eight different methods of separation on thin-layer plates described	Elution with dichloromethane: methanol (9:1)	74
Ergonovine, ergotamine, ergoscine, ergocristine, and mixed zone of ergocornine and ergokryptine	Silica gel plates; benzene-dimethylformamide (13:2), or ethyl acetate-dimethylformamide-ethanol (13:1.9:0.1)	Elution with methanol, deionized water, and glacial acetic acid. Determined with paradimethylamino-benzaldehyde reagent	75
Mixed zone from above	Alumina plates; chloroform-ether-water (3:1:1)		6
Opium			
Morphine, codeine, and thebaine	Silica gel plates; methanol-chloroform (1:9)	Elution and measurement of extinction	76
Papaverine and noscapine	Ethanol-benzene (1:4)		
Rauwolfia alkaloids			
Reserpine	Silica gel plates; methanol-methylethyl-ketone-heptane (8.4:33.6:58.0)	Location by UV light and elution with dioxane-ethanol (1:1); spectrophotometric determination	77
Tropane alkaloids			
Total alkaloids of *Datura alba*	Alumina plates; methanol	Elution of alkaloids with chloroform and acid-base titration	78
Various tropane alkaloids	Kieselgel G plates; combinations of ethyl-methyl ketone, methanol, water, and ammonia.	Planimetric determination	73

Some examples of the use of thin-layer chromatography for the quantitative estimation of alkaloids are given in Table 8.2.

10. Ion Exchange

As a result of its phenolic properties, morphine is retained on strongly basic anion exchange columns, whereas codeine is not. A mixture of the salts in water can be quantitatively separated by this means, methanol being used to elute codeine and 2% phosphoric acid to elute the morphine. In the method of Grant and Hilty[79] the separated codeine was determined by titration and the morphine spectrophotometrically at 285 mμ. By similar means the purified alkaloids of ipecacuanha can be separated into phenolic and nonphenolic groups.[3]

11. Sublimation

Microsublimation has been employed for the quantitative separation of the strychnine of nux vomica seeds.[7] A preliminary sodium carbonate treatment of the powdered drug converts alkaloidal salts into the free bases. Fifty mg of powder is heated at a pressure of 5 mm Hg, and the strychnine sublimate is collected at 175–185° (after discarding a small amount of brucine which sublimes at 160 to 165°). The strychnine is weighed.

8.4 QUANTITATIVE EVALUATION OF THE SEPARATED ALKALOIDS

Many methods are available for the determination of either the total alkaloids of a crude mixture or the individual alkaloids fractionated as in Section 8.3. Some methods have been in use for many years, but others have been recently introduced as a result of the development of microfractionation and advances in instrumental techniques. The latter methods are being increasingly adopted as "official" assay procedures.

A. DIRECT WEIGHING AND GRAVIMETRIC PRECIPITATION

The direct weighing of alkaloids is not commonly used in quantitative analysis because it is often difficult to obtain pure extracts and because the weights involved are often small. It is, however, employed for the assay of colchicum and quinine salts (see Table 8.3) and was used for rauwolfia and hemlock before other more satisfactory methods were developed. In cases where the product is relatively pure, for example, semimicrosublimates of caffeine, quinine, and strychnine, direct weighing can be used with accuracy. A more specific method is to precipitate quantitatively the alkaloid as a

TABLE 8.3*: Assay Procedures Employed by Various Compendia

Drug or preparation	Ref.	Method
Aconite	*BPC*	Total alkaloids by Stas–Otto fractionation; back titration
	PF	Same, but alkaloids finally precipitated with silicotungstic acid; precipitate ashed and anhydride weighed
Liniment	*BPC*	See Aconite, *BPC*
Aconitine	*PA*	Nonaqueous titration with perchloric acid
Apomorphine		
Hydrochloride	*BP, PA*	Nonaqueous titration with perchloric acid
Injection	*BP*	Extinction measured at 273 mμ after dilution
Tablets	*NF*	Stas–Otto extraction; back titration
Areca	*NF, PA*	Stas–Otto extraction; back titration
Arecoline hydrobromide		
Tablets	*NF*	Volhard estimation
Atropine	*BP*	Back titration
Atropine methonitrate	*BP*	Nonaqueous titration
Atropine sulfate	*BP, JP*	Extraction of base and back titration
	PA, USSRP	Dissolve in alcohol-chloroform and titrate with sodium hydroxide solution with phenolphthalein as indicator
Eye-drops	*BPC*	Alkaloid precipitated as tetraphenylborate and excess reagent determined with 0.005 *M* cetylpyridinium chloride
Eye-ointment	*BP*	Extraction of base and back titration
Injection	*BP, JP, USP*	Extraction of base and back titration
Lamellae	*BPC*	Bromothymol blue complex obtained in chloroform and ethylene chloride (3:2); extinction measured at about 420 mμ
Tablets	*BP, USP*	Extraction of base and back titration
	JP	Same but final determination by nonaqueous titration with perchloric acid in dioxane
Belladonna leaf, herb, root, and preparations thereof	*BP, NF, PA, PF, USP, USSRP*	Stas–Otto extraction; back titration of total alkaloids; volatile bases usually removed by drying the total alkaloid residue before titration
Cinchona	*BPC*	Total alkaloids determined by weighing
	PF	Total alkaloids determined by titration
Coca, Liquid extract	*PF*	Total alkaloids determined by weighing
Cocaine hydrochloride	*PA*	See atropine sulfate, PA
Eye-drops	*BPC*	See atropine sulfate eye-drops, BPC
Tablets	*NF*	Extraction of base and back titration
Codeine phosphate	*BP, USP*	Nonaqueous titration
	JP	Extraction of base and back titration
	PA, USSRP	See atropine sulfate, PA

TABLE 8.3 (continued)

Drug or preparation	Ref.	Method
Colchicine	*PA*	Methoxyl determination
Tablets	*BP*	Extraction of base and measurement of its extinction in dehydrated alcohol at 350 mμ
	USP	Extraction and weighing of alkaloid
Colchicum corm, liquid extract and tincture	*BP*	Extraction and weighing of alkaloid
Colchicum seeds	*FP*	Extraction and weighing of alkaloid
Coptidis rhizome	*JP*	Berberine isolated as acetone-berberine and weighed
Diamorphine		
Hydrochloride	*BP*	Nonaqueous titration
Injection	*BP*	Acid hydrolysis followed by colorimetric estimation with sodium nitrite
Emetine hydrochloride	*PA*, *USSRP*	See atropine sulfate, PA
Emetine hydrochloride and preparations	*BP*, *JP*, *USP*	Extraction of base and back titration
Ephedrine hydrochloride	*JP*,	Extraction of base and back titration
	PA	Titration with silver nitrate
Tablets	*BP*	Nonaqueous titration
Ergonovine maleate, Tablets and injection	*BP*, *JP*, *PA*, *USP*	Colorimetry or measurement of absorbance of solution at 550 mμ after treatment with *p*-dimethylaminobenzaldehyde reagent
	PA	Nonaqueous titration
Ergot	*BPC*, *JP*	Assayed for total alkaloids and water-soluble alkaloids with *p*-dimethylaminobenzaldehyde reagent
Ergotamine and preparations	*BP*, *JP*, *PA*., *USP*	See ergonovine, BP, JP, PA, USP
	PA	See ergonovine, PA
Gelsemium and tincture	*BPC*	Extraction of base and back titration
Homatropine		
Hydrobromide	*BP*, *USP*	Nonaqueous titration
	JP	Extraction of base and back titration
Eye-drops	*BPC*	See atropine sulfate eyedrops, BPC
Homatropinemethylbromide tablets	*NF*	Nonaqueous titration
	NF	Precipitated reineckate dissolved in acetone and absorbance measured at 525 mμ
Hordenine sulfate	*PF*	See aconite, PF
Hydrastis and liquid extract	*PF*	Alkaloids extracted and weighed
	BPC, 1949, *NFA*	Hydrastine isolated and weighed
Hyoscyamus and preparations	*BP*, *PF*	Stas–Otto extraction; back titration

TABLE 8.3 (continued)

Drug or preparation	Ref.	Method
Ipecacuanha and preparations	*BP, JP, PA, USP*	Stas–Otto extraction; back titration
	PF	Total alkaloids by titration; nonphenolic alkaloids removed in ether from the titration liquids made alkaline with sodium hydroxide; ether evaporated and residue back titrated
Lobelia and preparations	*BPC, PA*	Ether soluble alkaloids; titration
	PF	See aconite, PF
Lobeline hydrochloride	*JP*	Nonaqueous titration
	PA	See atropine sulfate, PA
Injection	*JP*	Extraction with ether and back titration of isolated alkaloid
Morphine hydrochloride	*BP, JP*	Salt in sodium hydroxide solution washed with chloroform; morphine extracted from the aqueous solution, to which ammonium sulfate added, with chloroform-ethanol; isolated morphine back titrated
	PA	Salt in alcohol-chloroform; titrate with 0.1 *N* NaOH using bromothymol blue indicator
	USSRP	Volhard determination
Solution	*BP*	Extraction from sodium bicarbonate solution with chloroform-ethanol; back titration
Injection	*JP*	Extraction from solution (pH 9.2–9.4) with chloroform-ethanol; back titration
Morphine injection	*USP*	Extraction from solution saturated with sodium chloride + ammonia with chloroform-isobutanol; back titration
Tincture of chloroform and morphine	*BPC*	Extraction of morphine; colorimetric analysis with sodium nitrite
Mixture of ammonium chloride and morphine	*BPC*	Morphine extracted and aqueous solution of alkaloid treated with iodic acid and then nickel chloride-ammonia solution; extinction measured at 670 mμ
Morphine sulfate	*BP*	See morphine hydrochloride, BP
Injection	*BP*	Extraction of alkaloid followed by colorimetry with sodium nitrite reagent
Tablets	*BP*	Similar to morphine hydrochloride, BP
	USP	See morphine injection, USP
Morphine and atropine		
Injection	*BPC, JP*	All depend on first extracting the atropine, then morphine
Tablets	*NF*	
Noscapine (narcotine)	*BP, NF*	Nonaqueous titration
Hydrochloride	*JP*	Extraction of base and nonaqueous titration

TABLE 8.3 (continued)

Drug or preparation	Ref.	Method
Nux vomica and preparations	*PA*	Stas–Otto extraction; total alkaloids by titration
	BP, JP, PF	Mixture of total alkaloids nitrated, strychnine unchanged and determined by titration
Nux vomica, dry extract	*BPC*	Alkaloids adsorbed on alginic acid and then eluted with 1 *N*-sulfuric acid; extinction measured at 262 and 300 mμ
Opium and tincture	*BP, JP, FP, USSRP*	Morphine extracted from opium by lime and water; solution treated with alcohol, ammonium chloride, and ether; the morphine crystals which separate are determined by titration
	USP	Similar, but lime added to the aqueous extract
	PA	Extract purified by chromatography on alumina; morphine extracted from eluate with sodium hydroxide solution; acid added and solution concentrated; morphine precipitated and weighed as the dinitrophenylether
Opium, camphorated tincture	*BP*	See morphine sulfate injection, BP
	USP	Extraction of morphine followed by back titration
Ipecacuanha and opium, powder	*BP*	Similar to opium, PA
	JP	Similar to opium, JP
Tablets	*BP*	Similar to opium, PA
Opium, various preparations with chalk, aspirin, etc.	*BPC*	Extraction of morphine followed by colorimetric analysis using sodium nitrite
Papaveretum	*BPC*	Morphine determination based on that of Opium, BP; codeine, papaverine, and noscapine isolated and weighed
Papaverine hydrochloride	*BP, JP*	Nonaqueous titration
	PA	See atropine sulfate, PA
Injection	*NF*	Extract base and weight
Tablets	*NF*	Extract base and weight
Phellodendron bark	*JP*	See coptidis rhizome, JP
Physostigmine salicylate	*PA*	See atropine sulfate, PA
Eye-drops (aqueous and oily)	*BPC*	See atropine sulfate eye-drops, BPC
Eye ointment	*BPC*	Dissolve ointment in light petroleum; extract alkaloid with water and measure extinction at about 298 mμ
Pilocarpine Hydrochloride	*PA*	See atropine sulfate, PA
	USP	Nonaqueous titration

TABLE 8.3 (continued)

Drug or preparation	Ref.	Method
Pilocarpine nitrate	*USP*	Nonaqueous titration, potentiometric end point
Eye-drops	*BPC*	See atropine sulfate eye-drops, BPC
Quinidine salts and preparations	*BP, JP, NF, USP*	Extraction and weighing of free base
Quinine salts, tablets, capsules, and injections	*BP, NF*	Extraction and weighing of free base
Quinine, ammoniated solution of	*BPC*	Extraction and weighing of free base
Quinine in ferrous phosphate with quinine and strychnine, syrup	*BPC*	Quinine, in dilute sulphuric acid, determined by quantitative fluorimetry;
Tablets	*BPC*	primary light source about 365 mμ; secondary radiation about 450 mμ;
Quinine in compound syrup of hypophosphites	*BPC*	Strychnine determination, see below
Rauwolfia	*BPC, NF*	Reserpine-like alkaloids separated from the strong bases and treated, in acid solution, with sodium nitrite reagent followed by sulfamic acid; extinction measured at 390 mμ; a reserpine standard solution is similarly treated
Tablets	*NF*	See *Rauwolfia*, NF
Rauwolfia, African	*BPC*	See *Rauwolfia*, BPC
Rescinnamine and tablets	*NF*	Nitrite method as employed in *Rauwolfia* assay
Reserpine	*BP, USP*	Nitrite method as above
	JP	Extinction of a chloroformic solution determined at 268 mμ
	PA; USSRP	Nonaqueous titration
Injection	*USP*	Nitrite method as used in *Rauwolfia* assay
Tablets	*USP*	Nitrite method as used in *Rauwolfia* assay
Scopolamine hydrobromide	*NF*	Extraction of base and back titration
	PA	Bromide determination
	USP	Nonaqueous titration
Eye-drops	*BPC*	See atropine sulfate eye-drops, BPC
Eye-ointment	*BP*	Extraction of alkaloid and back titration
Injection	*BP, USP*	Extraction of alkaloid and back titration
Tablets	*BP*	Extraction of alkaloid and back titration
Scopolia rhizome	*JP*	Stas–Otto extraction; back titration
Sparteine sulfate	*JP*	Extraction of free base; direct titration of base, in ethanol, with 0.1 *N* hydrochloric acid
Stramonium and preparations	*BP, PF*	See belladonna, BP

TABLE 8.3 (continued)

Drug or preparation	Ref.	Method
Strychnine nitrate	*PA*	See atropine sulfate, PA
	JP	Extraction of base and back titration
Phosphate	*NF* XI	Extraction of base and back titration
Sulfate tablets	*NF* XI	Extraction of base and back titration
Strychnine, mixture	*BPC*	Mixture diluted and extinction measured at 254 mμ
Strychnine and iron mixture	*BPC*	Strychnine extracted and extinction measured in acid solution at 254 mμ
Strychnine in syrup of ferrous phosphate with quinine and strychnine	*BPC*	Alkaloid mixture purified by chromatography; extinction of an acid solution of the alkaloid measured at 247, 254, and 262 mμ
Tablets	*BPC*	
Strychnine in compound syrup of glycerophosphates	*BPC*	Double precipitation of strychnine with potassium ferrocyanide followed by extraction and weighing of the base
Tubocurarine chloride injection	*BP*	Extinction of solution in water measured at 280 mμ
	JP	Chloride determination
	USP	"Head-drop" biological assay using rabbits
Yohimbine hydrochloride	*PA*	See atropine sulfate, PA

* *BP* = *British Pharmacopoeia*, 1963; *BPC* = *British Pharmaceutical Codex*, 1963; *PA* = *Austrian Pharmacopoeia*, 1960; *PF* = *French Pharmacopoeia* 1949 and Supplement; *JP* = *Japanese Pharmacopoeia*, 1961; *NF* = *National Formulary*, XII, 1965; *USP* = *United States Pharmacopeia*, XVII, 1965; *USSRP* = *Pharmacopoeia of the USSR.*, 1961.

complex; such precipitates need to be of reproducible, constant composition and sufficiently stable to allow washing and drying. The precipitates may be weighed or analyzed chemically. Alternatively, the excess reagent may be determined after the removal of the precipitate.

The quantitative evaluation of a number of alkaloids by means of the Caille and Viel reagent has been studied.[80] With this reagent, atropine, brucine, strychnine, and morphine give compounds of the formula $SbI_3Alk{\cdot}HI$, aconitine and emetine $SbI_3Alk{\cdot}2HI$, caffeine $(SbI_3)_2Alk{\cdot}$, and quinine and sparteine $(SbI_3)_2Alk{\cdot}2HI$. With Wachmuth's reagent (SbI_3 in ether) brucine, strychnine, and morphine give compounds of the same composition as above but quinine and sparteine give $SbI_3Alk{\cdot}2HI$. Bismuth iodide and silicotungstic acid in ether solution have been similarly employed in alkaloidal analyses. An amperometric evaluation of codeine phosphate, papaverine hydrochloride, and veratrine sulfate has been described in which silicotungstic acid is added dropwise to a solution of the alkaloid in hydrochloric acid. Electrodes are immersed in the solution, and no current flows until the alkaloid in solution is completely precipitated.

Phosphotungstic acid has been employed for the quantitative precipitation

of cocaine and ecgonine and for the turbidimetric analyses of the tropane alkaloids. The radiochemical determination of caffeine using P^{32}-labeled phosphomolybdic acid has been described. The alkaloid is precipitated from a dilute hydrochloric acid solution, and the activity of the complex is determined (see also strychnine and brucine in Table 8.1.) Mercurio-iodide complexes of alkaloids are well-known and have been employed for the assay of the lupin alkaloids. With zinc and cobalt thiocyanates, eserine, strychnine, and harmine form complexes of the type $Alk_2H_2[M(SCN)_4]$; M = Zn or Co. The precipitation of alkaloids with ammonium reineckate,

$$NH_4[Cr(NH_3)_2(SCN)_4]H_2O,$$

has been extensively employed; the complex can be weighed, redissolved in acetone, and determined colorimetrically or subjected to a thiocyanate determination.

Many alkaloids form insoluble tetraphenylborates, and these have been much used for preparations such as eye-drops which contain small quantities of alkaloid. The alkaloid can be precipitated by a standard solution of the sodium salt, and the unchanged precipitant can be back titrated with cetylpyridinium chloride (see Table 8.3). The same reagent has been used for the turbidimetric determination of submicro quantities of ajmaline.

Some reagents give quantitative precipitates of alkaloids in organic solutions, thus flavianic acid in ether-alcohol has been used for the determination of a number of alkaloids in ether or ether-alcohol.[81]

The formation of an insoluble dinitrophenylether by treating morphine with 1-chloro-2,4-dinitrobenzene or the corresponding fluorine derivative has been utilized for the gravimetric determination of morphine in opium.[82] The presence of other opium alkaloids does not appear to interfere with the assay.

B. TITRATION AND NONAQUEOUS TITRATION

Titrimetry has been commonly used in the past for alkaloidal analyses. The isolated bases can be dissolved in alcohol, a suitable indicator added, and the solution titrated directly with standard acid. For the evaluation of alkaloid fractions in organic solvents from chromatographic columns, two-phase titration is often useful. Ether and petroleum ether eluates can often be assayed with bromocresol green solution as indicator; chloroform presents some difficulty caused by the solubility of the indicator in it before the end point is reached. Back titrations in which the alkaloid residue is dissolved in standard acid and the excess acid then titrated with alkali are standard procedures (see Table 8.3).

Nonaqueous titration is now included in a number of pharmacopeias for the evaluation of alkaloidal salts. The technique has been dealt with in Chapter 6, and in Table 8.3 are listed some applications to alkaloids.

Some weakly basic salts can be titrated directly with alkali using phenolphthalein as an indicator, the salt of the weak base playing a minor role in the titration. Salts of stronger bases can be titrated in aqueous alcohol, but the end points of the titrations are often difficult to detect, owing to the buffering effect of the organic base. This, to some extent, can be overcome by adding a layer of chloroform to the aqueous titration liquid which removes the base as it is liberated. Also, by the use of potentiometric titrations, which are applicable to many alkaloid salts dissolved in aqueous ethanol, the difficult colorimetric end point can be entirely eliminated.[83] The titration of the solanaceous alkaloids with picric acid in chloroform has been employed in the investigation of *Duboisia* species.[84]

C. SPECTROMETRIC ANALYSIS

All compounds absorb electromagnetic radiations to a specific extent at characteristic wavelengths and absorption in the ultraviolet, visible, and infrared regions of the range is used extensively for the characterization, determination of purity, and assay of organic compounds.

For analytical purposes a compound must show some selective absorption at certain wavelengths, and in the ultraviolet and visible ranges this is associated with the presence of chromophoric systems within the molecule. Not all alkaloids contain these (for example, the pyrrolidine alkaloids hygrine, cuscohygrine, and stachydrine and the piperidine group coniine, conhydrine, coniceine, and tropine) and therefore exhibit no selective absorption in the range 200 to 800 mμ. Simple aromatic chromophores are present in some alkaloids (for example, substituted benzene in atropine and cocaine or a pyridine ring as in nicotine) and these absorb selectively at relatively short wavelengths (230 to 300 mμ) at "medium intensity." Other groups of alkaloids such as the isoquinoline, quinoline, and phenanthrene groups contain more complex chromophores. A few alkaloids (for example, berberine and serpentine), absorb light in the visible region and are consequently colored.

Quantitative measurements for alkaloids in the ultraviolet region are particularly valuable when small quantities only of alkaloid are available (for example, eluted spots of thin-layer or paper chromatograms), but a high degree of purity is necessary, since nonalkaloidal impurities may produce substantial extraneous absorption. At short wavelengths this has proved troublesome with eluates from thin-layer chromatograms. Examples of some applications to individual alkaloids will be found in Tables 8.1, 8.2, and 8.3. Individual alkaloids of a mixture may be determined by ultraviolet absorption, provided that the different alkaloids exhibit different absorption maxima. This has been applied by a number of workers to the analysis of the strychnine-brucine mixture present in nux vomica preparations. E (1%, 1 cm) values for

strychnine and brucine, respectively, are 318 and 314 at 262 mμ and 4.59 and 216 at 300 mμ. By measuring the extinction of the solution at these wavelengths, the two-point spectrophotometric assay can be used.[85] A similar method has been employed for the evaluation of mixtures of reserpine and rescinnamine by utilizing differences in absorption of trimethoxybenzoic and trimethoxycinnamic acids.

Colorimetry was employed long before the modern spectrophotometer was available. Most alkaloids and their simple salts are colorless, and for their quantitative determination either color reactions applicable to the particular molecule are applied or a colored salt-like complex is prepared which can then be isolated and examined. The specificity of many of the reactions makes the determinations less susceptible to irrelevant absorption than is the case with simple ultraviolet spectroscopy and hence more suitable for plant extracts.

Two characteristic color reactions of indole alkaloids are the deep blue produced with paradimethylaminobenzaldehyde and the red color with vanillin in concentrated hydrochloric acid, $\lambda_{max} = 532$ mμ. The former has been extensively used for ergot assays and the latter has been applied to reserpine. Another reaction for the estimation of reserpine is dependent on the greenish-yellow color ($\lambda_{max} = 390$ mμ) produced by the alkaloid and sodium nitrite in dilute sulfuric acid. Esters of tropic acid give an intense blue-violet color ($\lambda_{max} = 555$ mμ) when treated with fuming nitric acid followed by solution of the residue in anhydrous acetone and addition of potassium hydroxide in methanol (Vitali–Morin reaction). The reaction, which depends on the nitration of the aromatic nucleus has been much studied and numerous modifications proposed to improve color stability. Color reactions specific for individual alkaloids when in admixture are useful, for example, Folin–Ciocalteu reagent (sodium tungstate and molybdate in 85% phosphoric acid-concentrated hydrochloric acid mixture with added lithium sulfate) appears to be fairly specific for morphine and does not give colors with the other common opium alkaloids. This has been used in conjunction with adsorption chromatography for the assay of morphine in opium.[86]

A number of alkaloids form with some acidic dyes, salt-like addition compounds which can be extracted from the aqueous solution with certain organic solvents. These compounds can be decomposed with alkali and the colored aqueous solution determined spectrophotometrically. Bromocresol purple,[87] bromocresol green,[88] and bromothymol blue[89] have been employed for salts and preparations of tropane alkaloids; methyl orange for emetine,[90] reserpine,[91] and atropine;[92] bromophenol blue for reserpine in pharmaceutical preparations;[93] and eosin and erythrosin for a number of alkaloids.[92] The use of ammonium reineckate has already been mentioned; other reagents employed for the production of colored compounds with alkaloids include iodine and cisaconitic anhydride.

Infrared spectroscopy has been applied more to problems of authenticity and structure than to the quantitative analysis of alkaloids. The infrared absorption spectra of organic molecules is usually complex, but characteristic absorption maxima can be used for quantitative measurements in the same way as ultraviolet and visible absorptions. The possibilities of its quantitative applications were suggested in 1951 by Pleat and co-workers,[94] who examined some 22 alkaloids. It has since been applied to the evaluation of atropine[95] and reserpine[96] in tablets. The large number of, often characteristic, absorption bands exhibited by a single compound sometimes makes possible the quantitative analysis of the components of a mixture. Thus quinine and strychnine can be analyzed simultaneously by absorption measurements at 6.2 and 6.06 μ, respectively.[97] For mixtures of opium alkaloids, infrared spectroscopy has been used for the simultaneous determination of morphine and codeine as acetylated derivatives[98] and for noscapine, thebaine, and papaverine.[99]

D. FLUORESCENCE ANALYSIS

Some compounds absorb electromagnetic waves over characteristic wavelengths and then re-emit them at longer wavelengths. If the emission occurs in the visible region, during the period of excitation only, the phenomenon is known as fluorescence. Light of short wavelength and ultraviolet light are particularly active in producing fluorescence, and when the intensity of the emitted light obeys Beer's law with respect to the solute (over small concentration ranges) it can be used for quantitative evaluation. Modern spectrofluorimeters are available which accurately control the wavelength of the exciting rays and which analyze the spectrum of the emitted light. It is essential that extraneous substances which absorb the emitted light or which fluoresce in the same region as the compound to be assayed are absent or adequately accounted for. As with other methods of spectroscopic analysis, small quantities only of material are required.

Quinine in dilute sulfuric acid exhibits in ultraviolet light, a strong bluish fluorescence; for quantitative work the primary source should be in the range 300 to 400 mμ and the emitted light measured at about 450 mμ. The method is official for some quinine and strychnine preparations of the BPC (see Table 8.3). Hydrastine on oxidation with nitric acid yields hydrastinine which is strongly fluorescent. This has been utilized to determine the hydrastine content of the crude root in the presence of berberine and canadine.[100] Reserpine in acetic acid has a natural green fluorescence which can be intensified by treatment with hydrogen peroxide, whereupon concentrations as low as 0.2 μg reserpine per milliliter can be measured.[101] The method has been used for the assay of a number of species of *Rauwolfia*.[102] Other alkaloids which have been determined fluorimetrically include those of ergot, emetine hydrochloride after treatment with iodine (golden fluorescence), and yohimbine after treatment with hydrogen peroxide.

E. DENSITOMETRY AND AREA MEASUREMENTS

A quantitative determination of a separated solute on a paper or thin-layer chromatogram can be made by measurement of the size of spot, or density of the spot after spraying with a suitable reagent. In many cases a semiquantitative measurement can be made by visual comparison with standard reference spots produced under the same conditions. More refined techniques involve the use of planimeters and densitometers, the latter being available for automatically scanning a chromatograph at a given slit width. As with gas-chromatography records, the concentration of solute is proportional to the area under its respective peak on the graph. Some examples of the use of the method are included in Tables 8.1 and 8.2, and for some workers it is their method of choice.

F. POLAROGRAPHY

A number of papers report the use of the polarograph for alkaloidal analyses. Alkaloids which are reduced at the dropping mercury electrode present no special problems, but most common alkaloids are not of this type and produce catalytic waves which are influenced by a number of factors which must be carefully controlled. Kirkpatrick[103] has investigated these aspects and has applied the method to the separated, purified alkaloids of opium, ipecacuanha, hydrastis (hydrastine), nux vomica, cinchona, ergot, and other drugs. In this way it is of value for the analysis of injection solutions, pills, and tablets containing a single alkaloid. Šantavý[104] has used polarography for the determination of colchicine in colchicum seeds and tincture. The scope of the technique is widened by first converting the alkaloid to a reducible derivative, for example, the nitration of codeine and the conversion of ajmaline to its nitrosoderivative.

G. RADIOCHEMISTRY

Isotope-dilution analysis can be used for alkaloids provided a sample of the pure labeled alkaloid is available. A known weight of the radioactive alkaloid is added to the material to be analyzed, the total alkaloid extracted, and a pure sample of it prepared by repeated recrystallization to constant count. From the dilution of the radioactivity of the labeled alkaloid can be calculated the proportion of inactive alkaloid originally in the sample. The method has the advantage that quantitative isolation of the alkaloid is unnecessary but the isolated sample must be pure. Solutions present little difficulty but with dried plant materials the possibility of differential extraction of the added alkaloid compared with the inactive alkaloid bound in the plant tissues must be borne in mind.

N-methyl-C^{14}-caffeine has been used for the determination of caffeine, alone or in admixture with acetylsalicylic acid, theobromine, aminopyrine, or

acetophenetidine[105] and trimethoxybenzoyl-(carboxyl-C^{14})-methyl reserpate for the assay of reserpine in various species of *Rauwolfia*.[106] Caffeine has also been determined radiochemically by precipitation with P^{32}-labeled phosphomolybdic acid and the determination of strychnine and brucine with the same reagent after chromatography is indicated in Table 8.1.

H. HYDROLYSIS OF ESTERS AND THE DETERMINATION OF LIBERATED ACIDS

The total alkaloids (hyoscine and hyoscyamine) of the leaves of solanaceous plants can be determined by isolating the alkaloids and estimating the tropic acid produced by their hydrolysis.[107,108] By this means, tropine and oscine already in the plant material as a result of decomposition are not included in the assay. The weakly basic, therapeutically active alkaloids of rauwolfia are esters involving trimethoxybenzoic acid (reserpine, deserpidine) and trimethoxycinnamic acid (rescinnamine). Carol and co-workers[21] devised an assay for these alkaloids based on the hydrolysis of the separated weak alkaloids, extraction of the acids, and their determination by ultraviolet spectrophotometry at 270 and 300 mμ by means of a two-point correction procedure. A number of workers have used this method, but some work indicates that decomposition of the acids occurs in ultraviolet light making further investigation of the method necessary before it is generally adopted.[109]

I. BIOLOGICAL ASSAY

Biological assays are not commonly performed for alkaloids because chemical and physical methods are usually adequate and less time consuming. A biological technique is however used for tubocurarine chloride injection, USP, and dimethyltubocurarine iodide injection, NF XII. For this the "head-drop" method is used by which standard and assay preparations of the drug are injected into the marginal ear-veins of rabbits. The head-drop is considered complete when the rabbit, in response to a light tap on the back, is incapable of raising its head when it has fallen forward or of righting its head if laid on one side.

The mydriatic activities of the solanaceous alkaloids have been much studied, and the great sensitivity of the reaction made it suitable for the evaluation of very small concentrations of alkaloid at a time when physical methods of analysis were not extensively developed. Test animals for the mydriatic assay have varied from mice and cats to man. The method is advantageous in that it can be employed on mixtures without first separating other nonmydriatic alkaloids (for example, atropine in the presence of morphine). Miotic alkaloids (pilocarpine and physostigmine) can be determined in a similar manner, employing the constriction of the pupil of the eye as the test. Other biological methods for atropine involve the

measurement of blood pressure, salivation, and the degree of antagonism against parasympathetic stimulants.

Chemical assays of aconite root have not proved entirely satisfactory, and biological assay is probably of more value. Methods used include the comparison of LD_{50} doses of a reference standard aconitine solution injected intravenously or subcutaneously into mice with those of diluted tinctures. The MLD for guinea pigs has also been used.[110,111]

A number of biological assays have been employed for the evaluation of *Rauwolfia* roots and reserpine. The latter, administered to pigeons in parental doses of 0.06 to 0.12 mg/kg body weight produces a graded emetic response permitting its use as a quantitative assay. Rescinnamine and deserpidine will also produce emesis in pigeons within the same approximate dose range, and other emetics (for example, digitalis, veratrum, and quinacrine) must be absent from the preparation under test.[112] The ptotic activity, blood-pressure lowering, and sedation effects have also been used in mice, rats, and dogs for the determination of the reserpine-like activity of *Rauwolfia* species.

Other alkaloids and alkaloid-containing plants for which biological assays have been described include ergot, muscarine, nicotine, strychnine, *Veratrum*, and *Vinca*.

J. MISCELLANEOUS METHODS

Two methods employed in alkaloidal assays and not mentioned above are measurement of optical rotation (hyoscyamine admixed with atropine, mixtures of quinine and cinchonidine) and methoxyl group determinations (reserpine, quinine in totaquine, colchicine).

8.5 SUMMARY OF "OFFICIAL" METHODS

Table 8.3 summarizes the assays given in some recent pharmacopeias and allied works for the more common crude drugs, their alkaloids and preparations.

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