CERTAIN ASPECTS OF TOXICOLOGICAL ANALYSIS

Thesis

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CONTENTS

CHAPTER 1

INTRODUCTION

The publication of "Traite de Toxicologie" by Orfila in 1814 developed the idea of modern toxicological chemistry. Orfilla made the systematic experiments on animals with all the then known organic and inorganic poisons and described the symptoms and pathological changes in the internal organs produced by them. The first systematic treatise of the chemical tests and physiological effects for poisons was written by Christison (1829).

Two main problems are encountered in the isolation of the organic poisons from tissue. The first is to precipitate the tissue protein whilst leaving all toxic substances in solution and the second is to isolate the organic poisons from the deproteinized tissue extract.

Removal of Proteins and Fat

The first feasible method for the extraction of organic poisons from biological material was that proposed by Stas[1] (1852) when he was investigating the case of Count Hippolyte de Bocarne who was supposed to have been poisoned with nicotine. It was observed that the acid salts forming from alkaloids are soluble in water and alcohol, but that the alkaloidal bases liberated by alkali pass more or less completely and readily into ether or other immiscible organic solvents.

Otto[2] (1856) modified the Stas method, by treating the final aqueous liquid, while still acid, with ether to remove

fats and other substances (other than alkaloids) soluble in ether. Subsequently many workers described various modifications of the Stas-Otto process: Dragendorff[3] (1868) used aqueous acids for the extraction of alkaloids from biological material in contrast to the Stas procedure employing acidified alcohol; Autenrieth[4] (1928) on the contrary extracted the finely minced tissues with absolute ethyl alcohol and a few drops of tartaric acid, refluxing the mixture for about 10-15 minutes to obtain a more complete extraction.

Apart from the use of acid and alcohol, some workers tried to remove protein by chemical precipitation methods. Kippenberger[5] (1897) initiated the use of tannin and glycerine for 2 days at 40°C for the isolation of alkaloid from tissue, the alkaloid tannates being soluble in glycerine and the protein tannates insoluble in this solvent. Kesser, Oelkers and Raetz[6] (1933) used 29% uranyl nitrate as protein precipitant. Plant and Pierce[7] (1933) suggested various methods to separate the alkaloids from tissue proteins, by digestion with enzymes, by extraction with organic solvents and by heating and saturation with neutral salts. Alha and Lindfors[8] (1959) have recently published a method in which proteins are precipitated by acetone for use as a general procedure for the isolation of organic poisons from biological material.

The classical Stas-Otto extraction method at one time occupied a pre-eminent position for the isolation of organic poisons from biological material and in spite of the many modifications such as those listed, is still very useful and

widely used. Nevertheless the large number and tedious nature of the operations involved render it unsatisfactory for quantitative estimation of at least the less stable alkaloids. Moreover this method did not separate many volatile poisons and neutral organic substances. The increasing complexity of toxicological analysis demanded a rapid and satisfactory method for the isolation of organic poisons. Stewart, Chatterji and Smith[9] (1937) focussed the attention in the toxicological field by using trichloracetic acid as protein precipitant and following this by adsorption of the alkaloids on a solid medium (kaolin). Daubney and Nickolls[10, 11] (1938) used ammonium sulphate in presence of acetic acid for the precipitation of protein. Valov[12] (1946) utilized tungstic acid for the precipitation of protein in order to isolate barbiturate. Berman and Wright[13] (1953) used sodium tungstate for the final removal of protein to separate alkaloid. Gettler and Sunshine[14] (1951) precipitated the tissue proteins by passing steam through the homogenized liver acidified with tartaric acid.

Extraction from the Deproteinised Tissue Filtrate.

In the original Stas method the alkaloids were extracted from an aqueous alkaline solution by ether. This method of extraction was suitable for most of the alkaloids from plants whereas it was not very successful when the alkaloids were present in very complex organic mixture. Otto[15] (1856) utilized ether to remove fats and other substances from the final aqueous liquid, while still acid, and then made the

residual acid-aqueous phase alkaline. He used ether in general for the subsequent extraction of alkaloids, but advocated the use of amyl alcohol in place of ether for the extraction of morphine. Landsberg[16] (1880) and Marmé[17] (1885) extracted morphine from aqueous liquid, while still acid, with hot amyl alcohol, as the modification of the Stas-Otto extraction. Dragendorff[18] (1868) used chloroform for the extraction of alkaloids. Babel[19] (1904) suggested the use of chloroform as a universal solvent for all alkaloids but found difficulty in extracting morphine which contained some colouring matter. Daubney and Nickolls[20] (1938) also extracted the alkaloids from the protein-free filtrate with chloroform. Some workers used ethyl acetate for the extraction of morphine; Oberst[21] (1938) used ethyl acetate primarily as a rapid method of extracting morphine from urine and Roche Lynch[(22)] (1938) also selected ethyl acetate as a solvent for morphine. Bamford[23] (1940) agreed with this preference for ethyl acetate as extracting solvent for morphine on the grounds that though morphine was less readily dissolved in it than in an alcoholic chloroform mixture the ethyl acetate yielded a clearer extract, and he claimed that when the aqueous liquid was reduced to a very small volume, the relatively low solubility of morphine in ethyl acetate was less important. Kippenberger[24] (1897) extracted morphine by chloroform-alcohol mixture (9:1) after saturation of the aqueous residue with sodium chloride mixed with concentrated sodium carbonate. Oberst[25] (1942) employed a chloroform-alcohol mixture (3:1) to extract morphine. Kesser,

Oelkers and Raetz[26] (1933) used a chloroform-isopropyl alcohol mixture for the extraction of morphine. Rising and Lynn[27] (1932) considered a chloroform-acetone mixture as the best solvent for the extraction of morphine. When the alkaloid poison is unknown chloroform and ether are generally useful solvents for extracting them, but various other solvents may be preferentially employed by the analyst for individual alkaloids of known identity.

Direct Extraction of Alkaloid from Macerated Tissue

A direct extraction of the macerated tissue with organic solvent immiscible with aqueous phase has been tried by some workers. Roche and Wright[28] (1953) extracted buffered tissue extracts with chloroform. The residue from the evaporated chloroform extract was taken up in alcohol and examined by ultra-violet spectrophotometry. Smith and Macdougal[(29)](1953) utilized similar technique and published extensive data on the ultra-violet absorption curves of toxicological compounds.

Feldstein and Klendshoj[30] (1953) also used chloroform to extract organic poisons from tissue protein-free filtrate in a continuous liquid-liquid extractor. Umberger[31] described a method using continuous direct extraction of tissue with ethanol and tartaric acid. As a development of this procedure Curry and Phang[32] (1960) have designed a continuous extractor apparatus using ethanol under reduced pressure for the isolation of alkaloids, barbiturates and glycosides from viscera and this method, besides being more versatile, and giving a

reasonably pure extract in good yield, shortens the extraction time to 4 to 5 hours.

Separation of Alkaloids by Adsorption

Stewart et al.[33] (1937) initiated the use of kaolin for adsorbing alkaloids from trichloracetic acid filtrate. Later workers found that the adsorbing capacity of kaolin for alkaloids was not very high and this gave way to other adsorbents. Stolman and Stewart[34, 35] (1949) utilized the adsorption chromatographic method in the toxicological analysis for quantitative separation of mixtures of morphine, codeine, heroin and barbiturates, using Florisil columns. Ion-exchange resins and paper electrophoresis are promising techniques for the isolation and purification of organic poisons from biological material. No systematic approach to the general application of ion-exchange resins to poison separation has been described, although Kirk[36] (1955) pointed out that ion-exchange resins might be utilised to supplant or at least supplement the immiscible solvent separation in the investigation of individual poison.

The aim of this investigation is to utilize ion-exchange resins (Dowex 50 x 12 and Dowex 50 x 8) to isolate alkaloids direct from human liver extract. After adding the alkaloids in the liver, the protein was precipitated with $(NH_4)_2SO_4$ and the alkaloids adsorbed on Dowex 50. The isolated alkaloids were then purified by paper electrophoresis.

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CHAPTER 2

USE OF ION EXCHANGE RESINS FOR THE ISOLATION AND PURIFICATION OF ALKALOIDS

The alkaloids being bases existing in solution as cations are adsorbed by suitable ion exchange resins, and can therefore be separated from at least some of the unwanted constituents of alkaloid-containing mixtures.

An ion exchange resin has been described as a high molecular weight polymer containing ionic groupings as integral parts of the polymer structure. According to Kunin[1]:

> "An anion exchange resin was defined as a polymer containing amine groups as integral parts of the polymer lattice and an equivalent amount of anions such as the chloride, sulfate, hydroxyl etc. ions. The cation exchange resins were defined as polymers containing phenolic, sulfonic, carboxylic, phosphonic acid groups as an integral portion of the resin and an equivalent amount of cations. The polymeric portion of the resin is usually so highly cross-linked that solubility of the resin structure is negligible."

Dowex 50 is a synthetic cation exchange resin with a cross-linked aromatic hydrocarbon chain containing nuclear sulfonic acid groups as the only active cation-accepting group. Dowex 50 exhibits exceptional chemical stability which is complete at all pH values of both sodium and hydrogen forms. No chemical degradation of the resin occurs when any form is kept at 150°C for 16 hours in alkali, acid or neutralised water; the sodium form is stable in water at 175°C for the same length of time; and the resin is stable to dissolved oxygen and chlorine in water.

The discovery of sulfonated coals and of synthetic organic ion exchangers showed the efficacy of the new method in the isolation and purification of alkaloids from plant extracts and galenicals. However, the application of these exchangers was found to be of limited value in toxicological analysis although adsorption chromatography had been used successfully by Stolman and Stewart[2] and Calderon[3] in Florisil column for the purification of toxicological extracts.

Applezweig[4] studied the effective capacity of the "Zeo-carb" for quinine captured from sulphuric acid solution. The elution of alkaloid base from the column was carried out by ammonia-ethanol and this procedure simultaneously achieved the regeneration of the column. Applezweig[5] used the same technique for the isolation of atropine from Datura. Kingsbury, Mindler and Gilwood[6] used Zeo-carb for the separation of nicotine.

Jindra[7] percolated an alkaloidal salt solution through anion exchange resin Amberlite IR-4B and determined the base in the effluent by titration with acid using mixed indicator (methyl red and methylene blue). In this process he determined the salts of strychnine, atropine, morphine, brucine, ephedrine, quinine andcinchonine. Jindra and Pohorsky[8] developed the semi-micro and micromethod on ion-exchange by chromatography with Amberlite IR-4B for the determination of alkaloids in the alkaloidal salts, crude drugs and galenicals. This method was not satisfactory in case of apomorphine, physostigmine and ephedrine. Jindra and Rentz[9] studied the adsorption capacity for the salts of local anaesthetics, i.e. Procaine, Larocaine,

Tutocaine, Nupercaine, Amylocaine, Amethocaine and Diocaine on Amberlite IRA-400.

Saunders and Srivastava[10] described the adsorption capacity of quinine on Amberlite IRC-50 in its acid form from 50% ethanol solution. They observed that the adsorption capacity of the resin was increased by very fine granulation. Saunders and Srivastava[11] examined the equilibrium distribution of a number of organic bases between their aqueous and ethanol solutions and weak cation exchangers. Baggesgaard-Rasmussen, Fuchs and Lundberg[12] used Amberlite IRA-400 for the determination of natural alkaloids and other organic bases. They used free base for obtaining greater accuracy. Physostigmine, histamine, carbocholine and homatropine methylbromide gave no satisfactory results. Morphine due to its phenol groups was not employed on Amberlite IRA-400. Levi and Farmilo[13] employed the Amberlite IR-4B quite successfully for the analysis of narcotics which were quantitatively determined with a precision of $\pm$ 1.6%. According to Levi and Farmilo the success in the analysis depended on the choice of a solvent or solvent mixture which prevented precipitation of the free base during the ion-exchange process. Their method was found to be applicable to pharmaceutical preparations. Grant and Hilty[14] used a strongly basic anion exchanger Amberlite XE-75 for the separation of morphine from codeine. The phenolic structure of morphine causes the exchange process with Amberlite XE-75, whereas codeine passes through the column unadsorbed. The morphine was eluted from the column with dilute acid and estimated spectrophotometrically. Hamlow, DeKay and Ramstad[15] studied the behaviour of morphine

on several ion-exchange resins for the isolation and estimation from alkaloidal mixtures and opium tincture. Amberlite IR-120 adsorbed morphine quantitatively and the alkaloid was completely eluted from the resin by the use of 4N methanolic NH_3 as elutriant. Amberlite IRC-50 was found unsuitable for quantitative work for the salts of morphine. Amberlite IRA-400 adsorbed morphine from an alkaline solution and consequently was able to separate morphine from non-phenolic alkaloids. The ultraviolet spectra revealed that small amounts of non-alkaloidal impurities appeared in the eluate with morphine. In spite of this fact, according to the authors "the prospect of establishing a method for an exact morphine determination in opium preparations by use of ion-exchange resins is promising."

Huyck[16] used Dowex 50 and Amberlite IRC-50 for the adsorption of ephedrine from ethanol solution. Büchi and Furrer[17] used Amberlite IR-120 and Dowex-50 for adsorption of quinine, but they found that these resins were not suited for exchanging large cations. They declared that the sulfon type resin - Duolite C-10 was more suitable for the adsorption of quinine. The quinine was quantitatively exchanged from strongly acid as well as ethanol solutions and was eluted from the resin by 10% ammonia solution in ethanol. Achor and Geiling[18] used Nalcite SAR (Hydroxide form, dimensions 0.8 x 20 cm) and Amberlite IRC-50 for the isolation and purification of morphine. Morphine was extracted with H_2SO_4 and afterwards treated with saturated solution of barium hydroxide in 2% KoH prior to introduction to the column containing Nalcite SAR. This treatment removed the sulphuric acid and converted the alkaloid to

its anionic form. Morphine was eluted from the column by 0.1N hydrochloric acid. The Nalcite effluent containing morphine is adjusted to pH 7 with sodium hydroxide prior to passing through the Amberlite IRC-50, buffered at pH 7. The morphine from plant extracts was quantitatively recovered by these two resins.

Gunderson, Heiz and Klevstrand[19] focussed their attention on the choice of the strongly basic anion exchangers Dowex 1 and Dowex 2. They used Dowex 2 for the quantitative determination of salts of alkaloids and other organic bases. The base is eluted with 50 ml 70% ethanol. A large quantity of solvent is required for elution of amphetamine, whereas the base of atropine needed 96% ethanol for elution. Dowex 2 is unsuitable for the analysis of salts of phenolic bases (morphine) or of bases containing easily hydrolysible groups. Morphine and other bases of phenolic character are held in Dowex 2 as anions. This method was utilised to separate morphine from other alkaloids. Amphetamine and atropine were analysed under special conditions.

Van etten[20] studied the effects of degree of cross-linkage of the resin, the pH and the ionic strength of the elutriant on the elution of morphine from strong cation exchange resins Dowex 50 and strong anion exchange resins Dowex 1. Complete elution was observed from 1, 2 and 4% cross-linked cation resins with either ammonium or sodium hydroxide, but incomplete elution was seen from 8 and 16% cross-linked resins. In case of anion resin, only 1% cross-linked resin with acetic acid as the elutriant was found suitable for quantitative elution. Behaviour of other bases was described under same conditions of elution.

Van etten, Earle, McGuire and Senti[21] determined morphine

in papaver somniferum using cation exchange resin Dowex 50 x 1 (50 - 100 mesh) and the anion exchange resin Dowex 1 x 1 (50 - 100 mesh). The cation resin was in the hydrogen form and the anion resin in the chloride form. Finally the morphine was eluted by boric acid buffer (pH 9.4) from the cation exchange resin Dowex 50 x 1. The recovery of pure morphine was 98% with a standard deviation of 1.9. The presence of narcotine, papaverine and thebaine reduced the recovery of morphine to as low as 91% and codeine did not interfere.

Berggern, Björling and Willman-Johnson[22] studied the effect of varying degrees of cross-linkage of cation exchange resins (Dowex 50). They remarked that the elution rate was greatly decreased if the crosslinking was increased.

Sjöström and Randell[23] described the use of ion-exchange resin for the quantitative determination of microamounts of atropine and scopolamine in the presence of large quantities of morphine. They performed the experiments with Dowex 1 with various degrees of cross-linking having 50 to 100 mesh. The mixtures of morphine and atropine passed through the column containing resin Dowex 1 x 4. The morphine was taken up by the resin. The atropine was estimated from the effluent colorimetrically. It was observed that when solutions of relatively high methanol concentration (70% or more) were used the tropane alkaloids were not saponified by the strongly basic resin. On the other hand hydrolysis took place with the increasing water content and low results were obtained. The elution of morphine from the column was accomplished with boric acid in absolute

methanol. The technique was applied to the determination of tropane alkaloids in injection solutions.

Alha and Tamminen[24] used Dowex 2 (10 cm column, 5 ml resin, capacity 15 meq OH^-) for exchanging morphine. Morphine was isolated from urine and passed through Dowex 2, and was eluted from the column by formic acid in methanol and estimated (Spectrum = Morphine as formiate).

The isolation of alkaloids and basic drugs from plant extracts and galenicals by ion-exchange method was widely used by various workers. The application of ion-exchange to toxicological analysis has been very limited, so the present work is an attempt to utilize cation exchange resins (Dowex 50 x 12, mesh 200 - 400 and Dowex 50 x 8, mesh 200 - 400) in hydrogen form for the isolation of alkaloids from biological material. Codeine, strychnine and morphine were chosen for this study.

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CHAPTER 3

EXPERIMENTAL

Section A.

Colorimetric Determination of Alkaloid (Codeine & Strychnine) in acid eluates:

Benzene/Bromethylmol Blue/0.1N Sodium hydroxide method by El Darawy and Tompsett[1] was used, after slight modification.

Reagents:-

1. Benzene A.R.
2. Sodium hydroxide 30% Solution
3. Buffered Indicator Reagent (pH - 7.4)

   * Bromothymol blue - 0.25 gm
     Potassium dihydrogen Phosphate - 3.4 gm
     N. Sodium hydroxide - 15 ml.
     Water to 500 ml.

* The commercial bromothymol blue compound was purified by dissolving in 50% ethyl alcohol and filtered. The filtrate was evaporated to dryness. The clear residue was then ready for use.

4. Sodium hydroxide - 0.1N.

Procedure

Into a 50 ml glass stoppered measuring cylinder were measured 5 ml of acid eluate which was neutralised with $NaHCO_3$ and 1 ml 30% sodium hydroxide solution was added to it. The mixture was diluted to 10 ml with distilled water. 30 ml of benzene were added and the mixture shaken vigorously for 5 minutes.

25 ml of the benzene solution were transferred to another

50 ml glass stoppered measuring cylinder, 10 ml of buffered indicator solution was added to it and shaken vigorously for 4 minutes.

The benzene extract was separated and centrifuged. 15 ml of the benzene solution were shaken with 7.5 ml of aqueous 0.1N sodium hydroxide solution for 2 minutes. The blue aqueous solution was separated and read against an appropriate blank in a spectrophotometer at 600 mμ.

Standards

Standards containing 12.5, 25, 50, 100 and 200 micrograms of the appropriate alkaloid were prepared under the same conditions as the unknowns.

Modification:

When the determination was carried out on solutions containing high concentrations of ammonium salts, considerable amounts of free ammonia passed into the benzene extract and interfered with the subsequent reaction with the buffered indicator reagent. To overcome this difficulty the benzene extract was evaporated just to dryness in an all glass vacuum still, the residue was then redissolved in 25 ml of benzene which was treated with 10 ml buffered indicator reagent as described above. The modified procedure outlined here, in which the benzene extract was evaporated to dryness and then redissolved, was employed for all subsequent experiments.

Use of Dowex 50 x 12 Resin

The cation exchange resin Dowex 50 x 12 (mesh 200-400)*

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* Dow Chemical Co. Midland, Michigan, U.S.A.

Chromatographic Apparatus

was used for the isolation of alkaloids.

Packing and Preparation of Column

3 gm resin was weighed in a beaker and stirred with 10 ml distilled water and was packed into the column fitted with a sintered glass filter base (Quickfit and Quartz Ltd.) with the aid of mild suction. The dimensions of the operating column were: height - 50 mm, diameter 16 mm (vide apparatus). A small quantity of liquid should always be maintained above the surface of the resin in order to prevent the resin going dry. (A small piece of glass wool may be placed on the upper surface of the resin in the column to prevent any disturbance of the resin surface). The column was then washed with 75 ml distilled water and 75 ml N. Hcl. A flow rate of 35 ml/hr was maintained.

Adsorption of codeine from various concentrations of N. Hcl and elution of codeine from the column.

Prior to use the liver extract containing alkaloid, the alkaloid dissolved in N. Hcl, trichloracetic acid solution and saturated $(NH_4)_2SO_4$ solution respectively, then passed through the column containing resin. This was done so that one might study the effects of these substances on the adsorption of the alkaloid on the resin. Codeine phosphate was used as the test alkaloid.

3 mg of codeine phosphate in 100 ml N. Hcl was allowed to percolate through the column. The effluent was collected and examined. Elution was carried out with 80 ml of 2.5 N. Hcl, 80 ml of 5N Hcl and 80 ml of 8N Hcl successively. The eluates were examined as follows:

1) 5 ml N. Hcl effluent

2) 5 ml 2.5NHcl eluate

3) 5 ml 5N Hcl eluate

4) 5 ml 8N Hcl eluate

Each of the four eluates was neutralised with $NaHCO_3$ and afterwards treated with the procedure described in section A of this chapter. In each case a blank was prepared and the amount of codeine recovered was estimated by the modified benzene/ bromothymol blue/N/10 NaOH technique. The results are given in Table 1.

Since 5N Hcl eluted the largest quantity of codeine from the column, the above experiment was repeated to ascertain what volume of 5N Hcl would elute the codeine completely from the column. It was observed that 5 volumes, each of 50 ml quantity of 5N Hcl were required for complete elution. The results are given in the Tables 2 and 3.

To determine from how dilute a solution (in N. Hcl) the codeine could be satisfactorily adsorbed on the resin and then quantitatively recovered, 3 mg of codeine phosphate was dissolved in increasing quantity of N. Hcl. After passing the solution through a column, each column was washed with 50 ml N. Hcl and subsequently eluted by 250 ml 5N Hcl. Experiments were done in triplicate. Results are given in Table 4, which showed that the codeine could be adsorbed by the column from a solution containing as little as 3 mg per litre. This experiment revealed that alkaloid could be adsorbed by the resin from very dilute solution of tissue extract or urine in toxicological analysis.

In one experiment in which 4 mg and 8 mg of codeine was

adsorbed on the resin the recovery was 84% and 89%.

Adsorption of codeine from trichloracetic acid solution and saturated $(NH_4)_2SO_4$ solution with acetic acid.

These preliminary experiments showed that Dowex resin possessed the capacity of retaining codeine from N. HCl solution. The ability of Dowex resin to retain codeine from 10% trichloracetic acid solution and saturated ammonium sulphate with acetic acid was examined.

Four solutions were prepared.

1) 100 ml 10% CCl_3COOH solution (Blank)
2) 100 ml 10% CCl_3COOH solution + 3 mg codeine phosphate
3) 100 ml saturated $(NH_4)_2SO_4$ + 5 ml glacial Acetic Acid + 10 ml of 10N. HCl (Blank)
4) 100 ml saturated $(NH_4)_2SO_4$ + 5 ml glacial Acetic Acid 3 mg codeine phosphate+ 10 ml of 10N. HCl.

The above mentioned four solutions were passed through columns. The effluent of each solution was examined. After passing the solution, each column was washed with 50 ml N.HCl and subsequently eluted by 250 5N HCl. Codeine was determined from 5N HCl eluate. Results are given in Table 5.

The result of Table 5 led to an expanding knowledge of adsorptive conditions for alkaloid from biological material. Both trichloracetic acid and ammonium sulphate could be used successfully as protein precipitant. The deproteinized filtrate containing alkaloid obtained either by trichloracetic acid method of Stewart, Chatterji and Smith[2] or by saturated ammonium sulphate with acetic acid method of Daubney and Nickolls[3], could be passed through the column for capturing alkaloid, with this idea the experiment of Table 5 was performed.

Recovery of Codeine added to Liver

50 gm of liver was macerated in Waring Blendor and washed with distilled water. The volume of the solution was 140 ml. 5 ml glacial acetic acid and 3 mg codeine phosphate were added to 140 ml solution and shaken well and heated in a water bath up to 50°C. Then it was saturated with solid $(NH_4)_2SO_4$. The extraction was carried out by Daubney and Nickoll's method. The liver residue was taken out from the Buchner funnel and macerated with hot 1% acetic acid solution (65 to 70°C) and filtered with suction. This was repeated twice. The filtrates were collected together. It was turbid so it was again filtered. Total volume of the filtrate was 280 ml. The liver blank was prepared as above.

275 ml liver extract passed through each column. After passing the liver extract through the column, each column was washed with 50 ml N. HCl and the alkaloid was subsequently eluted by 250 ml 5N HCl. Codeine was determined in this eluate. The experiment was repeated many times and typical results are given in Table 6.

Above experiments showed that Dowex (50 x 12) possessed the capacity of adsorbing alkaloid from N. HCl, trichloracetic acid solution and ammonium sulphate solution. The percentage of recovery of codeine was given in the Tables 2, 3, 4 and 5. With these preliminary observations codeine was added directly to liver and extracted. The liver extraction passed through column containing resin. The recovery of codeine from Dowex 50 x 12 was only 40 - 41%. So an attempt was made to improve the yield of codeine by adsorbing on Dowex 50 x 8.

TABLE 1

Elution of Codeine by Increasing Concentration of HCl:

3 mg alkaloid in 100 ml N. HCl passed through the column containing resin (Dowex 50 x 12).

HCl Eluates	Codeine	%
N. HCl effluent	nil	nil
2.5 N HCl eluate	230 µg	7.6%
5N HCl eluate	2110 µg	70.3%
8N HCl eluate	850 µg	28.3%

TABLE 2

Fractional Elution of Codeine by 5N HCl:

3 mg alkaloid in 100 ml N. HCl passed through the column containing resin (Dowex 50 x 12)

5N HCl eluate	Recovery of Codeine	
1st fraction 50 ml 5N HCl	1200 µg	
2nd " " " "	700 µg	
3rd " " " "	440 µg	
4th " " " "	130 µg	
5th " " " "	100 µg	
Total	2570 µg	85.6%

TABLE 3

Elution of Codeine by 250 ml 5N HCl from the column containing resin (Dowex 50 x 12)

No. of Expt.	Codeine applied to the column	Vol. of 5N HCl for complete elution	Recovery of Codeine
1	3 mg	250 ml	84%
2	3 mg	250 ml	85%

*

TABLE 4

No. of Expt.	3 mg Codeine Phosphate in 250 ml N.HCl applied to the column		3 mg Codeine Phosphate in 500 ml N.HCl applied to the column		3 mg Codeine Phosphate in 1000 ml N.HCl applied to the column	
	Added	Recovery	Added	Recovery	Added	Recovery
I	3 mg	77%	3 mg	85%	3 mg	70% (App.)
II	3 mg	92%	3 mg	82%	3 mg	70%
III	3 mg	83%	3 mg	85%	3 mg	58%

* Resin used:- Dowex 50 x 12.

* TABLE 5

Recovery of Codeine from Trichloracetic Acid Solution and Ammonium Sulphate Solution

	Codeine Phosphate added	Recovery of Codeine
10% Trichloracetic Acid Soln.	3 mg	76.3%
Saturated $(NH_4)_2SO_4$ Soln.	3 mg	95.3%

* TABLE 6

Codeine Phosphate added to Liver

No. of Expt.	Weight of Liver	Amount of Codeine Phosphate added	Recovery of Codeine
1	50 gm	3 mg	41.3%
2	50 gm	3 mg	40%

* Resin used:- Dowex 50 x 12.

Section B

Use of Dowex 50 x 8 Resin

1. Treatment of Column

When 3 gm of Dowex 50 x 8 (mesh 200-400) resin was prepared for use in exactly the same manner as the Dowex 50 x 12 in Section A, the percentage of recovery of codeine (in acidified, saturated ammonium sulphate solution) from the column was between 71 to 78.5%. However, when the preparative treatment of this resin (Dowex 50 x 8) was changed to successive washings with 75 ml distilled water, 75 ml 4N HCl and then 150 ml N. HCl, the recovery of codeine was increased to within the range 83.3 to 92.6%. So in the subsequent experiments this modified method of column treatment was used.

It was observed that the elution with 250 ml 4N HCl gave maximum recovery of codeine from the column, codeine in acid eluate being determined as before.

2. Adsorption of codeine from trichloracetic acid solution and saturated ammonium sulphate solution.

In one comparative experiment 83.3% of codeine was recovered from the column treated in this way when codeine phosphate was adsorbed from an acidified saturated solution of ammonium sulphate, whereas only 56.6% of codeine was recovered from the column when codeine phosphate was adsorbed from 10% trichloracetic

acid solution. This experiment was done with the idea that the deproteinized liver extract could be prepared either by trichloracetic acid or by saturated $(NH_4)_2SO_4$, according to the analyst's individual preference. Clearly, however, ammonium sulphate precipitation of protein must be the method of choice.

Recovery of Codeine Added to Liver Extract

Preparation of liver extract:

50 gm of human liver macerated in Waring Blendor was transferred to a flask and made up to 140 ml with distilled water; 5 ml glacial acetic acid was added to it and the mixture was shaken well. The mixture was heated to about 50°C upon a water-bath. Then it was saturated with solid $(NH_4)_2SO_4$, the salt being added until even after thorough shaking some quantity remained at the bottom of the flask. Afterwards it was heated to between 75 and 80°C for 15 minutes (in the water bath) and filtered with suction. The residue was taken out from the Buchner funnel and macerated with 50 ml 1% acetic acid solution saturated with $(NH_4)_2SO_4$ having temperature 65 to 70°C and the mixture was again filtered with suction. This process was repeated twice. The filtrates were combined together.

For each experiment, 800 ml of liver extract was prepared from 150 gm of human liver as described above. This was divided into three aliquots each of 250 ml. One of these aliquots was reserved as 'blank'. To the second aliquot was added 3 mg of codeine phosphate and to the third aliquot was added 3 mg of codeine phosphate along with 25 ml of 10N. HCl. Each of these aliquots was passed through a prepared column of Dowex 50 x 8.

92.1% of codeine was recovered from the extract which contained 10N.HCl whereas only 68.1% of codeine was recovered from the liver extract to which no 10N. HCl was added (Table 7). On the basis of this clear superiority of recovery from extract strongly acidified with HCl (to give an approx. N.HCl solution) this procedure was adopted.

Recovery of Codeine added to Liver

50 gm of human liver was macerated with Waring Blendor and made up to 140 ml with distilled water, 5 ml glacial acetic acid and 3 mg codeine phosphate were added to it. Afterwards it was treated exactly in the same manner as described in 'Preparation of Liver Extract'. The liver extract was mixed with 10N. HCl (100:10) and passed through the column. The liver blank was done as above. Codeine was determined in 4N HCl acid eluate. This experiment was repeated several times. The results are given in Table 8.

1% Acetic acid solution used for extraction of liver residue and after extraction, the filtrate was saturated with $(NH_4)_2SO_4$.

Another experiment with 50 gm of human liver was performed in the same manner as described above except the treatment of liver residue. The liver residue was taken out from the Buchner funnel and macerated with 50 ml 1% acetic acid solution having temperature 65 to 70°C and filtered with suction. This process was repeated twice. All filtrates were combined together. Up to this point the procedure is exactly that described by Daubney and Nickolls. The filtrate, however, was often turbid and was not completely saturated with ammonium sulphate. Because such

solutions choked the column and interfered, therefore, with the adsorption on resin, a further step was introduced. The filtrate was saturated with ammonium sulphate, by addition of more solid salt, and filtered. This final, clear filtrate was mixed with 10N HCl as before and passed through the column. The recovery of codeine was as given in the Table 9 for a typical experiment.

It is clear from these experiments:

(1) Codeine added to a ready-prepared deproteinized liver extract was recoverable to the extent of 90%.

(2) Codeine added to macerated liver did not pass completely into the deproteinized extract.

(3) The use of Daubney & Nickolls' technique resulted in a turbid solution not suitable for application to the column and, when this was clarified, over half the alkaloid was lost.

(4) A technique which kept the concentration of $(NH_4)_2SO_4$ maximal throughout gave a clear solution and reduced the loss to about a third.

*TABLE 7

Codeine Phosphate added to Liver Extract

Quantity of Liver Extract	Amount of Codeine Phosphate added to Liver Extract	Recovery of Codeine
250 ml	3 mg	68.1%
250 ml + 25 ml 10N HCl	3 mg	92.1%

*TABLE 8

Codeine Phosphate added to Liver

No. of Expt.	Weight of Liver	Amount of Codeine Phosphate added to Liver	Recovery of Codeine
1	50 gm	3 mg	63.1%
2	50 gm	3 mg	61.8%

*TABLE 9

Codeine Phosphate added to Liver

Weight of Liver	Codeine Phosphate added to liver	Recovery of Codeine
50 gm	3 mg	40.4%

* Resin used: Dowex 50 x 8.

Section C.

Determination of Strychnine

Preliminary experiments were done with strychnine in the same manner as the codeine phosphate described in Section B. The method of column treatment was the same. It was found that 400 ml 4N HCl eluant was required for complete elution of strychnine from the column. 5 ml of 4N HCl eluate was neutralised with $NaHCO_3$ and treated with the modified technique of Benzene/Bromothymol blue/ N/10 NaOH to determine strychnine.

1. Recovery of Strychnine added to Liver Extract

The technique was exactly as described for codeine (Section B) and gave a 95% recovery of the 3 mg strychnine added to 250 ml of extract to which 10N HCl was added whereas only 81.1% of strychnine was recovered from the liver extract to which no 10N HCl was added. (Table 10).

2. Recovery of Strychnine added to Liver

The following five experiments were done, the first being a simple repetition of the experiment with codeine, and each of the others representing a modification of the original procedure in order to find a means of improving the recovery. The results were summarised in the table No. 11,

Expt. 1.

50 gm of human liver was macerated in Waring Blendor and made up to 140 ml with distilled water. 5 ml glacial acetic acid

and 3 mg strychnine were added to it. Then the extraction was carried out in the same manner as described in codeine phosphate in Section B. The liver extract was mixed with 10N HCl and passed through the column. Recovery was only about 20% - i.e. one third as good as with codeine.

Expt. 2.

50 gm of human liver was macerated with Waring Blendor and made up to 140 ml with 5% acetic acid solution. Extraction was carried out in the same manner as in Experiment 1 except the treatment of liver residue. The liver residue was macerated with warm 50 ml 1% acetic acid solution (65° to 70°C) then saturated with solid $(NH_4)_2SO_4$ and filtered with suction. This was repeated twice. The filtrates were combined together. The liver extract was mixed with 10N HCl and passed through the column. This gave no improvement and indeed the recovery was less than before.

Expt. 3.

50 gm of human liver was macerated in Waring Blendor and made up to 140 ml with 10% acetic acid solution. 5 mg strychnine was added to it. Afterwards done as it was described in Experiment 2. Again there was no improvement.

Expt. 4.

50 gm of human liver was macerated in Waring Blendor and made up to 140 ml with 5% acetic acid solution. 3 mg strychnine was added to it. The extraction was carried out by the process as

described by Daubney and Nickolls method. The whole liver extract was a bit turbid and it was saturated with $(NH_4)_2SO_4$ and filtered. The clear filtrate was mixed with 1ON HCl and passed through the column. This gave an improved recovery - about as good as with codeine under similar circumstances, but still less good than codeine gave with the method used in Experiment 1 of this series.

Expt. 5.

50 gm of human liver was macerated in Waring Blendor and made up to 140 ml. with 10% acetic acid solution. 5 mg strychnine was added to it. Afterwards done as it was described in Experiment 4.

This gave practically as good a result as did Experiment 4. Evidently, as was shown also by Experiment 3, the addition of extra acetic acid was not beneficial.

*TABLE 10

Strychnine added to Liver Extract

Quantity of Liver Extract	Amount of Strychnine added to liver extract	Recovery of Strychnine
250 ml	3 mg	81.1%
250 ml + 25 ml 10N HCl	3 mg	95.4%

* TABLE 11

Strychnine added to Liver

No. of Experiment	Amount of Strychnine added to 50 gm of human liver	Recovery of Strychnine
1	3 mg	19.9%
2	5 mg	14.6%
3	5 mg	13.8%
4	3 mg	37.8%
5	5 mg	34.4%

* Resin used: Dowex 50 x 8

Section D

Determination of Morphine

Colorimetric Determination of Morphine in an Acid Eluate

(Modification of the method used by Stolman and Stewart[4]).

Reagents:

1) 20% Sodium carbonate (Anhydrous) Solution
2) Folin & Ciocalteu's Reagent
3) Anhydrous Sodium Sulphate
4) Solid Sodium bicarbonate

Procedure

5 ml of acid eluate is neutralised with solid $NaHCO_3$, morphine was extracted from the neutralised eluate with 25 ml, 5 ml and 5 ml of a mixture of chloroform and ethyl alcohol (3:1) successively. The extracts were combined together and dehydrated with anhydrous sodium sulphate. After half an hour the extract was filtered and the residue was washed with small aliquots of the mixture of chloroform and ethyl alcohol. The filtrate was evaporated to dryness in an all glass vacuum still. The residue was dissolved in 5 ml distilled water and 0.25 ml Folin and Ciocalteu's reagent and 1 ml 20% sodium carbonate (anhydrous) solution added to it and placed in a boiling water bath for 1 minute. This was then allowed to cool at room temperature. After half an hour readings were recorded in the spectrophotometer at 600 mμ against an appropriate blank. The amount of morphine recovered was calculated from a standard curve.

2. Ion-Exchange Adsorption of Morphine which was added to Liver

50 gm of human liver was macerated in Waring Blendor and made up to 140 ml with distilled water. 5 ml glacial acetic acid and 3 mg morphine sulphate were added to it. Then extraction was carried out as described in Section B. The liver extract was mixed with 10N HCl (100:10) and passed through the column. It was impossible to determine whether morphine was adequately adsorbed by examination of the effluent, because the "blank" was too high. Some morphine was removed from the column when it was washed with N. HCl. This did not occur in the case of the other alkaloids and strongly suggests that adsorption was only partial.

The above experiment was repeated, the column was washed with 50 ml N. HCl, 100 ml N. HCl, and 100 ml N. HCl successively. These washings were analysed. The results were given in the Table 12.

Another experiment was done as described above. After passing the liver extract through the column, the column was washed first with 100 ml 3% acetic acid solution and then 200 ml N. HCl. These washings were analysed. The results were given in Table 13.

Comment on Result

The above experiments reveal that morphine was not completely adsorbed on Dowex 50 x 8 resin from a N. HCl solution.

* TABLE 12

Estimation of Morphine in N. HCl Washing

	N. HCl Washing	Morphine Found
1st fraction	50 ml	13%
2nd fraction	100 ml	22%
3rd fraction	100 ml.	16.6%

* TABLE 13

Estimation of Morphine in Acetic Acid and N. HCl Washing

	Volume of Washing Solution	Morphine Found
1st Washing	100 ml 3% Acetic Acid	12%
2nd Washing	200 ml N. HCl	28%

* Resin used: Dowex 50 x 8.

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CHAPTER 4

Application of Paper Electrophoresis for the Identification of the Isolated Alkaloids

Electrophoresis rapidly became an important tool in biological research after Tiselius developed the technique of free electrophoresis in 1937. The differences in electrophoretic mobility often make a separation and even an exact analysis of mixtures of ionizable substances possible. Tiselius' apparatus, however, is too expensive and complicated and besides considerable experimental skill is required to operate it. Hence electrophoresis remained of limited value as a routine procedure in clinical and toxicological laboratories.

In a recent review, Tiselius and Flodin[1] described the following advantages of electrophoresis on paper or in porous media over electrophoresis in 'free' solution.

"1) It is possible to obtain complete separation into zones of different migration and thus not only a boundary separation.

2) The so-called boundary anomalies interfere less in zone electrophoresis and therefore substances of low molecular weight (e.g. amino acids, peptides, nucleotides) may also be studied. In addition, zone electrophoresis (particularly in filter paper strips) requires only minute quantities of material and can be performed with simple and inexpensive equipment.

These advantages are gained, however, by the sacrifice of greater accuracy of the boundary method (particularly with regard to mobility and isoelectric point determinations). The supporting medium necessary in zone electrophoresis, introduces new factors which may influence the results in a way which is difficult to control."

The basic principle is simple, Ionic constituents of mixtures are separated by differential mobility in an electric field. Negative ions migrate towards the anode and positive ions towards the cathode, while non-ionic constituents do not move in the electrical field.

The satisfactory electrophoretic separation depends solely upon the following factors, i.e. the electrophoretic chamber, the paper, the buffer and the electrical field. The electrophoresis apparatus should have a small chamber with thin walls tightly sealed and equipped for easy levelling of buffer compartments. The paper strips should be placed horizontally at a moderate distance above the buffer level. Anticonvection media such as cellulose acetate, agar gel and starch gel have advantages for some types of work but paper has been most commonly used. Adsorption occurs between the solutes being separated and the anticonvection medium, and this may alter the electrophoretic velocity. Hence two particles with different mobilities on paper may happen to move together on agar gel and conversely two particles with an identical electrophoretic mobility on paper may migrate at different rates when adsorption becomes a factor. The pH and the ionic strength of the buffer are important in connection with mobility. With increasing ionic strength mobility decreases and salting out of protein can occur. On the other hand, mobility increases if the ionic strength is lowered, but this favourable effect has its limit. The electric field causes migration of the buffer ions simultaneously with migration of the macromolecules. The heat which is produced due to the electric field does not only increase the mobility of the charged particles but also evaporation and the concentration of the buffer. Durrum[2] pointed out that the migration distance

differed by less than 6% for change of 26°C at constant current, whereas at constant voltage the difference in migration is about 25% for change of 27°C. Hence constant current operation is preferred for routine work and for reproducibility.

Application of paper electrophoresis in the toxicological field has been very slow although there are a number of investigations of natural plant products and a few have been made to drugs.[3,4] Deckers and Schreiber[5] applied the Grassmann and Hannig apparatus[6] for the separation of scopolamine and hyoscyamine, using M/5 borate buffer at pH 8.6 for two hours and for strychnine and brucine using a citrate buffer at pH 3.5. Silveira[7] used sodium citrate buffer (pH 3.5) in ionophoresis for the separation of brucine and strychnine. Marini-Bettólo and Lederer[8] studied paper electrophoretic separation of the alkaloid chlorides of strychnos trinervis using M/5 borax and 25% acetic acid as an electrolyte. Burma,[9] employing McDonald's apparatus, separated two cinchona, five opium and two strychnos alkaloids. He noticed that the opiates are separated at pH 6 into three groups according to the increasing order of mobility, papaverine (slowest), narceine and narcotine (intermediate), morphine and thebaine (fastest).

Wagner[10] used S and S paper 2043B saturated with different buffers at various pH range for paper electrophoresis and chromatography to separate morphine, codeine, papaverine and narcotine along with opiates. Graf and List[11] used paper electrophoresis technique for the separation and quantitative estimation of morphine in tincture of opium. Farmilo et al.[12] studied the

electrophoretic separation and identification of alkaloids of raw opium and described a semi-quantitative method for determination of the origin of opium. Michl[13] applied electrophoresis for the isolation and identification of pruine base. He used acetic acid-pyridine solution at pH 4. Michl and Haberler[14] employed the same technique to the determination of pruines in caffeine containing drugs.

Wagner studied the paper electrophoretic separation of local anaesthetics[15] and sympathomimetics,[16] Kaiser and Haag[17] investigated the behaviour of synthetic basic drugs employing electrophoresis and chromatographic technique. Goldbaum and Kazyak[18] published a method for quantitative separation of morphine from urine applying electrophoresis. Brown and Kirk[19] used the electrochromatophoresis technique for the separation of unlike alkaloids from each other and from contaminating biological mixture. Buff et al.[20] identified the alkaloids which are generally encountered in toxicological analysis, according to their migration rate.

Kirk[21] pointed out that paper electrophoresis might be used for isolation and purification and preliminary identification of every type of toxic agent of interest to the toxicologist, aside from a few neutral compounds. Buff et al.[22] advocated the use of paper electrophoresis as it showed greater reproducibility and, therefore, more reliability for identification of alkaloids than did chromatography.

Experimental:-

Since quinine shows a blue fluorescence when examined in ultra-violet light and is therefore readily identifiable, this alkaloid was chosen as the first test substance. Quinine solution was mixed with serum (1 mg/ml). The mixture was put to the paper strip for electrophoresis. After electrophoresis the paper strip was dried and examined in ultra-violet light to see whether the quinine was separated from serum or not. It was of course observed that the separation of alkaloid (quinine) from serum depended upon various factors, i.e. composition of buffer solution, pH, voltage and time etc. These factors were investigated in a series of experiments to determine a set of conditions which could be applied for the separation of alkaloids. Examination was not solely confined by ultra-violet light, and to obtain more permanent records, the dried paper strips after electrophoresis were stained with Dragendorff's Reagent and Iodoplatinic acid reagent separately to locate the migrated alkaloid spot. Further the stained alkaloid was subsequently eluted and the amount of alkaloid estimated photometrically. In order to see the movement of serum protein, the dried strips were stained with lissamine green.

The Durrum apparatus was employed. The electrophoresis tank was cleaned and dried. The buffer solution was filled into the appropriate compartments of the tank. Three or more Whatman 3 mm chromatography papers (about 3.7 cm in width and 35 cm long) were draped over the wire with the ends dipping into the buffered

electrode compartments. The mixture of alkaloid and serum was applied across the strips at their supported points by means of a micro-pipette. The zone of application was kept as narrow as possible. The tank was closed and the current was switched on after the strips became saturated with the buffer.

After the time allowed for electrophoresis the strips were removed from the tank as soon as possible. They were dried at room temperature and examined in ultraviolet light. The bands were marked by pencil. The results are given in Table 14.

The strips of C and D (Table 14) were stained with lissamine green. It was observed that the region near the application of the mixture was stained with dye and marked portion (in U.V. light) remained unstained. It may be assumed that quinine had been separated from serum proteins by electrophoresis under these particular conditions. On the basis of this preliminary observation of separating quinine from serum after electrophoresis, other alkaloids - strychnine, brucine, atropine, codeine and morphine - were tried in the same way. It is seen from the table 14 that as the pH is decreasing the separation is improving.

It can be deduced from Table 15 that strychnine and brucine are separated from serum at pH 4.9. The mixture of strychnine brucine and serum is cleanly separated at pH 3.9, showing two distinct bands in ultra violet light, but the bands are not very compact in shape. Separation took place after a long time.

Atropine shows no band in ultra-violet light and different means must therefore be used to demonstrate its movement in

the electrical field. This was achieved when we stained the dried paper strips after electrophoresis with a modified Dragendorff's Reagent or Iodoplatinic acid Reagent.

Separation shows greater reproducibility at lower pH. The time of electrophoretic separation was maintained at 5 to 7 hours to obtain a compact band of migrated alkaloid-spot. Electrophoresis gave satisfactory separation using acetate buffer at pH 3.64, for 5 hours in case of strychnine, brucine, atropine, morphine and codeine (Table 16).

Under the same conditions a mixture of strychnine and brucine gave good separation in 7 hours, both from serum proteins and from each other. Two distinctly separated bands were seen when the paper was treated with Iodoplatinic acid reagent whereas in Dragendorff's Reagent the bands were not very clear. Iodoplatinic acid reagent is therefore preferable for staining the paper strip to detect the alkaloid band.

The stained alkaloid band could be eluted from the paper strip by any one of three methods.

1. Fisher technique[23]
2. Tryhorn-Curry technique[24]
3. Goldbaum and Kazyak technique[25]

The eluted alkaloid can then be estimated quantitatively by ultra-violet spectrophotometry.

When electrophoretic separation is used as a sequel to a preliminary chromatographic separation as described in this thesis (Chapter 3), a few ml of 4N HCl eluate is neutralised with $NaHCO_3$ and made alkaline, adding 1 ml 30% NaOH. The

alkaloid is extracted from the solution with organic solvent (benzene, chloroform, etc.). The solvent is evaporated to dryness. The residue is dissolved in alcohol. The alcoholic solution is applied cumulatively to the paper strip, each application being dried off before the next is added. After electrophoresis the paper strip is dried in room temperature as usual and stained with Iodoplatinic acid reagent. The spot is cut from the paper strip and the alkaloid is eluted from it using Goldbaum and Kazyak technique and afterwards identified and estimated by ultra-violet spectrophotometry.

TABLE 14

Separation of Quinine by Paper Electrophoresis

	Quantity µl	Buffer Soln.	Voltage	Time in hours	Examination in U.V. Light
A. Quinine sulphate solution (1 mg/ml) + serum equal volumes	20	Barbitone Buffer pH - 8.5	200	1	No separate band
				2	do.
				3	do.
				16 (overnight)	do.
B. Do.	40	Phosphate Buffer pH - 7.3	200	1	Band not very clear
				2	do.
				16 (overnight)	do.
C. Do.	40	Acetate Buffer pH - 5.6	200	1	Alkaloid migrated slightly from the point of application
				2	do.
				3	do.
				16 (overnight)	A single band seen some distance away from the point of application
D. Do.	40	Acetate Buffer pH - 4.92	200	16 (overnight)	A single separate band seen some distance away from the point of application
				16 (overnight)	do.

TABLE 15

Separation of Strychnine, Brucine or a Mixture of the two from Serum

	Quantity µl	Buffer	Voltage	Time in hours	Examination in U.V. Light
1. Strychnine (1 mg/ml) + Serum (1:1)	40	Acetate buffer pH - 4.9	200	16	One separate band seen some distance away from the point of application but not in compact form
				20	Do.
2. Brucine (1 mg/ml) + Serum (1:1)	40	Do.	200	16	One separate band seen some distance away from the point of application but not in compact form
				20	Do.
3. Strychnine (1 mg/ml) + Brucine (1:1)	40	Acetate buffer pH - 4.9	200	16	Two bands seen side by side some distance away from the point of application not in compact form
				20	Do.
4. Strychnine (1 mg/ml) + Brucine (1:1)	40	Acetate buffer pH (3.9 (3.85	200	16	Two clearly separated bands seen some distance away from the point of application, not very compact in form
5. Strychnine (1 mg/ml) + Brucine (1 mg/ml) + Serum (1:1:2)	80	Do.	200	16	Two clearly separated bands seen some distance away from the point of application - not very compact in form

TABLE 16

Separation of Alkaloids Using Acetate

Concentration of each Alkaloid - 1 mg/ml	Quantity µl	Buffer	Voltage
Strychnine + Serum (1:1)	60	Acetate Buffer pH 3.64	200
Brucine + Serum (1:1)	60	Do.	200
Atropine + Serum (1:1)	60	Do.	200
Codeine + Serum (1:1)	60	Do.	200
Morphine + Serum (1:1)	60	Do.	200
Strychnine + Brucine (1:1)	80	Acetate Buffer pH 3.7	200
Strychnine + Brucine + Serum (1:1:2)	80	Do.	200

* Dragendorff's Reagent.

Solution A.

Bismuth Subnitrate - 0.85 gm
Distilled water - 40 ml
Glacial Acetic Acid - 10 ml

Solution B.

Potassium Iodide - 8 gm
Distilled water - 20 ml

Solutions A and B are kept in brown bottles.

Spraying Reagent:- 5 ml of A and 5 ml of B are mixed with 20 ml of glacial acetic acid and 100 ml. distilled water.

The alkaloids form red spots on a pale orange background.

Buffer at pH 3.64 and 3.7

Time in hours	Dragendorff's* Reagent	Iodoplatinic** Acid Reagent
5	+	+
5	+	+
5	faint	+
5	+	+
5	+	+
7	Two bands are not distinctly stained	Two bands distinctly stained
7	Do.	Do.

** Iodoplatinic Acid Reagent:- Mix 45 ml of 10% potassium iodide, 5 ml of 5% platinum chloride and 100 ml of distilled water.

The paper is sprayed with iodoplate reagent to develop characteristic dark spots of the iodoplatinate complex of the alkaloids.

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CHAPTER 5

DISCUSSION

1. Exchange Resin

The object of the work described in this thesis was to investigate the utilization of ion-exchange resins (with Dowex 50 as the example) for isolating alkaloids which were added to biological material, and the use of paper electrophoresis to purify the isolated alkaloids. Three alkaloids were chosen, i.e. codeine, strychnine and morphine. The first of these is a simple base of average strength within the general range of alkaloids, the second is, among alkaloids, an exceptionally strong base, and in the third, morphine a phenolic group which profoundly affects the basic properties.

When alkaloid is present in biological material, the material must be deproteinized before any attempt is made to adsorb the alkaloid on a column. The omission of this step renders the adsorption of the alkaloid as a separate band extremely difficult, partly because of the formation of alkaloid-protein complexes and partly because the protein itself clogs the column. The chosen method for removal of protein has been in this work the precipitation of protein which can be achieved either by the trichloracetic acid method of Stewart, Chatterji and Smith, or by saturated ammonium sulphate in the presence of acetic acid, the method of Daubney and Nickolls. Prior to passing the liver extract (which was the experimental material)

through the column, the adsorptive conditions of the resins were investigated with respect to these two precipitants. Codeine was chosen as test substance.

When codeine was dissolved in either N. HCl or trichlor-acetic acid and saturated ammonium sulphate and adsorbed on Dowex 50 x 12, which was washed with 75 ml distilled water and 75 ml N. HCl before use, the recovery was 85%, 76% and 95% respectively (Tables 3 and 5).

On the other hand, when Dowex 50 x 8 was treated as Dowex 50 x 12 the recovery of codeine from saturated ammonium sulphate was 71 to 78%. But when the Dowex 50 x 8 was given a preliminary washing with 75 ml distilled water, then 75 ml 4N HCl and finally with 150 ml N. HCl, the recovery of codeine was increased to about 90%. These results indicated that the recovery of codeine was approximately 90% when it was adsorbed by the resin from saturated ammonium sulphate solution and that this medium gave better recovery than was obtained from trichloracetic acid solution.

These observations cleared the way for a study of adsorptive conditions for alkaloids which were added to liver extract or to macerated liver.

Codeine and strychnine added to deproteinized liver extract which was prepared by the technique that kept the concentration of $(NH_4)_2SO_4$ maximal throughout and was passed through the column containing Dowex 50 x 8 resin, gave a recovery of codeine and strychnine over 90% in the 4N. HCl eluate (Tables 7 and 10). When, however, the alkaloids (codeine and strychnine) were added

to macerated liver which was then extracted by Daubney and Nickolls method and the liver extract saturated with $(NH_4)_2SO_4$ and filtered, the recovery was only 40% in the case of codeine (Table 9) and 37% in the case of strychnine (Table 11, Expt. 4). Yet the effluent contained no appreciable amount of alkaloid, showing that adsorption was practically complete and the loss was in elution. However when codeine was added to macerated liver and extracted with the technique which kept the concentration of $(NH_4)_2SO_4$ maximal throughout and gave a clear solution, the loss was reduced to about a third (Table 8). These results suggested that, 1) Codeine and strychnine which were added to macerated liver did not pass completely into deproteinized extract, 2) the adsorbed alkaloids from the deproteinized extract on the resin were not completely eluted.

It may be suggested that this was due to the formation of an alkaloid-protein complex, largely precipitated by the ammonium sulphate, whilst that part which did reach the column was not eluted.

The behaviour of morphine with Dowex 50 x 8 resin was studied. The results (Tables 12 and 13) showed that morphine was either partially or not completely adsorbed on the resin from an approximately N. HCl. This characteristic of morphine on the Dowex resin indicated that this resin could possibly be used for the separation of morphine from codeine or other alkaloids.

As a general conclusion, it appears that, although Dowex 50 x 12 was not suitable, at least under the range of conditions tested, for the separation of alkaloids from biological material,

the resin Dowex 50 x 8 had distinct potentialities. An alkaloid of average strength as a base was well adsorbed and readily eluted, so that recovery, though not quantitative was sufficiently good to be useful. It is reasonable to expect that similar alkaloids - and this means most alkaloids - would show equally good results. Strongly basic alkaloids like strychnine were less successfully dealt with by Dowex 50 x 8, but the recovery was still enough for qualitative work. On the other hand, the poor results with the phenolic alkaloid morphine indicated that a negative result with Dowex 50 x 8 could not be held, in practice, as indicating complete absence of all alkaloids and that a further search would still have to be made (e.g. with another ion exchanger) for very weakly basic alkaloids.

There was evidence that part of the loss of alkaloid occurred during the deproteinization of the biological material and that improvement in this could well increase the efficiency of the over-all recovery of alkaloid. There was no opportunity during the present work to test methods of deproteinization other than precipitation by ammonium sulphate and trichloracetic acid. A next step in the investigation (which, the results so far indicate, is well worth continuing) should be to try the method of direct extraction from viscera by ethyl alcohol.

Zone Electrophoresis

Zone electrophoresis has previously been employed in toxicological analysis for the separation of alkaloids of

different mobilities but only after partial isolation by other means (see Goldbaum*). Under the right conditions it should be possible to separate alkaloids, with their small molecular weight and correspondingly high mobility, from proteins and, indeed Goldbaum notes this possibility. It would appear likely that for such a purpose electrophoresis would be most successful near the iso-electric points of the proteins where the mobility of these substances would be minimal whereas that of non-amphoteric alkaloids would still be considerable.

Separation of very small amounts of alkaloids, i.e. quinine, strychnine, brucine, atropine, morphine and codeine was achieved from serum protein by paper electrophoresis under such conditions (Tables 14 and 16). It was observed that as the pH of the buffer solutions was decreased from about 8 towards the iso-electric point of the proteins the separation improved (Table 14), satisfactory separation of alkaloids was possible by the careful control of the pH of the buffer solutions and even so closely related a pair of alkaloids as strychnine and brucine could be separated from serum proteins and from each other at pH 3.7 (Table 15), after 7 hours run. The migrated alkaloid band or bands after electrophoresis could be detected when the paper strip was stained with modified Dragendorff's reagent or Iodo-platinic acid reagent. Of these, Iodoplatinic acid reagent

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* Goldbaum, L. "Toxicology Mechanisms and Analytical Methods", Edited by C.P. Stewart and A. Stolman. Academic Press, New York and London, 1960. Vol. 1, p. 373.

is preferable for staining the paper strip to obtain clear band particularly in the case of strychnine and brucine (Table 16), whereas with Dragendorff's reagent there was undue diffusion and the bands were not distinctly stained. The stained alkaloid band could be eluted from the paper strip for identification and estimation. The elution of alkaloid from paper strip, however, involves some loss of alkaloid when adsorption became a factor.

Paper electrophoresis has some advantages for the identification of alkaloids in comparison of paper chromatography. Firstly alkaloids could be separated directly from serum protein. Secondly successful separation could be achieved in 5 hours, whereas 15 or 16 hours were required for paper chromatographic separation. Thirdly closely related alkaloids could be separated by suitable choice of pH.

CHAPTER 6

SUMMARY

1) Codeine in N. HCl at a concentration of 3 mg per 100 ml applied to column of Dowex 50 x 12 was recovered to the extent of about 85% by elution with 250 ml 5N. HCl. At lower alkaloid concentrations the recovery was lower, but at 0.3 mg per 100 ml it still averaged 66%.

2) In the presence of 10% trichloracetic acid the recovery at the 3 mg codeine phosphate per 100 ml level was only 76% whereas from acidified saturated ammonium sulphate it was over 90% when Dowex 50 x 12 resin was used.

3) Codeine phosphate (3 mg) was added to macerated liver and extracted by Daubney and Nickolls method. The deproteinized extract was filtered and passed through a column containing Dowex 50 x 12. The recovery of codeine was 40%.

4) Codeine phosphate (3 mg) was added to acidified saturated ammonium sulphate and passed through the column containing Dowex 50 x 8 (which was treated with modified method of column treatment, Section B). The recovery of codeine was within the range 83 to 92%. In one comparative experiment 83.3% of codeine was recovered from the column (Dowex 50 x 8) when 3 mg codeine phosphate was adsorbed from an acidified saturated solution of ammonium sulphate, whereas only 56.6% of codeine was recovered from the column (Dowex 50 x 8) when 3 mg codeine phosphate was adsorbed from 10% trichloracetic acid solution.

5) 3 mg codeine phosphate was added to the 250 ml liver extract prepared by the technique which kept the concentration of $(NH_4)_2SO_4$ maximal throughout and acidified with 10N. HCl. The whole solution passed through the column containing Dowex 50 x 8. The recovery of codeine was 92%. This technique was repeated exactly using strychnine as the alkaloid to be recovered and 95% recovery was obtained.

6) 3 mg codeine phosphate was added to macerated liver and extracted by the technique which kept the concentration of $(NH_4)_2SO_4$ maximal throughout. The liver extract was acidified with 10N HCl and passed through the column containing Dowex 50 x 8. The recovery of codeine was 63%. Applying same technique to strychnine the recovery was only 19.9%.

7) Codeine phosphate and strychnine were added separately to macerated liver and extracted by Daubney and Nickolls method. The filtrate was saturated with $(NH_4)_2SO_4$ and filtered. The clear liver extract was treated with 10N. HCl and passed through the column containing Dowex 50 x 8. The recovery in case of codeine was 40% and in case of strychnine was 37%.

8) The behaviour of morphine with Dowex 50 x 8 resin was studied. The results indicated that morphine was at best only partial-ly adsorbed by the resin.

9) Separation of alkaloids from serum protein by paper electrophoresis was investigated. Codeine, strychnine, brucine, atropine and morphine could be separated from serum protein using acetate buffer at pH 3.7 after 5 hours run. A closely related pair of

alkaloids, strychnine and brucine, could be separated from serum protein and from each other at pH 3.7 (acetate buffer) after 7 hours run.