REVIEW ARTICLE

TOXICOLOGICAL ANALYSIS

By A. S. Curry, M.A., Ph.D. (Cantab.)
Home Office Forensic Science Laboratory, Harrogate

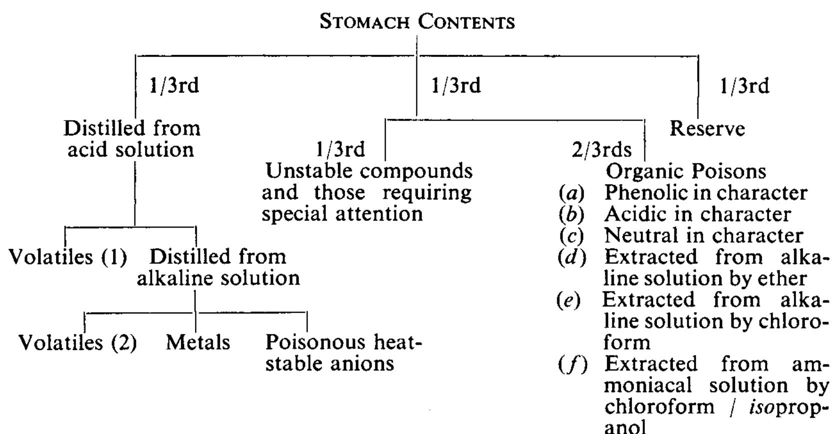
". . . Orfila first introduced and several authors have copied from him, a long methodical table of all poisons with their properties so arranged that a suspected substance might, by a successive comparison of its qualities, be referred to its proper head amongst them. But as in nineteen cases out of twenty the poison is presented to the medical jurist in so impure a state that this mode of examination is inapplicable; and as it is very doubtful whether an unknown poison is ever in practice found out in such a manner I have omitted the table altogether. By moral evidence and the consideration of the symptoms the particular poison is always indicated presumptively and the analysis directed by this presumption . . .".

So wrote Robert Christison in the preface to his *Treatise on Poisons* over 120 years ago. To-day the pendulum has been forced back to the Orfila philosophy. The cunning of modern poisoners is such that the tell-tale symptoms, especially of common poisons, are often well concealed. This is not the only factor with which the chemist has to contend. Since the nineteen-thirties the number of new compounds used in medicine, industry and the home has been increasing almost daily. The number of suitable substances readily available for the poisoner has correspondingly increased. Modern treatment also presents its own problems. As an example of this trend which illustrates the difficulties of the toxicologist, it is sufficient to quote the common occurrence of the injection of nikethamide as a medullary stimulant in instances of poisoning. This compound is extracted by the analyst together with the poisonous alkaloids. Indeed, the number of basic, naturally occurring and synthetic, nitrogen-containing compounds in use in medicine presents, in itself, a formidable problem in analysis.

To meet this growing challenge the newer techniques of electrophoresis, paper and ion-exchange chromatography, and ultra-violet spectrophotometry have arisen as the major techniques in use to-day. Infra-red spectrometers and X-ray diffraction cameras are within the reach of most toxicologists thanks to the ready co-operation of the universities and industry. The simpler techniques, however, are naturally in considerably wider use and this review will be concentrated in their direction.

Yet part of Christison's prefacory comment holds good, for the analysis is still guided by the symptoms and by the moral evidence, but in many cases these are either not available or have been deliberately disguised. Dr. H. Lederer and the author have recently reported a fatality in which the ingestion of a large quantity of a liquid arsenite produced no obvious post-mortem changes to suggest its consumption[1]. In these circumstances it is apparent that a systematic approach to the problem is essential.

The way in which the author approaches the analysis of, for example, a stomach contents is shown in the diagram.



The examination is conducted using the above general scheme: the results being noted on a standard printed form. Individual poisons are quoted by name on this form, the tests for the detection of the poisons being detailed on a "master plan". It is obvious that the master plan must be under constant review. Much work is done in an effort to find the most suitable and sensitive test for each poison or group of poisons. The application of the principles of operational research is an obvious necessity in any scheme of this type.

If the time that elapsed between the suspected ingestion of poison and death is known, this leads the analyst to his choice of organ to be examined first. If a sample of urine is available in sufficient quantity much useful information can often be gleaned by a process of spot testing. For example, a 30 ml. quantity of urine is sufficient to make tests for the following list of compounds: alcohol, acetone bodies, salicylates, chloral, chloroform, paraldehyde, formaldehyde, borate, bromide, iodide, chlorate, bromate, nitrite, phenacetin, sulphonamides, phenols and cresols, santonin, tetralin, naphthalene, arsenic and antimony.

Tests for these compounds are mainly old-established ones but for an up-to-date digest on laboratory tests for poisoning, the reader should consult the relevant section in von Oettingens book on *Poisoning* (Heinemann 1952). Tests of this type are of great value to the police toxicologist for he is usually required in a very short period of time to rule out any possibility of poisoning, or conversely, to produce positive evidence of poison. Thus it is of great value to the analyst to make use of simple rapid tests that can, in themselves, in a large number of instances, rule out poisoning by particular compounds. Positive evidence for the presence of any of a very large number of organic compounds can be obtained by extracting 5 ml. of urine with ether or chloroform from acid and alkaline solution and subsequently examining the extracts by ultra-violet spectrophotometry. The choice of solvents for this test is of importance.

Barbiturates, for example, will not show any characteristic inflections if ethanol is used as the solvent. A 0·5 N ammonia solution is therefore used by the author in the investigation of the "phenolic" extract from the urine. Thus with only a little over an ounce of urine it is possible to test for the majority of common poisons. The simplicity and high sensitivities of the tests ensure that there is the maximum chance of detecting poisoning.

The large number of investigations in which carbon monoxide and ethanol play not the dominant but the supporting role illustrate the need in all instances for the routine examination of blood samples for these compounds. Carbon monoxide determination by the reduction of palladium chloride have been extensively studied[2–4], while the examination of the spectra of blood samples at 540, 562 and 579 mμ also provides an accurate measure of carboxyhæmoglobin[5,6]. Simpson has shown[7], that if the Hartridge reversion spectroscope is used for the determination, then the sample need not be analysed with undue haste. Experiments by the author and Mr. S. S. Kind have shown that this does not appear to be true of blood samples from thin films— e.g. from blood-splashes. A lapse of a few hours is sufficient for great changes to be found. Simpson's results may be explained, either by a similar decomposition rate *in vitro* in bulk samples of both oxy-, and carboxyhæmoglobin, or to fortuitous spectral changes.

The determination of ethanol by dichromate oxidation methods has recently been studied by a panel of analysts of the Royal Institute of Chemistry[8]. Other chemical methods have also been reported[9–14], as has an enzymatic method for ethanol determination[15,16]. Perhaps the discovery with the widest medico-legal significance in the past decade has been that of the post-mortem production of many lower aliphatic alcohols in blood by fermentation with yeasts, fungi and bacteria[17–19]. Fortunately fluoride completely inhibits this alcohol production and this knowledge is now being put to practical use. Synergism and drug potentiation are growing problems for the toxicologist and emphasise the need for routine alcohol determinations. While the toxicity of methylpentynol is low, the increasing use of this drug and its alcohol type of action make it of particular significance. Analytical techniques for its determination have been devised[20,21] but its instability makes it one of the compounds for which a special search is necessary. Academic research has recently shown that the active constituents of water-hemlock and hemlock water-dropwort are also highly unsaturated alcohols[22]. The acetylenic links in these compounds are not able to form silver acetylides and although they possess characteristic ultra-violet absorption spectra it is probable that only the routine examination of stomach and intestine contents will give positive evidence for poisoning by these plants.

So it is that modern techniques, while making the task of the chemist easier, have not replaced the classical approach to the problem. The practical method of dividing poisons into the groups in which they are extracted from viscera is the approach that has guided the author in preparing this review. The first poisons to be considered therefore are those that can be separated from viscera by a process of distillation.

POISONS VOLATILE IN STEAM FROM ACID SOLUTION

One of the main requirements in any search for poisons is that the test applied should be sensitive enough to detect the quantities of poison likely to be present in the organs. "Approved tests" for this class of compounds are to be found in most textbooks. Those described below are not meant to be comprehensive but follow the trend of the review which emphasises the most recent aspects of the subject.

Tests for Alcohols

In tests for methanol little new has emerged in the past few years apart from variations in technique. The satisfactory method of controlled oxidation to formaldehyde and the determination of this compound with Schiff's or chromotropic acid still predominates[23]. Conway type of diffusion flasks have come into wide circulation for the determination of volatile poisons on a micro-chemical scale. Methanol[24] and cyanide[25] estimations are typical examples. The identification of aliphatic alcohols by the paper chromatographic separation of derivatives has been described[26,27] and there is also a method based on spectrophotometric measurements after conversion of the alcohols to the corresponding nitrites[28]. The differing toxicological properties of various glycols makes their identification important[29] and the separation by paper chromatography of the glycols present in foods, beverages and toothpastes[30] is of interest. The separation of polyhydric alcohols by paper electrophoresis[31] may also have some toxicological application as for example with erythritol derivatives.

Fujiwara's Test for Halogenated Hydrocarbons[32]

In this test 1 ml. quantities of distillate, together with 1 ml. of 20 per cent. sodium hydroxide and 1 ml. of pyridine are heated in a boiling water bath for one minute. Colours seen in the pyridine layer indicate halogenated aliphatic hydrocarbons. As there are 22 various halogenated hydrocarbons listed in a recent Government publication entitled *Toxic Chemicals in Agriculture*[33] which are being employed in this country as insecticides the significance of this test will be appreciated. Chloral, chloroform, methyl chloride, Frigen 12 and methylene dichloride give positive tests as does also trichloroethylene and its metabolites[34]. Chloral and chloroform can be determined together using this method[35]. Carbon tetrachloride does not give a positive result and the isolation of this compound and many other non-reactive volatile compounds is best approached by fractional distillation and determination of micro-scale boiling points using the techniques pioneered by Gettler[36].

Methyl salicylate, halogenated xylenols, cresols, and camphor are detectable in very small quanities if concentrated in the direction of an experienced nose. This may be done by extracting a portion of the distillate with ether and then carefully evaporating. The paper chromatographic separation of various cresols[37,38] is of interest in view of the continued popularity of the lysol type of disinfectant as suicide draughts.

Rothera's Test

This test has been recently re-investigated[39] and acetone, acetaldehyde and paraldehyde are of sufficient importance to warrant its routine inclusion in the scheme. Acetaldehyde disappears rapidly from the blood. Stotz, reporting a method for its determination[40], notes a 50 per cent. loss in 14 hours in a sample of blood kept in a refrigerator. In deaths from alcohol-disulphiram this is of importance. Methods for the determination of acetaldehyde as its 2:4-dinitrophenylhydrazone have recently been reported[41,42].

Measurement of the Ultra-violet Absorption spectrum

Measurement of the ultra-violet absorption spectrum constitutes a very sensitive test, suitable for eliminating from further consideration a vast number of compounds. In the toxicology section of a recent book on *Legal Medicine*[43] a table is given relating wavelengths maxima and minima with poisonous volatile compounds. The author feels that this list is a little unrealistic including as it does camphor which has $E^{1\ \text{per cent.}}_{1\ \text{cm.}} = 3$ at a wavelength maximum of 280 mμ. The only useful addition recently has been that of parathion. This compound has maximum absorption at 276 mμ and is readily detectable in concentrations of 1 mg. per 100 ml. This is the level likely to be found in accidental poisoning by this compound. Peaks about 270–280 mμ are not uncommon in distillates from stomachs and intestines from which no poison can be found. Heating the distillate with sodium hydroxide however will detect parathion, the yellow colour of *p*-nitrophenol being apparent in very low concentrations. Biggs uses this method for the determination of parathion from viscera[44].

Special Tests

(a) *Test for cyanide*[45,46]. A 1 ml. quantity of the distillate is put in a Gutzeit apparatus with some zinc and sulphuric acid. The test paper is made by dipping filter paper in slightly acid ferrous sulphate solution and drying. Before use the paper is dipped in 10 per cent. of sodium hydroxide and the excess removed by blotting. After 30 minutes in the Gutzeit apparatus it is dipped in concentrated hydrochloric acid containing a little ferric chloride. A blue stain is visible with only 0·2 μg. of cyanide. Concentrations of 0–14 μg. per cent. of cyanide have been reported recently in biological material[25].

(b) *Test for phosphorus.* Phosphorus poisoning presents special difficulties. Three murder trials, involving phosphorus, in the past two years in this country illustrate the toxicological importance of this element. Phosphorus is frequently concentrated in one section of the intestines, its length of travel depending on the length of time between ingestion and death. It is likely that if phosphorus is suspected then the whole of the intestine contents will have to be distilled if its detection is to be ensured. Distillation into silver nitrate traps the phosphorus and Kaye[47] claims a sensitivity of 10 μg. of phosphorus by converting this silver phosphide to phosphine in a Gutzeit apparatus and detecting it on a mercuric bromide paper. With the increasing availability of volatile organo-phosphorus

compounds the significance of the Mitscherlich test is increasing in importance.

While some indication of the general methods for detecting volatile poisons have been given, an example of a particular problem is not out of place. Small quantities of a lighter fuel had been added to a bottle of medicine. Chloroform and oil of anise in the medicine to a large extent interfered with the comparison, by classical methods, of control lighter fuel with that separated from the medicine. Vapour phase partition chromatography completely solved the problem as shown in Figure 1.

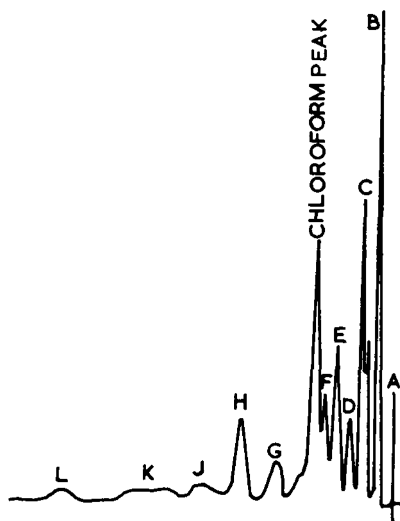


FIG. 1A. Control lighter fuel with added chloroform.

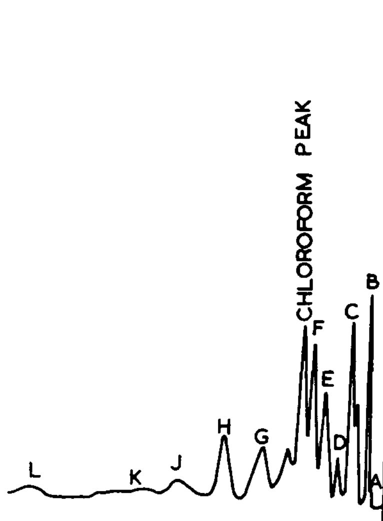


FIG. 1B. Liquid from medicine.

Poisons Volatile in Steam from Alkaline Solution

Nicotine is the most frequently met poison in this fraction. Amphetamine is an example of one of the modern drugs that also may be found in it. Evaporation of the distillate made acid with 0·01 N hydrochloric acid gives the solution for test. Because of the possibility of confusing amphetamine with naturally occurring amines it is convenient to consider here some compounds that are not steam volatile but which are found in the "alkaloid" fraction. Wickström and Salvesen have separated many sympathomimetic amines by paper chromatography[48]. One of their solvent systems, the common butanol 4 : water 5 : acetic acid 1 system, is closely related to the system used by Schwyzer[49] for the separation of many primary amines, including cadaverine and piperidine. Both these authors used as a general chromogenic spray an indicator solution, a sensitivity of 1 μg. being claimed. Many colour reactions suitable for distinguishing the various aliphatic and aromatic amines likely to be found in biological media have also been published[48,50,54]. R_F values in a variety of solvents have been extremely well covered[51–53]. Tests of this type are very valuable where small quantities of these compounds are present, for

example in horse-, and dog-doping cases. The quantitative determination of amphetamine in biological material has also been described recently[55,56]. The ultra-violet absorption of amphetamine is so low that this technique is of little value. Nicotine has $E^{1 \text{ per cent.}}_{1 \text{ cm.}}$ about 300 at approximately 260 mμ and so measurements in the ultra-violet are useful for detecting and assaying this compound.

The main bulk of organic compounds, however, have to be separated from viscera by a process of solvent extraction. It is convenient at this stage therefore to consider recent advances in isolation techniques.

Isolation Techniques

The Stas-Otto alcohol extraction process for the isolation of organic, solvent soluble, poisons is under considerable criticism at the present time when the increasing complexity of the analysis demands a corresponding increase in the speed of working. Such work as has been published on the quantitative isolation of poisons by this process also suggests that it is not as efficient as had previously been hoped. The tendency at present is towards a direct extraction of the macerated tissue with organic solvent immiscible with the aqueous phase. Roche and Wright[57], for example, extracted buffered tissue extracts with chloroform and examined the extract by ultra-violet spectrophotometry, while a similar technique was used by Smith and Macdougal[58] who also published extensive data of the ultra-violet absorption curves of compounds of toxicological interest. Feldstein and Klendshoj[59] in an important paper on the general isolation of poisons removed proteins by heat treatment and extracted the filtrate from acid and alkaline solution in a liquid-liquid continuous extractor. In contrast to this approach, methods are still being sought for a suitable protein precipitant.

Berman and Wright[60] in the extraction of alkaloids before ultra-violet spectrophotometry used a tungstic acid precipitation for the final removal of protein. Valov[61] also used tungstic acid for protein removal in the isolation of barbiturates. The author has used a similar method for the extraction of salicylates and phenacetin and has found that after the extraction of the acid-aqueous solution by ether many basic compounds can be extracted by ether and chloroform after making the solution alkaline with ammonia. Some, however, for example methadone, are co-precipitated with the protein, but their extraction is ensured if this protein precipitate is macerated with ammoniacal chloroform. The absence of difficult emulsions and the general cleanliness of the extracts suggests that this technique could be profitably investigated. Experiments by Mr. H. Powell and the author have shown, however, that chlorpromazine is not extracted using this method. The ammonium sulphate, acid extraction method of Daubney and Nickolls[62] has recently illustrated its wide application by the recovery of cantharidin from organs in a recent medico-legal case[63].

Whatever process or combination of processes is used the first group of poisons will be extracted from acid aqueous solution by ether. These can be subdivided in the following way.

Poisons Extracted from Ether by Sodium Bicarbonate Solution

The main bulk of toxicological specimens in this extract will be composed of aspirin metabolites. The colorimetric determination of salicylate has received its fair share of variations in the past few years[64–67]. Of interest to the toxicologist is the separation of aspirin metabolites by paper chromatography. Quilley and Smith[68] separated on Whatman No. 4 paper the compounds in Table I using a *n*-butanol 40 : water 56 : acetic acid 4 system.

TABLE I

R_F VALUES AND ULTRA-VIOLET DATA FOR SALICYLATE COMPOUNDS

Compound	R_F	Wavelength max. in mμ	Fluorescence in ultra-violet light
Salicylic acid	0·76	295	Violet
Salicyluric acid ..	0·65	320	Blue-violet
Gentisic acid	0·57	320	Intense blue
*Salicylamide	0·88	—	Blue-violet
*Gentisamide	0·75	—	Turquoise

* These compounds would not be found in this extract.

As individuals display a great variation in their sensitivity to aspirin it is important that the residues from a dose of 5 grains should be easily detectable. The paper chromatographic analysis, coupled with ultra-violet spectrophotometry, is capable of detecting microgram quantities of these compounds. The author has found, in agreement with other workers, that salicylic acid is normally the only acidic compound extracted from blood, brain and liver although the urinary excretion pattern on spraying with the ferric chloride, potassium ferricyanide reagent[69] shows a highly colourful picture. The determination of aspirin and salicylic acid in blood samples has recently been investigated[70]. Aspirin is the commonest poison in this group but many other compounds may also be found. One of these is diethylphosphoric acid from the hydrolysis of the insecticide tetraethylpyrophosphate. The author therefore puts an aliquot of this extract on a chromatogram, using a butanol-acetic acid system and detects by spraying with the Hanes and Isherwood[71] molybdate spray. The nitrophenols may also be found in this group, but by virtue of their colour are easily detected and determined. *p*-Nitrophenol is found as a metabolite of parathion. The dinitrophenols have caused many deaths of those accidently exposed to large concentrations; the observed dark colour of the urine of the victims has been exploited by malingerers. Picric acid, sometimes used in this way is readily identified by paper chromatography[72].

The naturally occurring organic acids sometimes cause difficulties of identification. The author has frequently isolated succinic acid from liver and intestines. The first time this compound was isolated its identification was delayed because of contamination with the anhydride after purification by a microsublimation method. Infra-red spectroscopy and paper chromatography finally led the way to its ultimate identification. A scheme for the identification of the common organic acids has recently been published[73] while organic acids in urine have been investigated by

ion exchange and paper chromatography[74]. Traces of hippuric acid in a urinary extract in an alleged case of poisoning recently aroused suspicions. The strong ultra-violet absorption showed its presence and identification was ultimately achieved by paper chromatography of the parent compound and of the glycine and benzoic acid formed by an acid hydrolysis. Hippuric acid is the normal excretion product after ingestion of benzoic acid and it is also found after exposure to toluene vapour. β-*p*-Hydroxyphenylpropionic acid, i.e. deaminated tyrosine, is also known to occur in exhumed bodies[75,76]. Most of the acids occurring in this fraction have a characteristic absorption curve and Table II may be of use in interpreting peaks found in this extract.

TABLE II

ULTRA-VIOLET DATA OF SOME ORGANIC ACIDS

Wavelength max. mμ	Compound	Solvent	Approx. $E^{1\ \text{per cent.}}_{1\ \text{cm.}}$	Reference
225	Aspirin	Ethanol	455	57
226	β-*p*-Hydroxyphenyl propionic acid	Ethanol	460	
227	Benzoic acid	Water	666	57
229–31	Opianic acid	Water	586	132
234	Meconic acid	Water	715	132
235	Salicylic acid	0·5 N HCl	640	57
238	β-*p*-Hydroxyphenyl propionic acid	0·1 N NaOH	370	
269	Cinnamic acid	Ethanol	125	57
272·5	*o*-Nitrophenol	Ethanol	400	57
275	β-*p*-Hydroxyphenol propionic acid	0·1 N H_2SO_4	71	
279	β-*p*-Hydroxyphenyl propionic acid	Ethanol	103	
282–4	Opianic acid		657	132
295	β-*p*-Hydroxylphenyl propionic acid	0·1 N NaOH	94	
300	Salicylic acid	0·5 N NaOH	260	57
303	Meconic acid	Water	440	132
361	2:4-Dinitrophenol	Ethanol	800	57

POISONS EXTRACTED FROM ETHER SOLUTION BY SODIUM HYDROXIDE

When the strong acids have been extracted from the ether solution by sodium bicarbonate, the much weaker acids may be extracted from the neutral compounds by a wash with aqueous sodium hydroxide. Thereafter, acidification and re-extraction, followed by evaporation leaves the crude poison.

Approximately 45 per cent. of all deaths from poison in this country, at the present time, are the result of barbiturate ingestion. The toxicologist's main difficulties in these deaths used to be the very low level of drug remaining in the body after several days spent in coma, and the presence of metabolites preventing crystallisation of the unchanged barbiturate. The use of ultra-violet spectrophotometry has solved the former problem while the presence of metabolites, instead of being a problem, now facilitates the identification of the ingested compound. Raventos, in a comprehensive review on barbiturate metabolism[77] focussed attention on the large amount of work being done at the present time and especially on the use of paper chromatography in this type of work. The identification of pentobarbitone from its urinary metabolites in a medico-legal death was reported recently[78], while the author has reported a urinary pattern seen only in cases of phenobarbitone poisoning[79], the

"extra spot" in this pattern being due to the excretion of *p*-hydroxy-phenobarbitone[80]. While the isolation of metabolites is in this way helping the toxicologist, the discovery of the rapid *N*-demethylation of, for example, *N*-methylphenobarbitone to phenobarbitone shows that very careful interpretation of results is essential. This work has shown that colour reactions for the identification of barbiturates from biological material are unreliable. While on this subject, reference should be made to a paper by Krauss and Grund[81] who have critically studied the theoretical background to colour reactions. Goldbaum's ultra-violet method[82] must also be used with extreme caution. The differing ultra-violet absorption of phenobarbitone and its urinary metabolite described by the author elsewhere, emphasises this point. The discovery of an interfering compound, most probably naturally occurring and seasonal in origin, drew attention to the need for ultra-violet spectrophotometry *after* purification by paper chromatography[83]. Many paper chromatographic systems have been reported and many methods used for the detection of barbiturates on paper. Generally the systems resolve themselves into two classes.

(1) Organic solvents saturated with ammonia or diethylamine. For a general review, see Raventos[77].

(2) Paper buffered with a basic buffer system with an organic solvent as the developing agent[84–85].

Raventos, has also reviewed the methods for detecting barbiturates on paper chromatograms. These can be classified under general headings as

(*a*) Formation of an insoluble metal salts, for example, mercury and silver salts, and the subsequent colorimetric detection of these metals.

(*b*) Formation of complexes, like cobaltamines and copper and pyridine complexes.

(*c*) Methods making use of the high absorption of the barbiturates in the ultra-violet.

The author as a general rule uses the *n*-butanol saturated with the 5 N ammonia solvent system of Algeri and Walker[86]. For showing the position of the spots a contact print of the paper is taken on Ilford Reflex Paper No. 50 using as the source of light a mercury ultra-violet lamp with no filters. This is the technique originally used by Markham and Smith[87] and Holiday and Johnson[88] for the detection of purines and pyrimidines on paper chromatograms. If the paper is exposed to ammonia vapour immediately before photographing then the increased absorption of the barbiturate at the more alkaline *p*H allows the ready detection of less than 10 μg. of barbiturate. The paper can then be cut and the spots eluted for quantitative ultra-violet spectrophotometry, or it can be dipped or sprayed to show the position of the spots as visible colours. The author prefers the mercury and diphenylcarbazone method and has used these techniques to separate phenobarbitone from diphenylhydantoin, compounds co-extracted, and frequently ingested together[89]. Using the modifications suggested by the author in this paper the background is made substantially colourless and the spots are stable for months and even years. Calculations which relate the ingested dose to concentrations in

the body at death are continually required of the toxicologist by coroners. For barbiturate drugs the accumulated experience acquired as a result of the very large number of suicidal and accidental overdose type of deaths encountered annually in such a laboratory as one of the Home Office Forensic Science Laboratories means that doses can be estimated in this way. It has been known, however, for a number of years that identical quantities of barbiturate given to different individuals will produce widely differing levels in the blood.

The identification of barbiturates by infra-red[90–92] and X-ray diffraction[93,94] analysis has also been reported.

The use of modern techniques for the analysis of barbiturate from viscera can be quoted as an example of a revolution in the last few years. One has only to investigate a death involving the simultaneous ingestion of two or three barbiturates and to develop the chromatograms, giving the distribution, and by elution and ultra-violet spectrophotometry the quantitative distribution, of each barbiturate in each organ to realise the tremendous analytical advances that these techniques have brought. It may well be that with the discovery of new drugs for the treatment of barbiturate poisoning and with the advent of improved hypnotics the phase of the barbiturates as the most common poisonous drug is passing.

It is unavoidable that so much of this section should be concerned with the barbiturates but the other compounds in this group must not be forgotten.

The application of paper chromatography to the detection and identification of hashish has been reported[95] and the ultra-violet absorption curves of the plant extracts before and after their extraction from viscera have been described[96]. The phenols derived from the anthraquinone type or purgatives may also be found in this fraction and paper chromatography is extremely useful for separating the complex mixtures of phenols found in these types of drugs. The author uses two systems, *n*-butanol-ammonia and butanol-acetic acid, for investigating these mixtures. Colours with ammonia vapour are striking but even more so are the fluorescent spots observed under ultra-violet light. Comparisons of samples of aloes, cascara, and rhubarb are simply made, using this technique.

Butazolidine, being a weak acid soluble in organic solvents, is extracted in this fraction. Methods for its determination have been published[97–99], and its distinctive ultra-violet spectrum ensures the detection and identification of very small quantities of this compound. The new rodenticide Warfarin also has a very characteristic absorption spectrum and the quantitative analysis of this compound using this spectrum has been reported[102]. Once the presence of Warfarin has been proved colorimetric methods for its determination are also available[100,101]. The ultra-violet spectrum of this compound is shown in Figure 2.

Non Basic Poisons not Extracted from Ether by Alkali

The number of poisons found in practice in this group is not great, but the number potentially to be found is very large indeed. The author

has shown the presence of phenacetin in extracts from liver after ingestion of tablets containing aspirin, phenacetin and codeine. The only published work of which the author is aware for the detection of phenacetin poisoning refers to the detection of *p*-aminophenol in the urine. The detection of unchanged phenacetin by its characteristic 250 mμ peak in these extracts was therefore especially interesting. Apiol may also be found in this fraction and the investigation of this compound by paper chromatography has recently been reported[103]. The long chain acetylenic alcohols and related compounds from *Oenanthe crocata* have been referred to, as has the analysis of 3-methylpentynol. The chlorinated organic neutral insecticides are well represented in toxicological literature by dicophane (DDT). Levels of 3·6 mg. per cent. in the liver and 2·7 mg. per cent. in the kidney were obtained in a fatal case reported by Luis[104]. The ultra-violet spectrum of this compound has been described[57] while its separation and identification on paper chromatograms has also received much attention. Mitchell and Patterson have dealt with this subject at length[105], while Mitchell has separated Prolan from Bulan[106], the isomers of benzene hexachloride[107], and aldrin, isodrin, dieldrin and endrin[108]. The determination of gamma benzene hexachloride from its absorption at 284 mμ has also been reported[109].

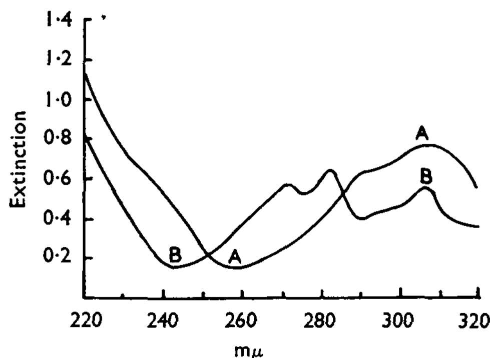


FIG. 2. Ultra-violet absorption curves for Warfarin 1·7 mg. per cent.
A in 0·1N sodium hydroxide
B in ethanol.

If an aliquot of this neutral extract is chromatogrammed and sprayed with the Hanes and Isherwood molybdate spray this ensures the detection of a number of neutral organic phosphorus insecticides. Methods for the detection and identification of these compounds are gradually being published; paper chromatography and ultra-violet spectrophotometry are again the main tools of this type of analysis[110,111,112,44]. While on the subject of insecticides reference must be made to review articles dealing with these compounds. Unfortunately the vast majority of the published methods of analysis are only of use when *the compound is known*. The main task of the toxicologist is to identify any abnormal analytical finding. The quantitative determination rarely presents difficulty. This is a fact very often not realised by those not actively engaged on this type of work.

The use in medicine of the substituted glutarimides in the treatment of barbiturate poisoning and as new hypnotics implies that these compounds may be found in this fraction. Already the isolation and identification and an urinary metabolite of bemegride (β-ethyl-β-methylglutarimide) has been reported[113].

Khellin, also found in this fraction, has been assayed from its ultra-violet spectrum and colorimetrically[114,115]. Santonin may also be conveniently detected and assayed by its ultra-violet spectrum[57] as may naphthalene[116], mephenesin[117], acetanilide[57], and phenacetin[57]. All these compounds show high absorption and are readily detectable in 10 μg./ml. concentrations.

While the identification of poisons is the main concern of the police toxicologist, there are many times when the identification of various pills and tablets assumes importance. The ready estimation and identification of the œstrogens and androgen preparations from ultra-violet spectra, coupled with paper chromatography, have led to the collection of control curves and patterns in the author's laboratory. So much work has been reported in the past few years in this field that it is obviously impossible in this short review even to begin to survey it. The toxicologist must have at least a passing acquaintance with the work of the clinical biochemist in this field. The publication of the *British Medical Bulletin* on Chromatography was especially useful[118]. The use of cortisone and hydrocortisone in horse- and dog-doping is a particularly pertinent example of the meeting of clinical biochemistry and toxicology. Methods for the differentiation of these two compounds have been reported[119,120]. The next group to be considered consists of poisons of basic character extractable from aqueous solution by ether.

Poisons Extracted from Ammoniacal Solution by Organic Solvents

The author prefers to extract first with ether. While the majority of alkaloids are successfully extracted using this solvent many other compounds, such as certain alkaloids, glycosides, aglycones and the rodenticide α-naphthylthiourea, are only extracted by chloroform or chloroform and ethanol mixtures. In this way an initial separation of these groups of poisons is possible. So much information has been of interest in the last few years that no review of this size can adequately cover even the outskirts of the field.

If a search for one, or a particular group of poisons, is requested it is a simple matter to turn to the relevant section of textbook and card index and perform the necessary analytical procedures that have been found to work for that particular compound or particular group of poisons. It is not unusual, however, to find that the deceased has had all the advantages of modern medicine before death, and consequently techniques that were suitable for academic studies on animals can not be used in the presence of, for example, procaine penicillin to quote only one commonly met antibiotic. In other instances the police may request a search for all poisons. It is at this stage that the author would echo Dr. Turfitt's sentiments in a similar review to this four years ago, "the actual problems of toxicological analysis are rarely appreciated except by those intimately connected with the field"[121].

The first stage in the analysis of poisons in this group is the separation of the poison from metabolites, from compounds that occur naturally in

the body and from any medicines administered in hospital. For analysis on a microchemical scale the author chooses paper chromatography. A system of *n*-butanol 100: water 100: citric acid 2, using Whatman No. 1 paper buffered with 5 per cent. sodium dihydrogen citrate was developed as a suitable system giving maximum resolution of poisons likely to be found in this group[122]. In passing it should be noted that Whatman No. 1 paper is essential for the production of really good spots. Whatman No. 4 paper gives only trails with many alkaloids. In the report of this work we gave a map reference for the poisons; by arranging them in order of ascending R_F it was possible to some extent to answer a single need of the police toxicologist—a rapid method of showing the absence of a vast number of poisons and at the same time concentrating attention on any abnormal finding.

After development the next stage is to show the position of the now separated compounds on the paper. To do this and at the same time cover as wide a field as possible, four methods are used by the author. Known alkaloids are put on the chromatogram as markers together with aliquots from the extract under investigation. If necessary, tests 1 and 4 can be carried out on one aliquot and tests 2 and 3 carried out in sequence on another aliquot.

The tests are as follows:

(1) The paper is photographed in ultra-violet light using the method as described under barbiturates. The sensitivity of this method for many compounds is high—10 μg. of many alkaloids being easily detectable. The paper is also inspected under filtered ultra-violet light. The fluorescence of quinine is particularly striking.

(2) The paper is sprayed with 0·2 per cent. solution of ninhydrin in acetone and heated at 80° C. for 3 minutes. This treatment ensures the detection of such compounds as ephedrine that have very weak ultra-violet absorption and do not react with the alkaloidal reagent in test 3.

(3) The paper is then dipped in a potassium bismuth iodide solution, or sprayed with potassium iodoplatinate. These general alkaloidal reagents detect 20 μg. quantities of most alkaloids.

(4) When the position of the spots has been revealed by one of the above tests quantitative ultra-violet spectrophotometry is carried out on relevant spots which are eluted from the paper and re-extracted.

The alkaloid complexes formed in test 3 can be cut from the paper, decomposed with ammoniacal chloroform, and the now pure alkaloid can be investigated by classical colour tests and micro-crystallography. Mannering and his colleagues in a paper on the use of paper chromatography applied to the detection of opium alkaloids in urine and tissues[123] give an excellent account of this type of scheme applied to a particular problem. Dependent on the type of isolation method used a "normal pattern" is soon established and any abnormality is cause for further investigation.

One disadvantage of the use of buffered paper is the decrease of sensitivity found with ninhydrin and Dragendorff reagents. While 30 μg. of ephedrine give an exceptionally good spot with ninhydrin using plain paper

and a butanol-acetic acid system only a faint spot is obtained with this quantity on citrated paper. In extracts from human viscera this degree of sensitivity is often acceptable. In other cases, however, especially if the analysis of a known compound is required, the advantage of the great resolving power of the citrated paper may not be required and by using systems not requiring buffered paper greater sensitivity is possible. The author obtained a very good ephedrine spot (approx. 10 μg.) using a butanol-acetic acid system for development and ninhydrin for detection of a known non-toxic dose, in an extract from only 2 ml. of urine. In this instance approximate quantitative measurement was made by the comparison of the ninhydrin colours with those from control quantities of ephedrine.

It is apparent that for test 4 to be of real value a great amount of relevant information about the ultra-violet absorption of compounds of toxicological interest has to be abstracted from the literature. The publication of logarithmic curves and those calculated from known molecular weights, coupled with the use of large number of various solvents, does detract from the value of some of this data. The amount of relevant information is now so large that for it to be of maximum value a card index system is really essential. Nevertheless, while the identification of ultra-violet peaks is often difficult, it is none the less rewarding. Even the apparent absence of absorption, in the presence of a positive Dragendorff reaction at a particular R_F, can point to the presence of a particular alkaloid, as for example, a member of the belladonna group.

Since the publication of the report of this citrate system a considerable number of additional compounds has been positioned on the map. Particularly useful is the way in which compounds like caffeine with their very high interfering ultra-violet absorption are separated one from another and from the majority of the common alkaloids.

There have been reports of confusion between the newer synthetic drugs and the alkaloids because of similar reactions with colour reagents, for example chlorpromazine with strychnine[124]. Paper chromatography was used to resolve this difficulty[125]. Morphine poisoning presents its own difficulties. Morphine has been found as a metabolite of codeine, while the use of nalorphine in the therapeutic management of morphine poisoning illustrates yet again the problem facing the toxicologist. With morphine and nalorphine an examination of the ultra-violet curves is of no avail, but X-ray, crystal formations and paper chromatography readily distinguish them[126]. In this last paper Pedley makes the very important point that while microcrystalline comparisons are readily reproducible on pure compounds in known concentrations, in the case of specimens extracted from viscera where the concentrations are not known and impurities are present completely different types of crystal formations can be, and are, obtained. This is a fact that has been very largely ignored in many publications and must to some extent detract from the value of this type of analysis. The same phenomena are repeatedly met with the barbiturate drugs.

So with this particular group of poisons, paper chromatography is

proving to be an invaluable tool. The great problem of separating microgram quantities of poison from the bulk of interfering material has been largely overcome. Obviously personal preference and experience have entered into the above description of the *modus operandi.* The foundation of this work is of course laid on the vast amount of work that has been published in the last few years on the chromatography of alkaloids, and especially on the work of Munier and his colleagues[127,128]. Once again the toxicologist is faced with the problem of abstracting from the whole field of chemistry, biology and medicine that which is particularly suited to his needs. It is with that problem in mind that the author has compiled Tables III and IV which summarise some of the more recent references that will be of interest in this field.

TABLE III

AIDS TO IDENTIFICATION

Compounds	Method	Reference
Selected alkaloids	Flavianic acid salts	129
Selected alkaloids	General crystal formation	130, 143
Narcotics	Reinecke salts	131
Narcotics	Ultra-violet	132
Narcotics	Infra-red	133
Narcotics	X-ray diffraction	134
Aliphatic amines	Colour reactions	50
Aromatic amines	Colour reactions	54
Local anæsthetics	Crystals	135
Local anæsthetics	Vacuum micro sublimation	136
Local anæsthetics	Paper chromatography	137
Antihistamines	H_2PtCl_6 crystals	138
Antihistamines	Colour reactions	139
Antihistamines	Picrate crystals	140
Antispasmodics	Colour reactions and crystals	141
Atropine and hyoscine	Infra-red	142
Methadone	Colour tests and crystals	143
Morphine and nalorphine	X-ray diffraction and crystals	126
Scopolamine	Crystals	145
Pethidine	Styphnate crystals	146
Opiates	Colour tests	147
Morphine	Mayer reagent	144

The extraction of the bulk of the alkaloids and synthetic basic compounds by ether does to some extent divide these from other alkaloids, the xanthines and other compounds only extracted to any real extent at the next stages. These are extracted by chloroform and chloroform-*iso*propanol. The author invariably uses for this last stage a liquid—liquid continuous extractor. While morphine and the xanthines are readily identifiable the detection and identification of the chloroform soluble glycosides present a rather more difficult problem. Current literature again shows that paper chromatography has proved invaluable for the separation of the various digitalis glycosides. Similarly the use of Carr Price reagent has been shown to give differential colours with various digitalis glycosides and their aglycones in concentrations of 0·5 μg./sq.cm.[179]. The methods for detecting extremely small quantities of these compounds

are therefore available and, coupled with paper chromatography, are potent tools not only for the pharmacologist but also for the toxicologist. The literature on this topic is so extensive that there is not space to do justice to it here. The author was particularly grateful that some ultra-violet data on these compounds had been published because a successful detection of digoxin was initiated by a peak observed at 217 mμ.

TABLE IV
METHODS OF QUANTITATIVE DETERMINATION

Compound(s)	Methods used	Reference
Some alkaloids	Methyl orange reaction	148
Alkaloids	Chemical reactions, colorimetric	149, 150
Selected narcotics	Ion exchange	151
Local anæsthetics	Ion exchange and titration	152
Analgesics	Reinecke salt	153
Antihistamines	Ultra-violet	154
Belladonna	Colorimetric	155
Tropine alkaloids	Colorimetric	156
Brucine	Ultra-violet	157
Caffeine and theobromine	Ultra-violet	158
Cocaine	Ultra-violet	159
Codeine and morphine	Ultra-violet	160
Colchicine	Colorimetric	161
Ergot	Review	162
Hyoscine and hyoscyamine	Paper chromatography and colorimetric	163
Methadone	Reinecke salt	153
Morphine	Colorimetric	164, 165, 166
Morphine	Ultra-violet	157
Morphine	Paper chromatography and colorimetric	167
Nalorphine	Ultra-violet	168
Phenadoxone	Ultra-violet	169
Prantal	Colorimetric	170
Pilocarpine	Colorimetric	171
Procaine	Ultra-violet	172
Procaine	Ion exchange	152
Quinine	Ultra-violet	173
Quinine and strychnine	Ultra-violet	173
Strychnine	Ultra-violet	173
Strychnine	Colorimetric	174
Reserpine	Electrophoresis and ultra-violet	175
Veratrum	Infra-red	176
Strychine and brucine	Ultra-violet	177, 178, 157

IONISED COMPOUNDS THAT ARE NOT EXTRACTED FROM AQUEOUS SOLUTION BY SOLVENTS

This section is not devoted exclusively to inorganic compounds because the quaternary ammonium and quaternary pyridinium compounds are becoming increasingly important in medicine. Two main methods are available for the determination of these types of compounds. One is based on the extraction of dye-complexes[180,181] and the other on the formation of Reinecke salts[182,183]. The former is capable of high sensitivities suitable for biological media. To identify members of this group paper chromatography has been used[184] while the analytical properties of some methonium compounds[185] have been published as have those of some synthetic curarising agents[186]. The amino-acids fall into this chemical

group and of particular interest is the discovery of abnormal urinary amino-acid chromatograms in those with lead poisoning[187]. Blood cholinesterase determinations and their significance in poisoning, especially by the organic phosphorus insecticides, also underline the contribution of biochemistry to toxicology.

Activation analysis has been used to show the distribution of arsenic along the length of hair[188] and ^{75}As has been used to follow the uptake of arsenic by hair immersed in arsenical solutions[189,190]. This is of particular medico-legal interest.

Numerous poisons available to the general public are sold as rat poisons. Sodium fluoroacetate is one of the new rodenticides that is toxic to man. The detection of this compound and of inorganic fluoride has recently been reported by Goldstone[191]. Thallium compounds form the basis of other rat poisons and the detection of thallium by paper chromatography[192] and by radioactivation methods[193] again illustrates the application of modern methods to toxicology. The destruction of biological matter before analysis for metals has also received attention recently[194].

SUMMARY

This review has encompassed a wide range of analytical techniques. It will be noticed that instrumentation has entered into toxicology to a tremendous degree in the past few years. Only four years ago Turfitt wrote: "physical methods have been used only to a relatively limited extent in toxicology". In the author's laboratory the Unicam SP500 in the last year was in operation for nearly 400 hours.

While infra-red, ultra-violet and X-ray spectrophotometry are now accepted tools in this type of work the simpler techniques of paper chromatography, paper electrophoresis and ion exchange chromatography are playing an increasing role in the isolation, purification and analysis of poisons. Paper electrophoresis apparatus is very simple to construct and has yielded valuable analytical data on a wide variety of subjects in this laboratory.

Toxicological analysis is a specialised science in its own right, as the author hopes he has demonstrated in this review. While the only collected work on some modern methods to be published so far is in a text-book on *Legal Medicine* it is now certain that this subject merits greater attention than it has received in the past.

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