Progress in Chemical Toxicology

VOLUME 1

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Edited by

ABRAHAM STOLMAN

Toxicological Services Section
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Contributors

Numbers in parentheses are the page numbers on which the author's contribution begins.

*ELVERA J. ALGERI (157), *Department of Legal Medicine, Harvard University Medical School, Boston, Massachusetts*

A. S. CURRY (135), *Forensic Science Laboratory, Harrogate, Yorkshire, England*

ABEL M. DOMINGUEZ (11), *Toxicology Laboratory, Armed Forces Institute of Pathology, Washington, D. C.*

CHARLES G. FARMILO (199), *Organic Chemistry and Narcotic Section, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada*

MILTON FELDSTEIN (297, 317), *Bay Area Air Pollution Control District, San Francisco, California*

ROBERT B. FORNEY (53), *School of Medicine, Indiana University, Indianapolis, Indiana*

HENRY C. FREIMUTH (1), *Chief Medical Examiner's Office, State of Maryland, Baltimore, Maryland*

KLAUS GENEST (199), *Organic Chemistry and Narcotic Section, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada*

LEO R. GOLDBAUM (11), *Toxicology Laboratory, Armed Forces Institute of Pathology, Washington, D. C.*

ROLLA N. HARGER (53), *School of Medicine, Indiana University, Indianapolis, Indiana*

†ARTHUR J. McBAY (157), *Chemical Laboratory, Massachusetts State Police and Department of Legal Medicine, Harvard Medical School, Boston, Massachusetts*

FREDRIC RIEDERS (191), *Division of the Medical Examiner, Department of Public Health of the City of Philadelphia, Pennsylvania*

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* Present address: Department of Biological Chemistry, Harvard University Medical School, Boston, Massachusetts.

† Present address: Law-Medicine Research Institute, Boston University, Boston, Massachusetts.

EUGENE L. SCHLOEGEL (11), *Toxicology Laboratory, Armed Forces Institute of Pathology, Washington, D. C.*

ARTHUR E. SCHWARTING (385), *School of Pharmacy, University of Connecticut, Storrs, Connecticut*

VARRO E. TYLER, JR. (339), *College of Pharmacy, University of Washington, Seattle, Washington*

Preface

This volume is one of a continuing series covering developments in toxicology. Since the publication of the treatise "Toxicology: Mechanisms and Analytical Methods," new literature has appeared containing much valuable information for toxicologists and scientists in allied fields.

"Progress in Chemical Toxicology" will provide source material on selected subjects in which the most information is available. Each subject area selected is reviewed and discussed by experienced workers. The objectives are to report on the usefulness of the newly developed techniques and methods with necessary modifications for toxicological studies and to supply related information for the proper evaluation of the results obtained.

This book maintains the aims of the original treatise. Information on the main topics such as isolation procedures, alcohol, barbiturates, and narcotics and related bases is developed further. Knowledge accumulated on other subjects including ataraxics, air pollutants, mushrooms, opium, and poisonous seeds and fruits has developed to a stage where separate chapters are required for each subject. Stress is laid on details of practical analytical significance and those which may be of importance in arriving at a decision as to the toxic effects of drugs.

The Editor wishes to express his sincere thanks to the authors for their patience, their cooperation, and the willingness with which they shared their experiences and accomplished their tasks. He also acknowledges gratefully the assistance and guidance of his associate, Dr. C. P. Stewart. Finally, the publishers of the book deserve special recognition and thanks for their tireless cooperation.

ABRAHAM STOLMAN

August, 1963

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Isolation and Separation Techniques for Identification of Poisons

by HENRY C. FREIMUTH

Office of the Chief Medical Examiner, State of Maryland, Baltimore, Maryland

I. Acetone Extraction of Organic Substances

The classical Stas–Otto procedure for the isolation of nonvolatile organic substances from tissues has been found, through the years, to have many shortcomings. To cite only two of these, there may be mentioned the relative lack of purity of the extracted compounds and the poor yields of many of these compounds. Because of these factors, various modifications of the Stas–Otto technique have been proposed (F2). A further modification has been suggested by Alha and Lindfors (A2) in which acetone has been used, instead of ethanol, as the protein precipitant and solvent for the organic substances. This procedure was first employed by Chéramy and Lobo (C1, C2) for the isolation of barbiturates from biological material.

The procedure developed by Alha and Lindfors uses 100 gm of minced tissue which is made acid (pH 4) with aqueous tartaric acid. To this, there is then added 200 ml of 70% acetone. The mixture is heated on a water bath at 60°C, using a reflux condenser, for ½ hour. It is then allowed to stand at room temperature for several hours and preferably overnight. The acetone is filtered and the residue washed with 70% acetone with the washings being added to the original acetone filtrate.

The acetone is then distilled from the filtrate by vacuum distillation until approximately 50 ml of liquid remains in the distilling flask. After cooling, the residue in the distilling flask is rinsed into a beaker with

water, made alkaline with ammonia, and then reacidified with tartaric acid. The precipitate formed at this point is removed by filtration and the residue is washed with water which is added to the filtrate.

Approximately 400 ml of acetone is added gradually to the aqueous filtrate and the mixture is allowed to stand at room temperature until the precipitate formed has settled to the bottom of the container. The mixture is filtered and the residue is washed with acetone. The filtrate (plus the washings) is subjected to vacuum distillation until 10–15 ml of liquid remain in the distilling flask. After cooling, another precipitation is carried out with 400 ml of acetone. The precipitate is allowed to settle and the mixture is filtered as before. The filtrate (plus washings) is again distilled until 10–15 ml remain. To this, 50 ml of hot water is added and, after cooling, the aqueous solution is filtered and the filtrate, which should still be acidic, is subjected to extraction procedures with immiscible solvents. The separation of four fractions is accomplished: (1) substances soluble in ether from acid solution; (2) substances soluble in chloroform from acid solution; (3) substances soluble in ether from NaOH solution; and (4) substances soluble in a mixture of $CHCl_3$ containing 10% CH_3OH from a solution which is alkaline with $NaHCO_3$.

The authors report that the solvent-extraction steps are most efficiently accomplished by using liquid-liquid extractors with a capacity of 100 ml of aqueous solution. In each group, 75 ml of solvent is used. However, the authors also used a simple shaking procedure employing three 25-ml portions of solvent. The latter procedure did not give as good recovery as the former.

Alha and Lindfors (A2) also used a simplified acetone extraction procedure in which the acidified minced tissue is treated with undiluted acetone, heated, and filtered as in the procedure described above. The filtrate is treated with 25 gm of anhydrous sodium sulfate, shaken mechanically for ½ hour, allowed to stand several hours at room temperature, and then filtered. The residue is washed with acetone and the combined filtrate and washings are then vacuum distilled until 10–15 ml of solution remains in the distilling flask. The flask is rinsed with hot water to make 50 ml of solution. After cooling, this solution is filtered and extracted with immiscible solvents as above.

Using these procedures, the authors report recoveries ranging from approximately 40% for morphine to 95% for carbromal plus phenobarbital.

II. Separation of Alkaloids from Tissue

A. Cation-Exchange Columns

Tompsett (T1) has reported a technique for separating morphine and other alkaloids from tissue using a cation-exchange column. The pro-

cedure uses 100 gm of minced tissue to which are added 500 ml of water and 100 ml of 10 *N* HCl. This mixture is heated to boiling and maintained in a boiling water bath for 1 hour. It is then cooled, diluted to 1000 ml with water, and filtered. The residue is washed with 500 ml of 1 *N* HCl and the washings are added to the filtrate which is then passed through a cation-exchange column. The latter is 140 mm long and 15 mm in diameter and contains Dowex 50 × 12, 200–400 mesh. After the filtrate has passed through the column, the latter is washed with 500 ml of 1 *N* HCl followed by 500 ml of water. The column is then eluted with 300 ml of 6 *N* ammonia. This eluate constitutes the morphine fraction.

The column is next washed with 200 ml of water and 200 ml of 1 *N* HCl. The more basic alkaloids are then eluted with 200 ml of 8 *N* HCl.

Tompsett's data show morphine recovery in a concentration of 4 mg% to be quantitative as well as that of codeine, brucine, and strychnine.

B. Acetonitrile Extraction

A method for isolating organic bases from tissues using acetonitrile and ether has been suggested by Abernathy *et al.* (A1). They have applied the method to liver, bile, and urine and have used it for the isolation and identification of morphine, nalorphine, codeine, meperidine, Methadone, amphetamine, quinine, caffeine, quinidine, colchicine, and phenothiazines. Recoveries of 75–80% were reported for codeine, morphine, strychnine, meperidine, and Methadone. Caffeine, amphetamine, and promazine were recovered in amounts in excess of 50%

The standard procedure results in the separation of two fractions, i.e., a "standard basic fraction" and a fraction designated as "solvent soluble salts." The latter contains Methadone, caffeine, and colchicine. The procedure, in detail, follows:

Reagents:

(1) A-E Solvent: 1 volume acetonitrile and 2 volumes ethyl ether.
(2) Alcoholic chloroform: 1 volume ethanol and 9 volumes chloroform.
(3) Salt buffer: 20 gm sodium borate and 200 gm sodium chloride made up to 1 liter with water.

Unhydrolyzed Tissue: 100 gm of liver are homogenized with 50 ml of salt buffer. This is extracted with 500 ml of A-E solvent, in three portions, by mixing in the same homogenizer. It is suggested that a rheostat control on the homogenizer will prevent spilling of solvent into the motor. The extracts are decanted into a beaker to allow further settling of the aqueous phase, after which the solvent is decanted into a separatory funnel. The solvent is washed twice with one-twentieth of its volume of salt buffer. One fourth volume of petroleum ether is added and the mix-

ture is extracted twice with one-twentieth of its volume of 1 *N* H_2SO_4. The combined acid extracts are washed with 2 volumes of A-E solvent and once with 2 volumes of chloroform. The original A-E solvent and the two solvent washes are combined and reserved for later extraction of the "solvent soluble salt" fraction. The aqueous phase above is adjusted to pH 8.6 and extracted twice with 3 volumes of alcoholic chloroform. The combined solvent phase is filtered, a few drops of HCl are added, and the solvent is evaporated using an air jet and mild heat. The residue is the "standard basic fraction."

The "solvent soluble salt" fraction is obtained by adding to the combined solvent one-twentieth of its volume of saturated sodium bicarbonate solution. The mixture is shaken and the aqueous layer is discarded. The solvent is decanted into a beaker and evaporated to near dryness in the presence of an excess of dilute HCl, using an air jet and mild heat. The residue is rinsed into a separatory funnel with 50 ml of water and 50 ml of ether. This is shaken and the ether phase is discarded. If necessary, any emulsion at this stage is broken by centrifuging. The ether wash is repeated after which excess sodium carbonate is added. The mixture is extracted twice with three times its volume of chloroform. The combined chloroform extract is filtered and, after addition of a slight excess of dilute HCl, the solvent is evaporated to dryness using an air jet and mild heat.

Urine samples may be extracted by first saturating 50 ml of urine with sodium chloride, adjusting to pH 8.6, and extracting twice with 3 volumes of A-E solvent. Thereafter, the procedure for tissue is followed beginning with the salt buffer washing.

For morphine and other conjugated compounds, hydrolysis is first carried out with sulfuric acid, with this procedure varying somewhat with the type of sample used. For tissues, 100 gm of homogenized tissue are treated with 18 *N* sulfuric acid until the mixture is 1 *N* in sulfuric acid. This is autoclaved for 15 minutes at 20 lb pressure. Urine samples of 50 ml are treated in the same way. Bile, which is used primarily for morphine, is treated by adding 5 ml of 2 *N* sulfuric acid to 5 ml of bile and autoclaving in the same manner as tissue. After cooling, the hydrolyzed bile is filtered and the residue is boiled briefly with 5 ml of 1 *N* sulfuric acid. This is cooled and filtered into the first filtrate.

To the hydrolyzate or filtrate obtained above, a few crystals of sodium sulfite are added to prevent oxidation of morphine. The mixture is then adjusted to pH 8.6 and extracted twice with three times its volume of A-E solvent. The solvent layer is washed twice with one-twentieth of its volume of 1 *N* H_2SO_4. The acid layer is washed once with 2 volumes of A-E solvent and once with 2 volumes of chloroform.

One milligram of sodium sulfite is added to the aqueous phase and the pH is adjusted to 7.0 with 50% NaOH. Five drops of NaOH are added in excess and the mixture is extracted twice with 3 volumes of chloroform. The latter is filtered, and, after addition of a few drops of hydrochloric acid, evaporated to dryness, using an air jet and low heat. The residue is the basic fraction.

The aqueous phase remaining after the chloroform extraction is brought to pH 8.6 and extracted twice with 3 volumes of alcoholic chloroform. The extract is filtered and evaporated as above, leaving morphine in the residue.

III. Two-Stage Extraction

A. Acid Spot Extraction—Paper Chromatography

An elegant method for the rapid isolation and detection of organic bases in urine has been described by Morgan (M1). The method is especially suited for use with paper chromatographic methods of identification. In applying the method, oxygen-free nitrogen is used in both the extraction and isolation procedures. The apparatus used is shown in Fig. 1.

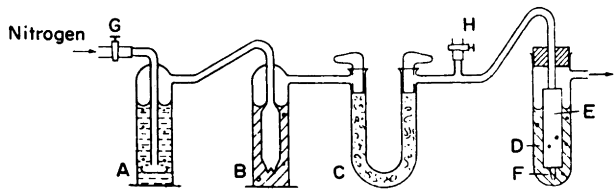

FIG. 1. Diagram of acid spot extraction apparatus.
A = Washing bottle containing chromous sulfate solution
B = Washing bottle containing concentrated sulfuric acid
C = U-tube containing self-indicating silica gel
D = Solvent extract
E = Paper strip with acid spots
F = Tip of gas delivery tube
G = Screw clip
H = By-pass to flowmeter

The chromous sulfate solution is prepared in the gas-washing bottle by mixing 12 gm of powdered chromic potassium sulfate, 50 ml of water, and 5 ml of concentrated sulfuric acid. When the solid has completely dissolved, the solution is cooled by the addition of 10–15 gm of crushed ice and the mixture is poured into 6 gm of zinc dust contained in the bottom of the gas-washing bottle. The inlet tube is immediately inserted and air is expelled by passing a small stream of nitrogen through the bot-

tle. Complete reduction is indicated by a pale blue solution which will darken as oxygen is absorbed.

For extraction, the urine sample is first adjusted to a pH of 9 by adding 20% sodium hydroxide. One-fifth of the volume of chloroform is introduced into a separatory funnel and the urine is carefully poured onto the chloroform so that no emulsification occurs. A slow stream of nitrogen (less than 50 ml/minute) is then passed into the chloroform layer and the rate of gas flow is adjusted so that the coarse emulsion which forms at the interface does not extend to the tip of the gas-delivery tube. After 20 minutes the gas supply is shut off and any residual emulsion that does separate on standing is broken up as far as possible by gentle stirring of the chloroform layer. The latter is filtered through a dry filter paper into a test tube of such diameter that the height of the liquid is 5–7 cm.

A strip of Whatman 3MM filter paper 7 cm by 1.5 cm is spotted with drops of 0.25 and 0.5 *N* sulfuric acid, 2 cm from one end of the paper. Morgan indicates that it is advantageous for the sulfuric acid to contain thymol blue indicator. Such solutions can be prepared by dissolving separate 4-mg portions of thymol blue in 0.5 gm and 1.0 gm of concentrated sulfuric acid, respectively, in 50-ml stoppered flasks. These solutions are set aside overnight and diluted to 40 ml with distilled water. These are then used for spotting the filter paper as indicated above. It should be mentioned that the filter paper should first be washed chromatographically with chloroform followed by water before it is cut into strips. The acid spots can be applied with a loop of platinum wire adjusted to produce spots 3–5 mm in diameter.

Immediately after the paper has been spotted with the acid, the strip is placed in the chloroform extract so that the spots, and preferably the entire strip, are completely immersed. The extract is agitated with a stream of dry oxygen-free nitrogen flowing at a rate of 100 $\pm$ 50 ml/minute/20 ml of solvent. The gas passes into the test tube through a glass delivery tube whose lower end is below the acid spots. The latter should not come into contact with the delivery tube or the walls of the test tube.

After some minutes, the spots will become transparent owing to loss of water. If a high concentration of basic substances is expected the strip may be removed from the chloroform at this point. In any event, 15 minutes of agitation is sufficient to extract alkaloids which were originally present in the urine sample in a concentration of 1 ppm. Morgan indicates that lower concentrations can be detected after longer agitation or by simply immersing the paper strip in the chloroform extract overnight without passing nitrogen through it.

After the gas flow has been stopped, any part of the paper strip not immersed in the solvent is rinsed with the extract by shaking for a few

minutes. Excess chloroform is removed by shaking the strip and exposing to a current of air. The dried strip is then suspended in a flask over a few drops of concentrated ammonia until the spots change from pink to yellow or blue. This will take about 15 minutes.

The ammonia vapor is removed from the paper in a current of air and the resulting mixture of the sulfates of organic bases extracted from the urine is developed on the paper strip as a paper chromatogram by any convenient ascending solvent technique.

B. Protein-Free Filtrates—High Temperature Chromatography

A method for the simultaneous detection of alkaloidal, neutral, and acidic poisons in human tissues has been described by Street (S1). This procedure involves the separation of various toxic substances by the use of high temperature, reversed phase paper chromatography. The chief advantages for such a system are: (1) the use of a single solvent system and the same paper for the separation of a mixture of compounds having widely different properties; (2) the time required for chromatography is only 20 minutes rather than hours; (3) the chromatographic spots show little diffusion, (4) the solvent, which is *M*/15 phosphate buffer pH 7.4, is more stable than the usual organic solvents used in chromatography.

In the procedure described, a protein-free filtrate of blood or tissue is prepared by adding to 10 ml of blood (or 10 gm of tissue) 63 ml of distilled water and 20 ml of 10% (w/v) sodium tungstate. The mixture is homogenized and 7 ml of 10% (v/v) sulfuric acid are added while stirring vigorously. The acidified homogenate is placed in a boiling water bath for 15 minutes and then filtered while hot through Whatman No. 1 filter paper on a Buchner funnel using gentle suction.

The cooled filtrate is then treated as outlined in Fig. 2. This results in five fractions, each of which is dissolved in 2–3 drops of ethyl alcohol.

Each of these solutions is applied to a 6¼ by 7 inch sheet of Whatman No. 1 filter paper which has previously been dipped in 10% glycerol tributyrate (tributyrin) in acetone and dried. Suitable marker solutions are also applied as separate spots adjacent to the appropriate fraction extract.

In preparing the paper, an even distribution of the ester over the paper is obtained by placing the treated sheet between several sheets of filter paper immediately after it is removed from the ester solution and then pressing the top sheets with a 10-inch rubber roller. In cutting the paper, a portion is left at one corner to form a tongue which is used in clipping the paper in cylindrical form for chromatography.

The paper cylinder with the various fractions is then placed in a

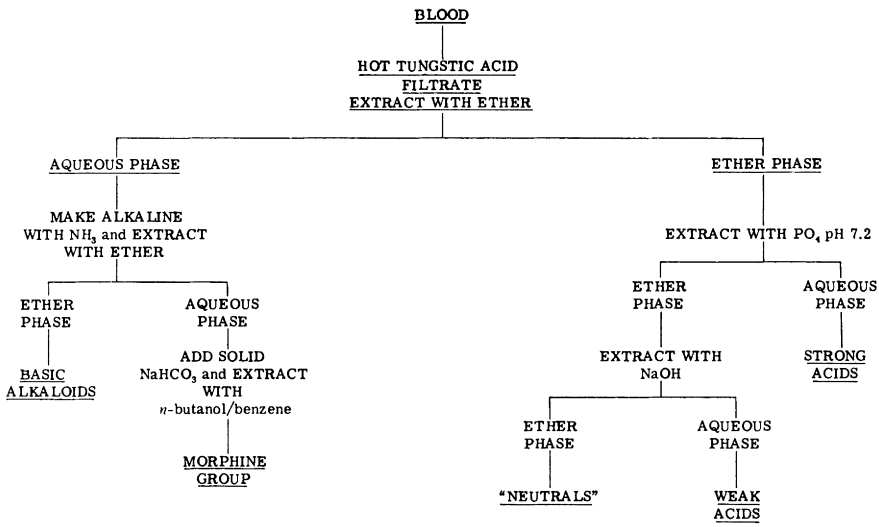

FIG. 2. Scheme showing the treatment of the protein-free filtrate in order to produce the various fractions for application of high temperature, reversed phase paper chromatography.

cylindrical jar fitted with a ground glass lid. Silicone grease is used to seal the lid of the jar. The jar is slightly larger in diameter than the paper cylinder and contains *M*/15 phosphate buffer, pH 7.4. The jar and solvent are maintained in an oven at 86°C before introducing the paper cylinder. Ascending chromatography is then carried out at 86°C for 20 minutes.

After chromatography, the wet paper is examined under ultraviolet light at 2540 Å. Any absorbing or fluorescent spots and their colors are noted. The paper is then exposed to ammonia gas and any additional absorbing spots are noted. The paper is subsequently dried with a hair dryer and re-examined in ultraviolet light. After this examination, the paper is cut into strips, thus isolating the various fractions.

The basic alkaloids and the morphine group of alkaloids are developed by dipping the appropriate strips in the iodoplatinate reagent of Farmilo and Genest (F1). The strips containing the neutral fraction may be treated with 0.1% potassium permanganate which will develop ethinamate (Valmid) as a yellow spot on a pink background. The same permanganate solution may also be used to locate those barbiturates having unsaturated groups in the side chain on the strip containing the weak acids. A typical chromatogram produced by this procedure is shown in Fig. 3.

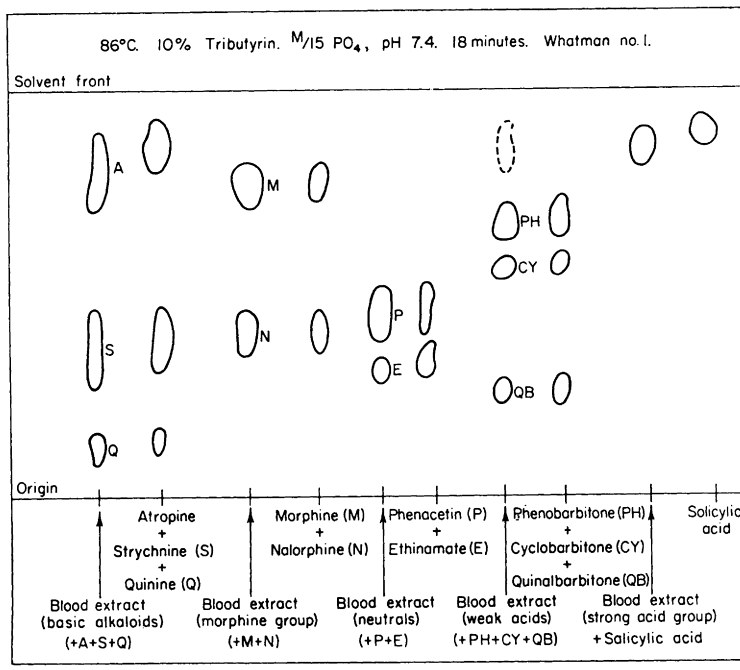

FIG. 3. Tracing of composite chromatogram obtained by chromatography of various fractions isolated from blood to which the compounds referred to on the chromatogram had been added. The dotted area in the weak acid fraction is due to a trace of salicylic acid, present because of incomplete extraction by the phosphate buffer at pH 7.2. Tributyrin-impregnated Whatman No. 1 paper; solvent, *M*/15 phosphate buffer, pH 7.4, temperature, 86°C; time, 18 minutes.

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