UTILIZATION OF ION-EXCHANGE CHROMATOGRAPHY AS A MEANS OF SEPARATING FIVE COMMONLY USED 5-5 SUBSTITUTED BARBITURIC ACIDS

by

Wesley Ray Jacobs, B.S.

A THESIS

Presented to the Department of Pharmacology
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Master of Science

May 1960

Acknowledgments

The author wishes to express his sincere appreciation to Dr. Norman A. David for his guidance and instruction during the past four years, especially in regards to the investigation and preparation of this thesis. The advice and suggestions of Drs. David Jackson and Elton McCawley are also gratefully acknowledged. The author also wishes to thank Mrs. Kathryn Bittick for the artistic preparations and to Miss Betty Jacobs for typing and assembling the manuscript.

The work of this thesis was supported by funds supplied by the Multnomah County Coroner's Office.

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

TABLE OF CONTENTS

INTRODUCTION

Few drugs enjoy as widespread clinical use today as do the barbiturate hypnotics and sedatives. Barbital, introduced in 1903 as Veronal(R) and phenobarbital, discovered in 1912, are extensively employed as safe and effective sedative agents for the management of a wide variety of nervous disorders. With the development of the short-acting barbiturates such as pentobarbital, amobarbital, secobarbital and, later, the ultra short-acting thio barbiturates, the scope of usefulness of these drugs has been greatly widened. These newer barbiturates serve as potent hypnotic agents, relieve agitation, are routinely used for pre-medication and, as the ultra short-acting barbiturates are most frequently employed for surgical anesthesia. There are, however, certain serious drawbacks attending their clinical use. These are habituation, poisoning from overdosage, and a rather high incidence of untoward reactions. Because of these disadvantages substitute drugs such as the non-barbiturate hypnotics, the antihistamine sedatives and the tranquilizing agents have been introduced. Nevertheless, the present-day therapeutic supremacy of the barbiturates remains unchallenged (11).

With the goal of developing barbiturates which are safer, are less likely to cause habit formation and which can readily be detoxified within the body should overdosage occur, the laboratory study of these drugs has been mainly concerned with their metabolic fate in the body. Much investigative work has been done and the distribution of the more important barbiturates has been studied in considerable detail by numerous

workers and has been reviewed by Porter (28). Unfortunately, most investigators express their results as quantitative total amounts including both the barbiturate metabolites and the unmodified drug. The reason for this is explanable: at present chemical methods for the accurate and quantitative separation and identification of barbiturate metabolites are unsuitable and difficult to perform. It is evident that much more information must be obtained from laboratory investigations in order to elucidate the metabolic fate, distribution and excretion of the barbiturates.

Of chief concern to this writer - and one of the reasons for undertaking the research work described in this thesis - is the need for improved methods for determining blood and tissue barbiturate levels. With the introduction of more potent, rapidly acting barbiturates there has been an increased incidence of barbiturate overdosage (11,23) and accidental deaths. In overdosage or death these barbiturates appear in such low concentrations in body tissues and fluids that they are extremely difficult to isolate and identify. Chemical means are the only ways of establishing death due to barbiturate poisoning since no anatomical lesions are characteristic (36). Another goal may be achieved through further work dealing with the fate and distribution of the barbiturates. Such studies may lead the way to finding more specific antidotes for treatment of overdosage or for speeding the recovery and lessening the incidence of untoward actions in patients undergoing general anesthesia with the barbiturates. Also, studies dealing with metabolic fate of these drugs may provide a possible answer as to why continued use of the short-acting barbiturates leads to addiction. With better understanding of the metabolic

role these drugs play in the body, ways and means to forestall the addictive propensity of the barbiturates may be forthcoming.

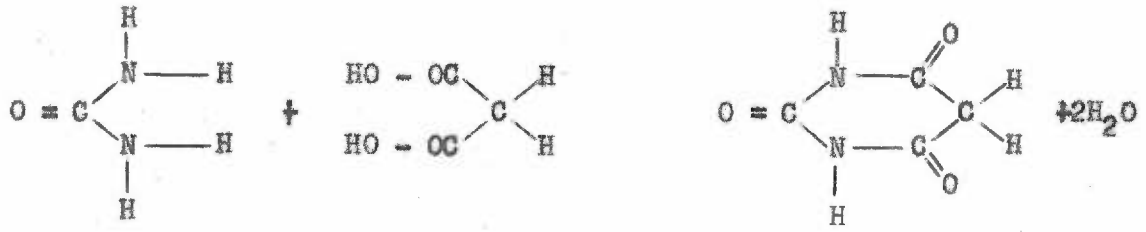
PURPOSE OF RESEARCH INVESTIGATIONS DESCRIBED IN THIS THESIS

The researches described in this thesis were undertaken for the purpose of reviewing, through laboratory experiments, the chemical methods now available for the identification and quantitative separation of the barbiturates and their metabolites in body tissues. While the writer has used most of these procedures in the actual performance of work dealing with the toxicologic analysis of tissues for barbiturates, only the methods further explored in this thesis work will be described in detail here.

1. Chemistry of the Barbiturates.

Some understanding of the chemical features of the more important barbiturate compounds is necessary at this time if the problems involved in their identification and separation in body tissues is to be appreciated.

The basic molecule of all barbiturates is the barbituric acid structure. Barbituric acid is a diureid formed as the condensation product of urea and malonic acid (figure 1) (12).



Urea + Malonic Acid → Barbituric Acid (Malonylurea) + $2H_2O$

Figure 1

The diureides differ from the mono ureids in that in the latter only one amino group of urea is condensed with a carboxyl group. Barbituric acid, as such, has no pharmacologic action and is not a central depressant.

The various barbiturate analogs are formed by substitution of various radicals at different positions on the barbituric acid nucleus. The general formula for these substitutions is shown in figure 2 (12).

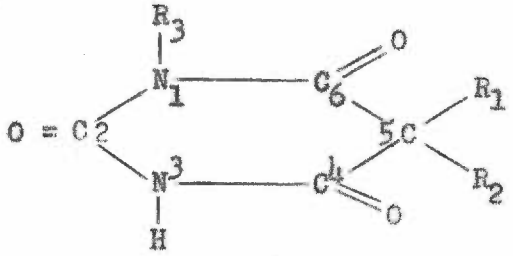


Figure 2

Substitutions in the barbituric acid nucleus may be made at R_1, R_2, or R_3. The thiobarbiturates have a sulfur molecule substituted for the oxygen in the urea portion.

The substitutions made for several of the barbiturates are tabulated below:

Barbiturate	at R_1	at R_2	at R_3
Amobarbital	ethyl	isoamyl	H
Barbital	ethyl	ethyl	H
Pentobarbital	ethyl	1-methyl butyl	H
Phenobarbital	ethyl	phenyl	H
Secobarbital	allyl	1-methyl butyl	H

The largest and most important group of derivatives are the 5-5 substituted barbituric acids. The other two major groups are the N-alkyl barbituric acids which have a methyl group on a nitrogen (mephobarbital and methabarbital) and the thio barbiturates which contain a sulphur on the number two carbon.

The barbiturates are acid in reaction by the fact that the keto form of the molecule (-CO-NH-) is in equilibrium with the enol form (C OH :N), the hydrogen of which is readily dissociated allowing formation of salts and alkalis (12).

Except in the N-alkyl substituted barbiturates, the hydrogen atoms capable of ionization are attached to both nitrogens of the ring. In 1940, Krahl (19), determined the ionization constants for 30 substituted barbituric acids. In physiologic pH ranges, the barbiturates act as mono basic acids as the second hydrogen is not significantly ionized below pH 10 (figure 3).

pK Values At 25° For The Barbiturates Used In This Project, Adapted From Krahl (19)

Barbiturate	pK
Phenobarbital	7.41
Barbital	7.91
Amobarbital	7.94
Secobarbital	8.08
Pentobarbital	8.11

Figure 3

2. Identification of Barbiturates in Biologic Material.

Many methods have been devised for isolation and identification of barbituric acid derivatives in biologic material. One of the first was the classic Stas-Otto procedure (37) which used organic solvents for separation and a very crude colormetric means of detection. This procedure requires the extraction of large volumes of blood or organs and is very time consuming. About twenty years ago a colormetric method was introduced by Koppanyi, Murphy, Krop (18) which utilized the formation of a cobalt complex with the barbituric or thio barbituric acid as the means of identification. Both methods have the disadvantage of low specificity and low sensitivity, (7,40) also "false positives" may result due to impurities in the biologic extracts used as the initial sample specimen (17,31).

More recent methods for separating and identifying the barbiturates include chemical (24,34); titration (32,35); X-ray (16,27); infra-red

(5,29); paper chromatography (2,25,28,40); and ultraviolet spectrophotometry (10,22,36).

Of these many methods, the only two presently in wide spread use are ultraviolet spectrophotometry and paper chromatography with its several variations. Ultraviolet spectrophotometry analysis is based on the fact that all of the 5-5 substituted barbiturates have a characteristic ultraviolet absorption spectrum (10,22).

The thio and N-methylated derivatives represent a special group and have their own ultraviolet characteristics (8,13,36). However, as long as the basic ring structure of the barbituric acid nucleus remains intact, identification of the specific derivatives from the remainder of the group is impossible (30). This is a considerable drawback since the barbiturates vary considerably in speed of action, potency and metabolism.

3. Theoretical Considerations Pertaining to the Use of Ultraviolet Absorption and Variation in pH for Isolation and Identification of the Barbiturates in Biologic Materials.

The ultraviolet absorption spectrum of the barbituric acid derivatives has been well established by numerous workers. Various authors have described the maximum absorption of the non thio derivatives from 239 millimicrons to 255 millimicrons (30). Walker, Fisher and McHugh (36) have made clear these variations on the basis of pH which affects the degree of ionization of the molecule. In strongly acid solutions there is no tendency to absorb ultraviolet light at 239 millimicrons and this feature has been called by Walker and co-workers (36) the acid phase. As the pH is increased above pH 7, ultraviolet absorption at 239 millimicrons is increased to a maximum at pH 9.5-10. This Walker calls the first alkaline phase which corresponds to ionization of the first hydrogen. At pH 12 the

absorption spectrum shifts toward the visible region of the spectrum, the absorption band broadens at the base, and the molecular extinction decreases, (figure 4). Of course, this second alkaline phase does not exist in the N-substituted derivatives since there is only one ionizable hydrogen.

As pointed out by Lous (22) and others (13,39) the barbiturates follow Beer's Law and, therefore, can be quantitated if the specific barbiturate being examined is known since variation in extinction coefficients occurs within the group (4). This markedly restricts the ability to obtain quantitatively valid tissue or blood levels in clinical or toxicologic situations unless the barbiturate in question can be identified by history or by physical evidence. Thus it is evident that even though ultraviolet spectrophotometry is highly sensitive for the identification of barbiturates as a group, it has a complete lack of specificity and, therefore, is of limited value (40).

4. Use of Paper Chromatography For Identification of Barbiturates.

In an attempt to overcome the lack of specifity for distinguishing the barbiturates or their metabolites by ultraviolet spectrophotometry, the principle of paper chromatography was introduced in 1946 (2). Since this time, various alterations and modifications have been made (1,3,38,40). The technique of Walker and Algeri (1) has been used in this laboratory and seems to give the best results. A subsequent report by Walker and Algeri (2) confirms the simplicity of their method, and its sensitivity to 50 ug. of drug in some instances under ideal conditions. Unfortunately, three commonly used drugs amobarbital (Amytal^{R}), hexethal (Ortal^{R}) and pentobarbital (Nembutal^{R}) were not differentiated by this technique. It

should also be pointed out that in Walker and Algeri's method the means for locating the absorbed material are not specific for barbiturates and that the changes produced by the color developing reagents destroy the drugs for further analysis (25). Sectional elution of the chromatogram followed by ultraviolet spectrophotometry gives the most reliable information, but the multiple technical procedures decrease the sensitivity.

5. Densitometry Technique For Barbiturate Isolation.

In 1956 Algeri and McBay (25) introduced a method of densitometry which combined the advantages of spectrophotometry and chromatography into a single procedure. This method, however, like the other lacks specificity in that three commonly used drugs, amobarbital (Amytal^{R}), secobarbital (Seconal^{R}) and pentobarbital (Nembutal^{R}) are indistinguishable. When a single known barbiturate and its metabolites are being examined and separation from other barbiturates is not a problem, the method is adequate and not too difficult technically (25).

6. Infra-Red Spectrophtometry.

In addition to ultraviolet spectrophotometry and chromatography the method of infra-red spectrophotometry deserves mention since it has been suggested as a possible procedure for accurate barbiturate separation. However, in 1954 (29) Price et al showed that the difference in frequencies of the carboxyl absorption bands between the members of the series was too small to allow adequate separation. Infra-red spectrophotometry also has the disadvantage of requiring a high degree of purity, and the apparatus needed and operation is expensive.

Thus, it is readily apparent that there is as yet no single method or combination of methods which will separate from biologic materials or

mixtures all the members of the substituted barbituric acids with an adequate degree of sensitivity and selectivity. Until this is done, little progress can be made in the more important inquiry concerning the nature and identification of the metabolites of the barbiturates formed in the body.

* * * * * * * * * * * * * * * * * *

The difficulties and disadvantages inherent in the various methods for isolating and identifying the individual barbiturates in biologic material have been described. It seemed worthwhile, therefore, to further examine some of these or other techniques in the laboratory in the hope that through modification or further development, a more accurate and suitable method for barbiturate analysis would be obtained. In pursuing this objective, the qualifications for an ideal method were kept in mind. These are:

1. High degree of specificity and sensitivity.
2. Technically easy to perform in a short period of time.
3. Requires the minimum of expensive equipment and reagents.
4. Easily reproducable in other than a research laboratory.

Basic Ion-Exchange Chromatography: Its Use For Separation of Barbiturates?

A survey of the literature failed to reveal the use of ion-exchange chromatography as a means of isolation and identification of the barbituric acid derivatives. Ion-exchange chromatography is a technique now commonly employed in biochemistry and certain phases of pharmacologic study. Hayaishi and Kornberg (15) used ion-exchange in 1951 to separate the bacterial enzymatic oxidation products of thiamin and uracil which formed

5-methyl barbituric acid and barbituric acid respectively. The uracil product was compared with pure barbituric acid. This is the only report the writer has been able to find relative to the use of ion-exchange for separation of substances related to barbiturates. This lead appeared promising and the investigation of basic ion-exchange chromatography as a method for separating other barbiturates was undertaken.

Chromatography, in general, has been defined by Cassidy (6) as "A separation process applicable to essentially molecular mixtures which relies on distribution of the mixture between an essentially two-dimensional, or thin, phase and one or more bulk phases which are brought into contact in a differential counter current manner." Cassidy points out that these two phases are commonly referred to as the stationary and mobile phases. In ion-exchange chromatography the stationary (two-dimensional or thin) phase is the surface of an ion-exchange resin. These resins are comprised of polymeric structures with fixed charge sites in the neighborhood of which oppositely charged ions reside. These latter ions may be exchanged with similarly charged ions in the mobile phase. The resin may have fixed anions or fixed cations and thus are referred to as cation and anion exchangers, respectively. The resins are named by the type of ion they exchange rather than by the fixed ion composing the resin. The bulk phase or mobile phase in this case is a liquid which transports the ions over the stationary phase. Separation with ion-exchange, as well as all types of chromatography, depends on the differential distribution of solute that occurs between mobile and stationary phases. The success of chromatography depends on influencing this distribution process, so as to drive the solutes selectively into one or the other phases at the proper stage in the separation

process. This distribution depends on molecular inter-action within the system.

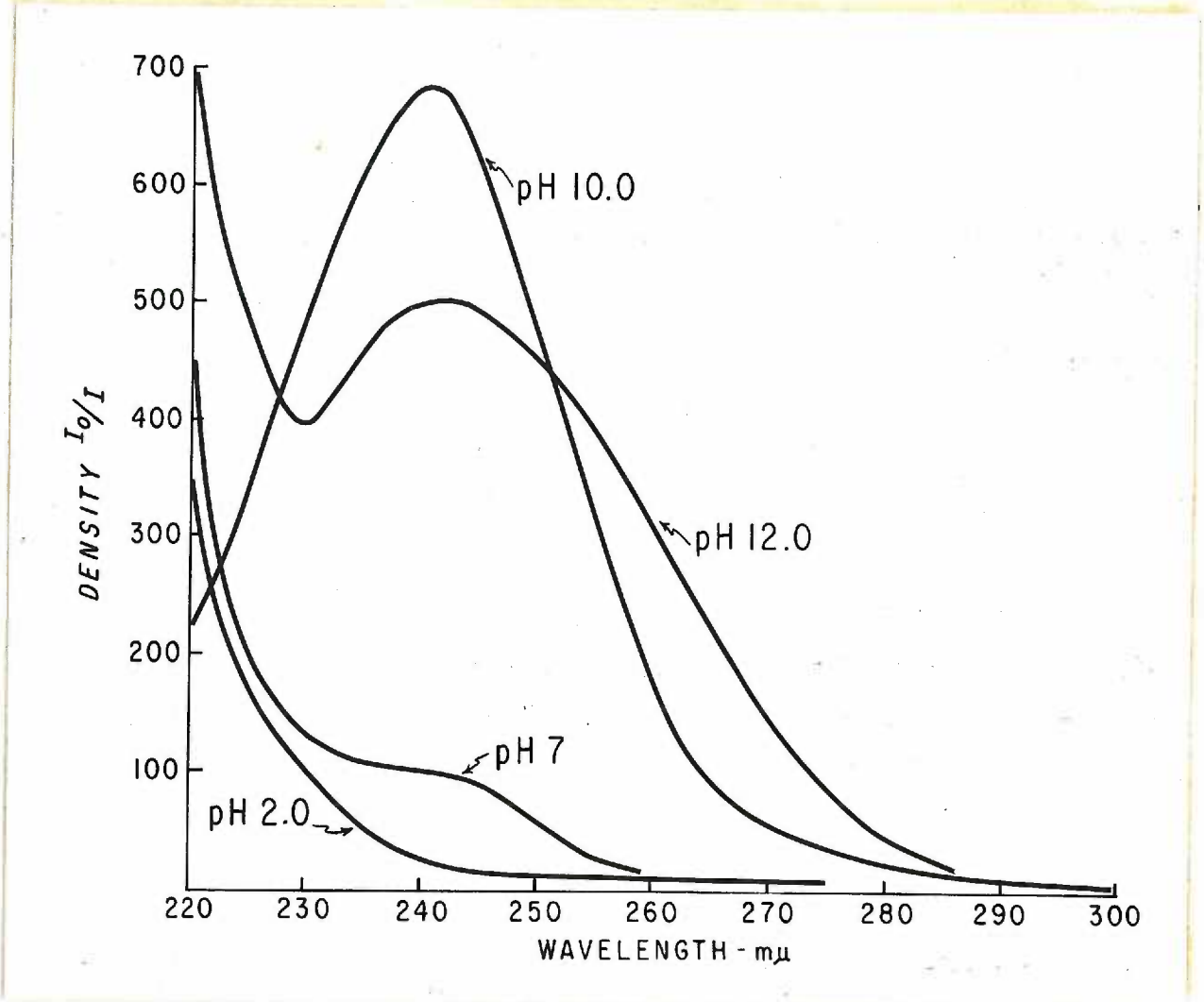


Figure 4. - Ultraviolet absorption spectra of barbital (12.5 gamma/ml.) in aqueous solution of various degrees of pH (36).

METHODS AND MATERIALS

1. Basic Concepts of Ion-Exchange Procedures: Selection of Procedure for Barbiturate Identification.

As mentioned previously, Hayaishi and Kornberg (15) used ion-exchange to separate the bacterial enzymatic oxidation products of thiamin and uracil which formed 5-methyl barbituric acid and barbituric acid respectively, the uracil product being compared with pure barbituric acid. Their system, Dowex-1 (Cl^- form) was used in a 9.5 x 1 cm. glass tube column. Dowex-1 is a strong base anion exchange resin composed of quaternary ammonium groups on a styrene divinyl benzine matrix (6). Using this column they then buffered the liquid-resin system at pH 8.7 with 0.2 M trix-(hydroxy-methyl)-amino methane buffer. The reason for choosing this pH is not made clear by the authors but at this pH, which is considerably above the pK of barbituric acid (3.98) (21), the barbituric acid molecule should be significantly ionized and carry a net negative charge. Under these circumstances, the molecule should be firmly attached to the positively charged resin by replacing equivalent amounts of chloride so as to maintain electro-neutrality. Hayaishi and Kornberg then eluted the column containing this mixture with NH_4-OH-NH_4Cl buffer (0.2 M, pH 9.9). The barbituric acid was recovered between 265 and 360 cc. of the effluent. The authors do not make clear the rational for eluting with this mixture but it is assumed that pH 9.9 was chosen because at this pH there is maximum ultraviolet absorption of barbituric acid, a fact mentioned previously in this thesis in the discussion of barbituric chemistry. Ionization of the barbituric acid molecule should be more complete at this pH than at time of sorption which seemingly should increase the attraction of the molecule to the resin. Therefore, it is

suggested that electro-chemical change is not the primary factor involved in removal of the barbituric acid molecule from the resin. It was felt that this effect is most likely the result of mass action with excess chloride shifting the equilibrium reaction to the left in the following equation (6):

$$R\text{-}B_1^+ + A_1^- + B^+ + A_2^- \rightleftharpoons R\text{-}B_1^+ + A_2^- + B_2^+ + A_1^-$$

$$R\text{-}B_1^+ + Cl^- + H^+ + B.A.^- \rightleftharpoons R\text{-}B_1^+ + BA^- + H^+ + Cl^-$$

In the equation, R is a polymeric structure to which a cationic group B_1^+ is covalently attached (quaternary ammonian groups in the case of Dowex-1) while A_1^-, A_2^- and B_2^+ are ions, in this case Cl^-, BA^- and H^+ respectively. It is realized that there are a multitude of factors (33) which affect the interaction of the individual molecules with the resin. It was hoped there might be significant differences in each of the substituted barbiturate molecules to allow separation from each other using Hayaishi and Kornberg's scheme as a starting point.

An alternate procedure avoiding the necessity of removing the barbiturates from the column by a mass action effect was considered. It was felt that they might be eluted by increasing the relative positive charge of the barbiturate molecule so that there would be less attraction between the barbiturate molecule and the positively charged resin (Dowex-1). This is the general theory of the procedure used by Moore and Stein (26) for the separation of the acidic and neutral amino acids. In this method the amino acids are placed on a cation exchange resin, Dowex-50 (a nuclear sulfonic acid groups on styrene DVB matrix - $R\text{-}SO_3^-$) at pH of 2. At pH 2 the amino acids are only slightly ionized and carry a relative positive charge and are, therefore, firmly held by the negatively charged sulfonic acid resin.

With increased pH the alpha-carboxyl of the amino acids becomes more ionized and the proportion of molecules having a net positive charge decreases. Thus, the amino acids are less firmly attached to the resin and tend to move with the buffer. The pK (alpha-carboxyl) values of the neutral and acidic amino acids vary slightly at any pH, therefore, the extent of ionization will vary and the net charge will differ allowing different flow rates (9) at any pH.

Example:

(glycine pK 2.4) $+ NH_3\ CH{\cdot}R{\cdot}COOH \rightleftharpoons + NH_3\ CH{\cdot}R{\cdot}COO^- + H$

To apply this theoretical procedure to isolation of the barbiturates using a Dowex-1 column carrying a positive charge, the pH would necessarily have to be decreased below the pK, thereby decreasing the degree of ionization. With decrease in the degree of ionization, the molecule would tend to become more positive and thus less firmly attached to the positively charged resin. Theoretically, separation could occur because of the differences in pK., other factors being equal. However, the effluent then would be less than pH 7 and, therefore, would necessitate the addition of a suitable buffer to increase the pH to 9.9 for maximum ultraviolet absorption needed for identification.

On the other hand, if a cation exchange resin (Dowex-50) were used as in the method of Moore and Stein, the barbiturates, in a relatively unionized state, would carry a net positive charge and be attracted to the SO_3^- portion of the Dowex-50 of the column. As the pH was increased above the pK, the barbituric acid molecules would tend to become more

ionized and thus carry a relatively greater negative charge and have less electro-static attraction to the resins. Since the individual barbiturates have different pK's, at any pH the extent of ionization will vary. Thus, the barbiturate-resin attraction will vary, theoretically allowing different flow rates.

In Moore and Stein's procedure, the effluent would be alkaline and require less adjustment of pH to obtain maximum ultraviolet absorption. As pointed out by Moore and Stein, the pK of the amino acids is not the sole factor regulating the exchange. Also, the order of elution may be different than predicted on the basis of pK alone. They point out that general size, shape and chemical characteristics of the individual molecular types play a part in rates of exchange. There is no reason to believe that these factors would not also affect the barbituric acids since there is a great variance in the side chains of the individual drugs.

2. Selection of Suitable Resins for Eluting Barbiturates in Ion-Exchange Procedures.

The resins used in this project were Dowex-1 (chloride form) and Dowex-50 (hydrogen form). As mentioned, Dowex-1 was used by Hayaishi and Kornberg (15) and their methods were used as a starting point for the present investigation. Dowex-1 is a strong base anion exchanger made of quaternary ammonium groups on styrene divinyl benzene matrix. Several mesh sizes of this resin were used but 100-200 mesh was found to give the best flow rates in the column sizes used. The resins are also available in various degrees of cross-linking. Cross-linking is a characteristic of resins which primarily controls the degree of swelling in water and the accessability of the interior of the polymer to ions of different size (6).

A lesser degree of cross-linkage, (i.e. a molecule which is structurally less dense), permits exchange with larger ions, however, swelling is increased in water. The majority of the commercial resins are about 8 per cent cross-linked (6).

Dowex-50 was also used and was selected as the cation exchanger since this was the resin used in the amino acid separation of Moore and Stein discussed in the preceeding section. Dowex-50 is composed of nuclear sulfonic acid groups on styrene divinyl benzene matrix. Eight per cent cross-linking was used throughout this project. Lack of sufficient research time was the only factor which prevented further evaluation of Dowex-50 using various degrees of cross-linkage. The 100-200 mesh particle size was used since flow rates were most easily controlled with this size.

3. Preparation of Resins.

(A) Anion Exchange Resins (Dowex-1). The chloride form of these resins were used. The resins were obtained in a moist condition in plastic bags. The resins, although already in the form desired, were soaked in a solution of 4N hydrochloric acid. The acid was decanted off and new acid added until the decanted acid became clear. This procedure removes impurities such as rust flakes found in the resins (33). Although other agents are suitable, Samuelson (33) suggests hydrochloric acid because it is inexpensive and easily obtained in pure form. The resin was stored in 1 N HCl until its use at which time it was washed on a Buchner funnel until the effluent was negative for chloride, as detected by addition of silver nitrate solution. At this point, the resin was ready to be poured into the columns.

(B) Cation Exchange Resins (Dowex-50). Initially the Dowex-50 was treated with 4 N HCl in the same manner and for the same purpose as the Dowex-1. The 3-4 N HCl was also found by Samuelson to allow for maximum regeneration of the resin so that the Dowex-50 was stored in 4 N HCl. Prior to its use in the column it was washed with distilled water until free of excess chloride. If thorough washing with distilled water was not carried out completely it was found that excessive amounts of buffer would be required to obtain desired pH of the column. In all cases where Dowex-50 was used, the columns were buffered prior to the sorption step.

4. Reagents, Chemicals and Drugs.

(A) Reagents and Chemicals - Standard commercially available laboratory reagents and chemicals of analytical grade were used.

(B) Barbiturates - The following is a list of the six barbiturates used in this project:

1. Barbituric Acid, mol. wt. 128.09
2. Barbital, N.F., mol. wt. 184.19
3. Phenobarbital, U.S.P., mol. wt. 232.33
4. Pentobarbital, U.S.P. (Nembutal(R), Abbott), mol. wt. 248.26
5. Secobarbital, U.S.P. (Seconal(R), Lilly), mol. wt. 260.27
6. Amobarbital, U.S.P. (Amytal(R), Lilly), mol. wt. 226.27

5. Technique.

The techniques of ion-exchange chromatography used in this project were adopted from Samuelson's textbook, Ion-Exchangers in Analytical Chemistry (33). In the chapters on ion-exchange technique he points out that "any general rules for obtaining accurate results in the shortest possible time

cannot be given." However, he also points out that there are some general indications which can be given.

(A) Choice of Operation - Column operation was used in favor of batch operation since this is the method employed by Hayaishi and Kornberg (15) whose work is being used as a starting point. This is also the operation which Samuelson considers most applicable under the majority of circumstances.

(B) Apparatus - The columns used were commercially made 20 cc. burettes fitted with a stopcock (60 cm. length and 1.2 cm. diameter). A small plug of glass wool was placed in the bottom to support the resin. A glass wool plug was also placed on top of the resin to prevent disruption of the resin when solutions were poured onto it. The resin was introduced into the column as a slurry prepared by mixing the resin about half and half by volume with distilled water. The resin was allowed to settle with the stopcock open but never allowing the water level to fall below the resin so as to prevent air from entering the column and causing "channeling". This can be prevented by keeping the outlet above the level of the resin bed. When the column length desired was obtained, the excess water was removed from the top of the resin bed by suction and the desired buffer solution was introduced and allowed to run through the column until the material coming off the column (effluent) was the same pH as the buffer (influent).

When the column had been properly buffered, the barbiturate(s) to be analyzed was placed on the column in volumes of 1 cc. or less. After the drug had flowed onto the resin, the side walls of the tube were washed with water and then the water was suctioned off. When this was completed, the

elution buffer solution was added to the top of column to the height which would allow proper flow rate. When the proper level was obtained. a resorvoir of buffer was placed at the same level and connected with the tube by means of a siphon hose so as to provide nearly the same level throughout the procedure.

The effluent from the columns was collected in various size fractions with a Misko (Microchemical Specialties Co) 72 position fraction collector and timer. When the flow rate and size of fractions were determined, the timer was set to collect separate fractions on the basis of the time interval. Since this method of collection provided consistant collection columns, no other means of fraction collection was attempted.

After the fractions were collected, they were analyzed for presence of barbiturate in a Model DU. Beckman Spectrophotometer with photomultiplier attachment. If the effluent was not already at pH 9.9; it was adjusted to this by use of a small amount of concentrated buffer solution of pH 9.9. Each fraction absorbed the ultraviolet light at this wave length, the specimen was then scanned between 220 and 250 MU to see if the characteristic barbiturate curve was present. This was done to rule out any contaminating substance which might absorb ultraviolet light at 240 but not yield the characteristic curve. All fractions were examined against a blank of the eluting buffer which has been run through a column without the addition of any drug.

(C) Rate of Flow - The rate of flow depends upon a number of factors; 1) particle size, 2) length of column, 3) viscosity of fluids being passed through column, and 4) height of the liquid column above the resin (33). Sameulson points out that ideal flow rates will certainly vary with each

operation but a "normal" flow rate for cation exchange columns is 3-10 ml. per square cm. per minute and 3-5 ml. per square cm. per minute for anion exchange resins. Flow rates for all columns were kept within these limits unless otherwise noted. For the sake of economy and convenience, only one resin size, 100-200 mesh, was used for all of the columns. Since columns of varying lengths were used, the flow rate was maintained within limits by varying the height of the liquid column about the resin. This was found to be an effective method; however, in several cases an additional length of glass tubing had to be added to the burett by means of a short piece of Tygon tubing.

(D) Concentration of Drug Solutions - In most cases, ion-exchange chromatography is best suited for solution of relatively low ion concentration, however, they must not be so dilute so as to interfere with accurate identification. The drug solutions used were at a concentration of 1 mg./cc. of distilled water. Since the barbiturates used range in molecular weight from 164.04 to 248.15 the ion strengths are no greater than $\frac{1}{128}$ or about 0.008 M.

(E) Temperature - All operations in this project were operated at room temperature. Samuelson feels that most cases of analytical ion-exchange can be best operated at this temperature. Even though this becomes a variable in some conditions of ion-exchange no attempt was made to evaluate the effect of temperature change in this project.

RESULTS

Since two basically different ion-exchange systems were employed, each one will be presented individually.

I. Method A Ion-Exchange System.

A. Reduplication of the work of Hayaishi and Kornberg. - The first step of the project was to attempt to reproduce the results of Hayaishi and Kornberg using chemically pure barbituric acid. A column was set up and operated as described previously under methods and materials. The only modification on the procedure was the use of a 10 cm. column instead of a column of 9.5 cm. length. The eluent was 0.2 M NH_4OH-NH_4Cl buffer adjusted to pH 9.9. Using this system inconsistent results were obtained on four trials. Figure 5 is a graphic representation of these results compared with the results of Hayaishi and Kornberg which are shown by the shaded area.

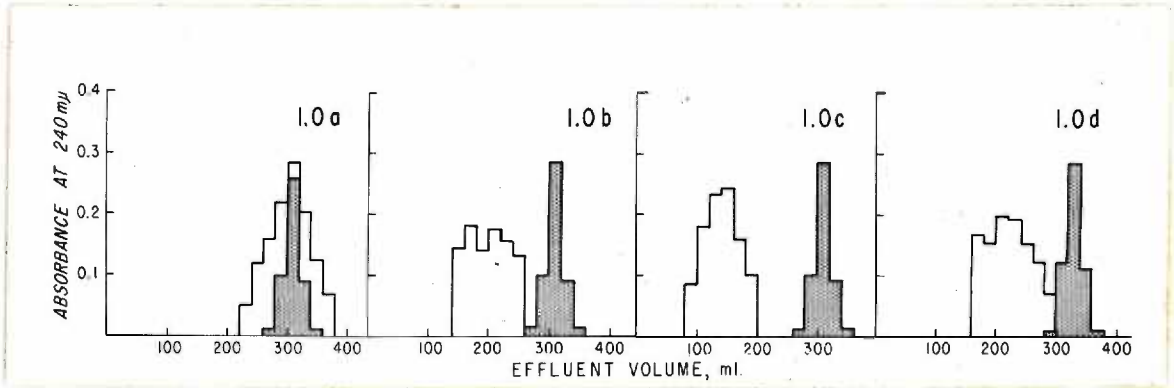


Figure 5. - Barbituric acid 10 cm. column with 0.2 M eluent. Shaded area represents results of Hayaishi and Kornberg.

However, by increasing the eluent concentration to 0.4 M and leaving all other factors constant, relatively consistent results were obtained on three runs as shown in figure 6.

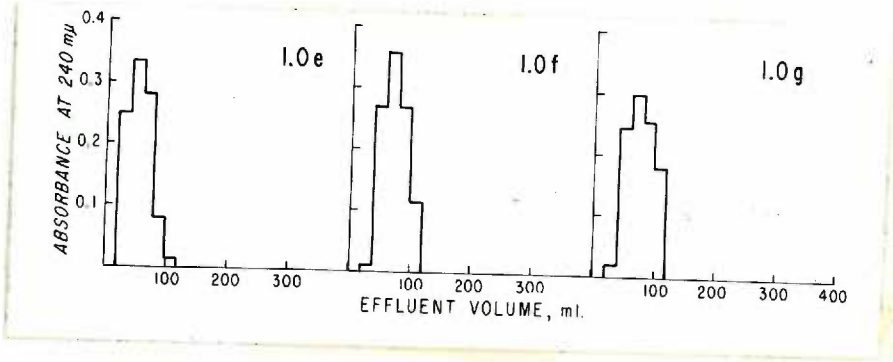


Figure 6. - Barbituric acid, 10 cm. column with 0.4 M eluent.

In order to become better acquainted with some of the factors which affect drug elution, column length and eluent molarity were varied. The column length was increased from 10 cm. to 20 cm. and eluents of 0.4 M and 0.5 M were used. Barbituric acid, 0.5 mg., was used in all trials except run 2.0-a where 0.1 mg. of barbituric acid was used. This was done to see if major differences would occur in the elution curve because of the increased drug amount. Results are shown in the figure 7 graphs on the next page.

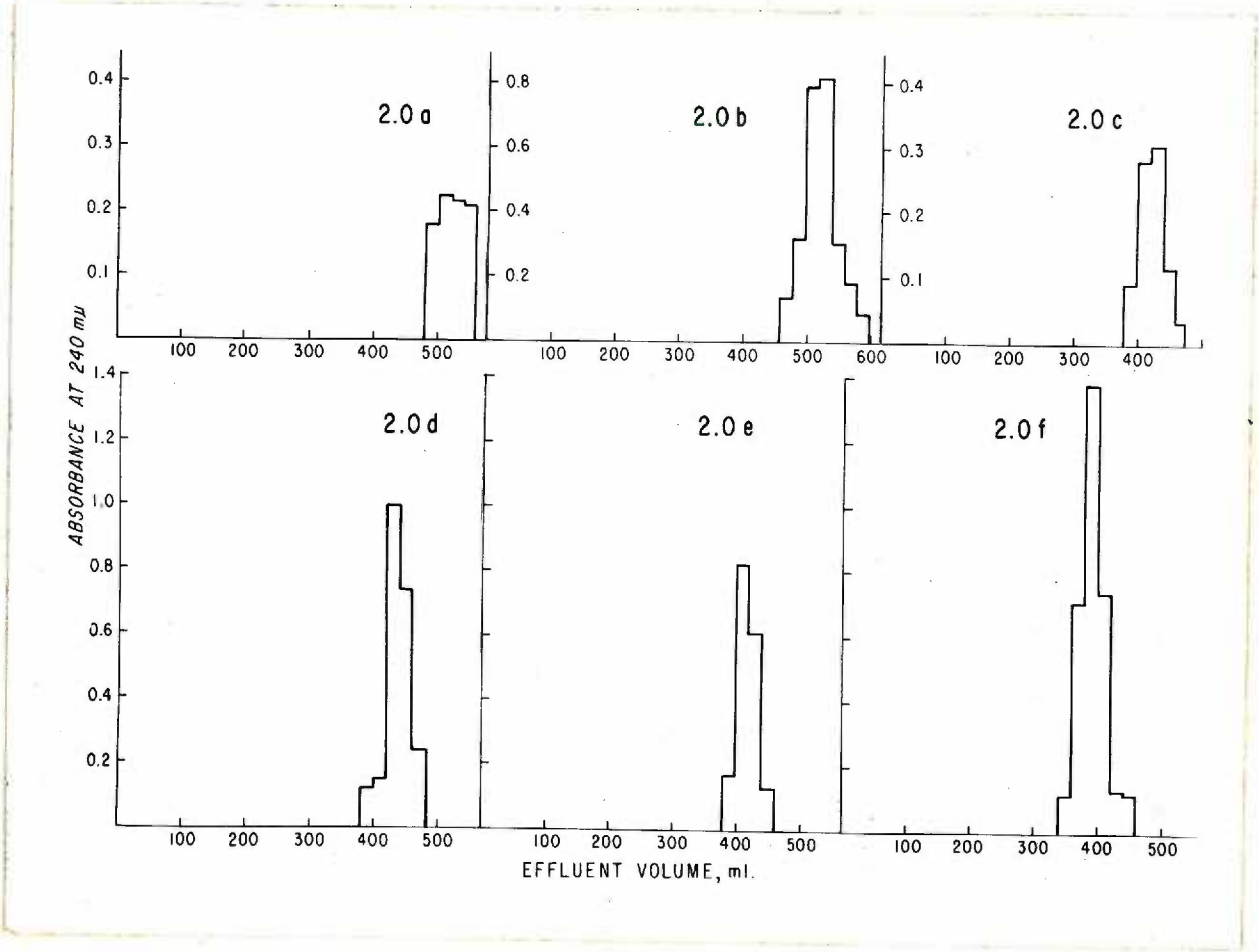


Figure 7. - Barbituric acid, 20 cm. column with 0.4 M eluent in 2.0 a and 2.0 b, and 0.5 M eluent in 2.0 c, 2.0 d, 2.0 e, and 2.0 f.

The second step was to use the same column set-up but to use the 5-5 substituted barbituric acids in place of plain barbituric acid. A buffer of NH_4OH-NH_4Cl buffer adjusted to pH 9.9 was used throughout this phase of the experiment for the eluent and will be referred to as "eluent" of a certain concentration for convenience. Unless otherwise stated 1 mgm. of drug was used in all trials. As before, only 10 and 20 cm. columns were used. A total of 1000 cc. of the effluent was arbitrarily set as the maximum to be used in any one run. If no drug was recovered within this volume, the concentration was increased.

B. Analysis of the 5-5 substituted barbituric acids individually using the ion-exchange system of Hayaishi and Kornberg.

1. Barbital - Barbital was the first drug to be analyzed using an 0.4 M eluent. No drug was recovered within 1000 cc. and the column was regenerated with 4N-hydrochloric acid. The eluent concentration was increased to 1 M and effluent curves were obtained within 1000 cc. on 10 and 20 cm. columns. The procedure was repeated using 2 M eluent also. In run 1.1-d, the drug amount was decreased to 0.1 mgm. (Figure 8).

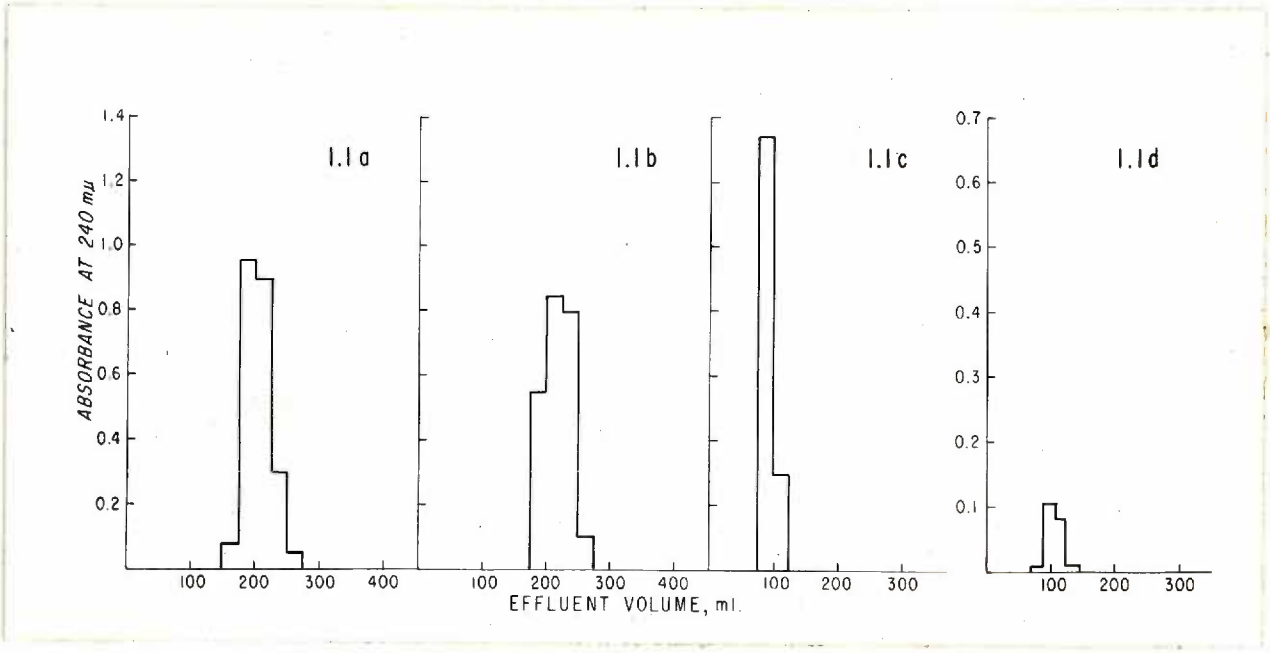


Figure 8a. - Barbital, 10 cm. column with 2 M eluent.

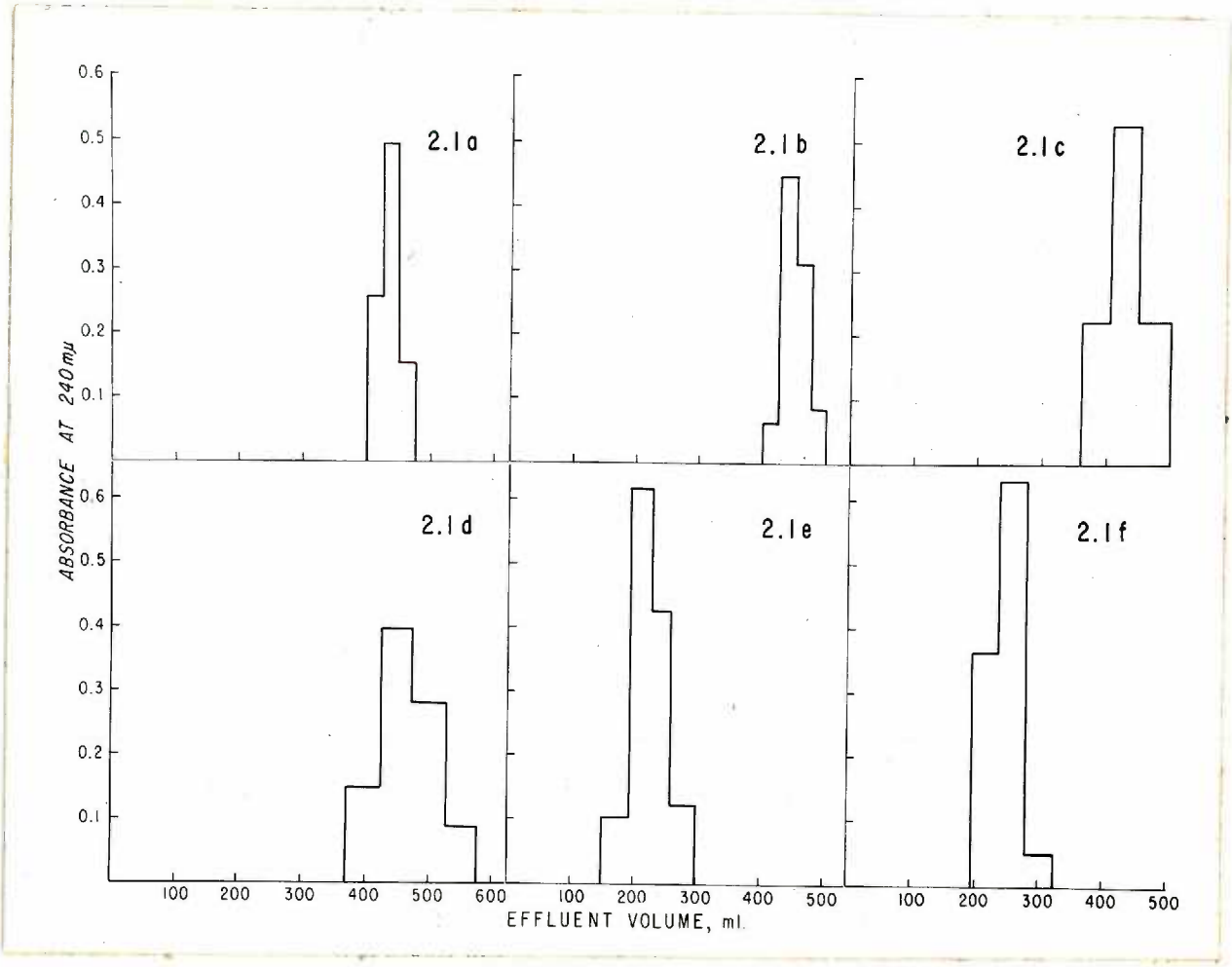


Figure 8b. - Barbital, 20 cm. column with 2 M eluent.

2. Phenobarbital - The initial run employed a 10 cm. column and 1 M, 2 M, 3 M, and 4 M eluent were used but all failed to remove the phenobarbital within the arbitrary limit of 1000 cc. It was not until 6 M eluent was used that satisfactory results were obtained. This was repeated on four trials. In run 1.1-c and 1.1-d, the drug amount was decreased to 0.5 mg. (figure 9). Since phenobarbital was removed with such great difficulty on a 10 cm. column, its separation was not attempted on a 20 cm. column.

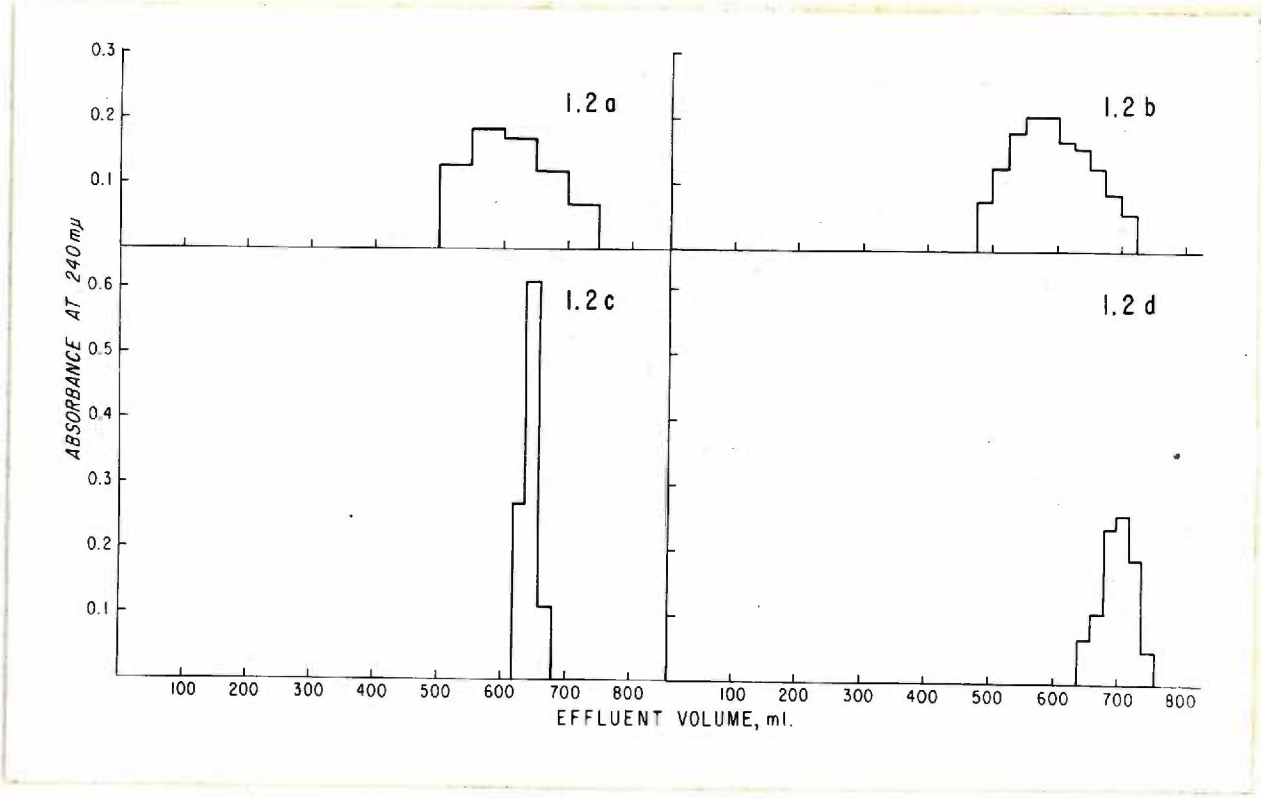


Figure 9. - Phenobarbital, 10 cm. column with 6 M eluent.

3. Pentobarbital - Effluent curves were established for pentobarbital in the same manner, beginning first with 1 M eluent on a 10 cm. column. This was unsuccessful. 2 M, 3 M, and 4 M eluent was successful on single runs (figure 10). It was during this time in the

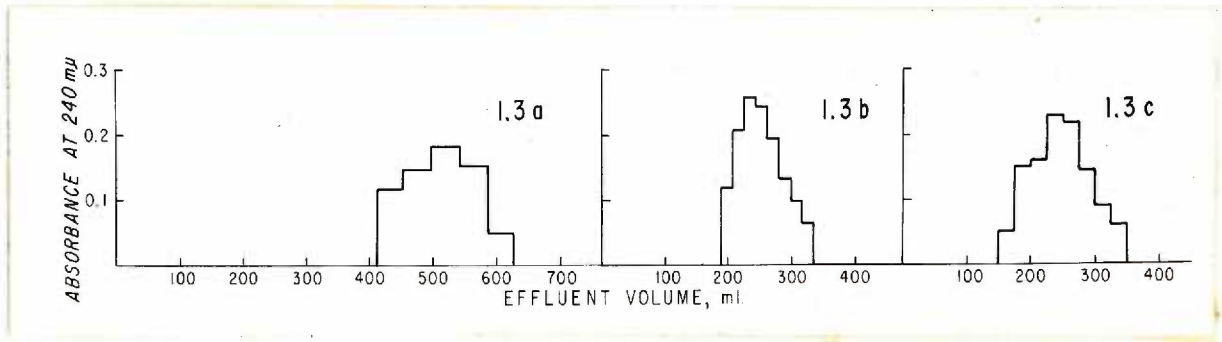


Figure 10. - Pentobarbital, 10 cm. column with 2 M eluent in 1.3-a, 3 M eluent in 1.3-b and 4 M eluent in 1.3-c.

project when we obtained Dowex-1 in two other degrees of cross-linkage, 4 per cent and 10 per cent, (8 per cent Dowex-1 had been used to this point). Accordingly, pentobarbital was used to evaluate any variations due to cross-linkage. Until this time, all molarity values of eluent had been increased stepwise by one unit but, by mistake, 2.5 M eluent was prepared and used. Since this molarity demonstrated the effects of cross-linkage, it was not repeated. Only a single run was made using each of the three degrees of cross-linkage, (figure 11a).

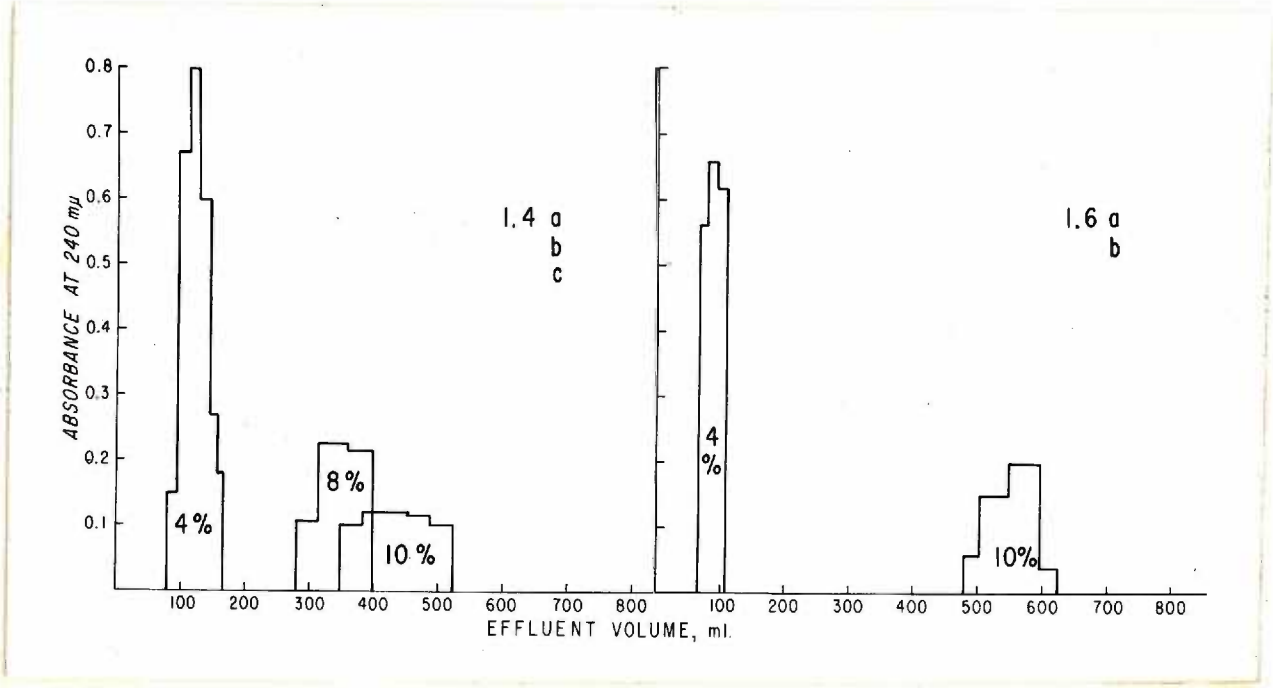


Figure 11a. - Pentobarbital, 10 cm. column with 2.5 M eluent using three different degrees of resin cross-linkage as noted.

11b. - Secobarbital, 10 cm. column with 2.5 M eluent using two different degrees of resin cross-linkage as noted.

4. Secobarbital - This drug was run four times on a 10 cm. column using 1 M, 2 M, and 3 M eluent. The last three eluents removed the drug within the 1000 cc. volume (figure 12).

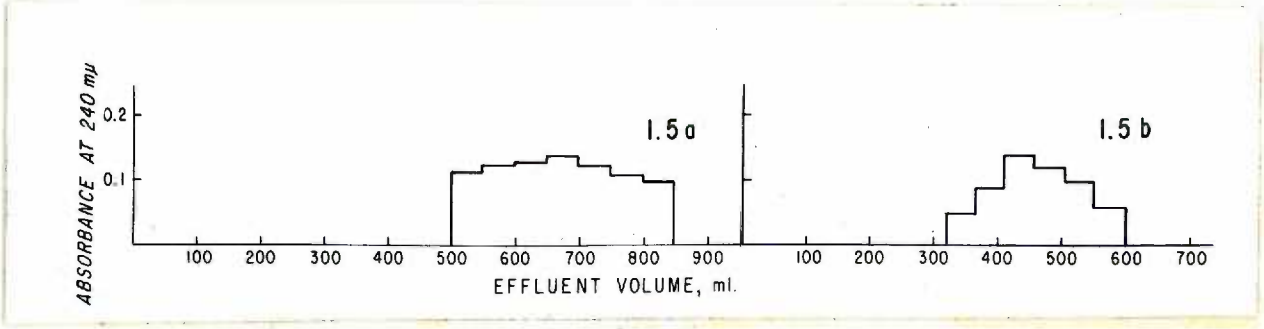


Figure 12. - Secobarbital, 10 cm. column with 2 M eluent in 1.5-a and 3 M eluent in 1.5-b.

Also, secobarbital was used with 4 per cent and 10 per cent cross-linkage with 2.5 M eluent in the same manner as was pentobarbital (see figure 11-b on previous page).

5. Amobarbital - This was the last barbiturate tried individually. Only one run on a 10 cm. column with 3 M eluent was made (figure 13).

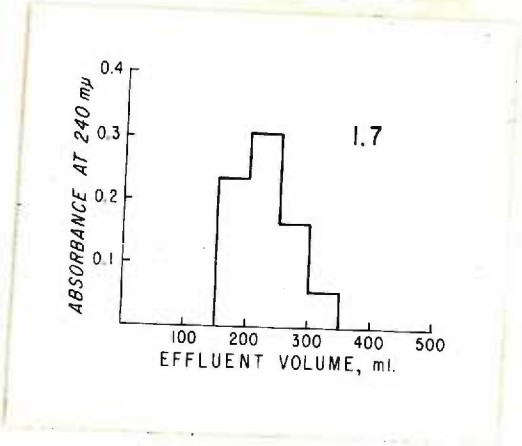


Figure 13. - Amobarbital, 10 cm. column with 3 M eluent.

C. Separation of Barbiturate Combinations - At this point in the investigation, the data were analyzed to see if one column length and one concentration of eluent was capable of removing all of the drugs at different effluent volumes. Since barbituric acid can be easily identified by its ultraviolet absorption spectrum, which is different from the substituted barbiturates, it was not considered. A 3 M or 4 M eluent will remove all of the drugs from a 10 cm. column within 1000 cc. except phenobarbital which requires 6 M.

1. Amobarbital-Secobarbital - Rather than attempt to separate all five drugs initially on the same column only two drugs, amobarbital and secobarbital, were selected for analysis. While these two barbiturates were eluted at distinctly different column lengths, nevertheless, they were relatively close together in relationship to the other drugs. The initial run employed a 10 cm. column and 4 M eluent. The drugs were separated completely but each drug occupied a large fraction of the effluent. In an attempt to reduce the size of the drug fractions, a 20 cm. column was run under the same conditions. This improved the results somewhat (figure 14).

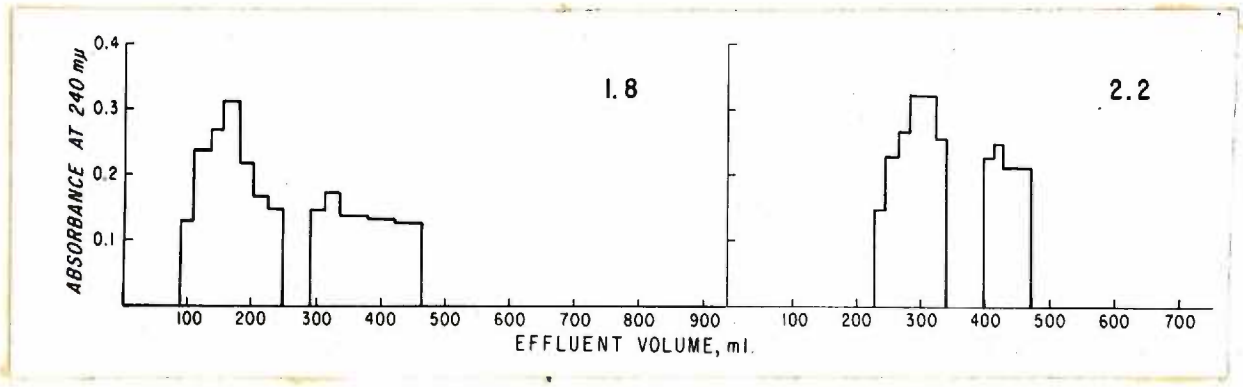


Figure 14. - Amobarbital-Secobarbital, 10 cm. column with 4 M eluent in 1.8 and a 20 cm. column with 4 M eluent in 2.2.

2. Pentobarbital-Secobarbital - These two barbiturates were run under the same conditions and similar results were obtained with only a 20 cm. column being used. The eluent was 4 M (figure 15).

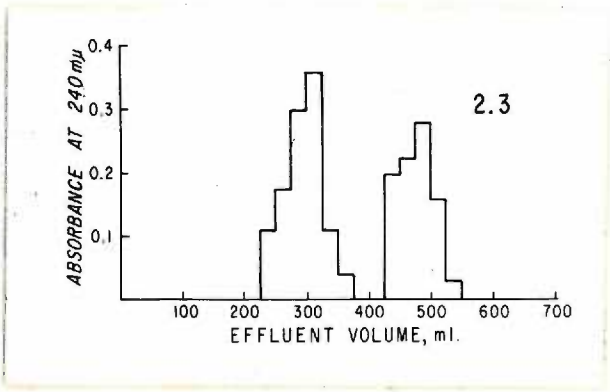


Figure 15. - Pentobarbital-Secobarbital, 20 cm. column with 4 M eluent.

3. Pentobarbital-Amobarbital - When these drugs were analyzed individually, their elution curves were similar. Separation was attempted using 10 and 20 cm. columns with 3 M and 4 M eluent. No separation occurred (figure 16).

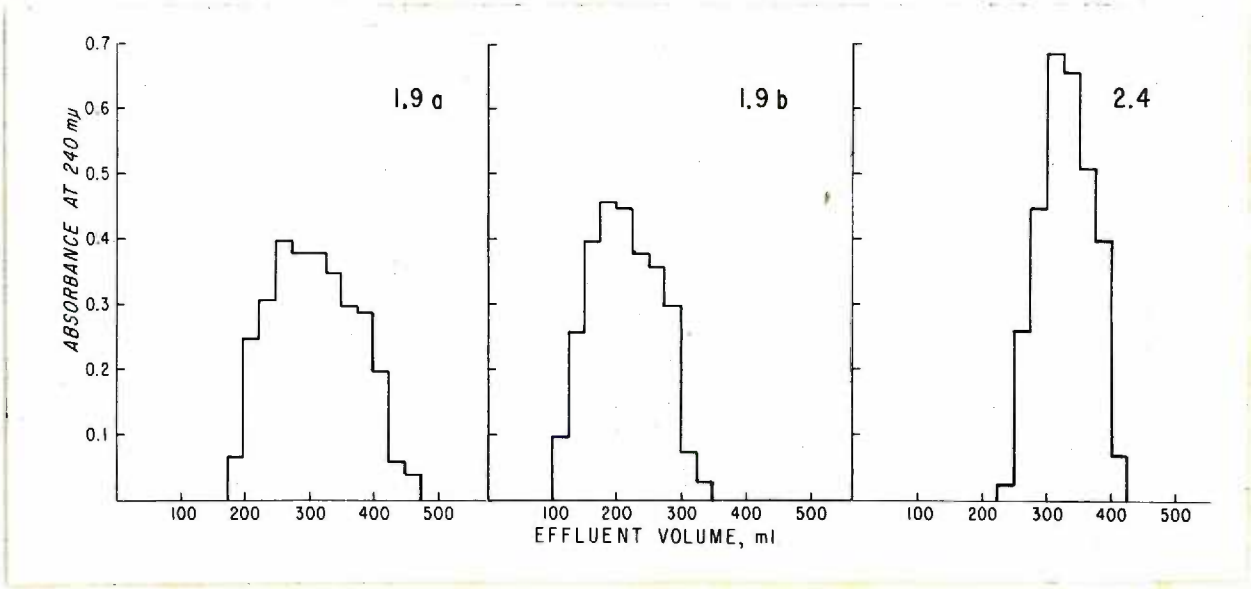


Figure 16. - Pentobarbital-Amobarbital, 10 cm. column with 3 M eluent in 1.9-a, 10 cm. column with 4 M eluent in 1.9-b and 20 cm. column with 3 M eluent in 2.4.

4. Barbital-Phenobarbital - Barbital and phenobarbital were separated on a 10 cm. column by eluting initially with 2 M eluent and then increasing to 6 M eluent after the first fraction (which is presumed to be barbital on basis of data from runs with these drugs used individually) was completely removed. Surely the barbital would have been removed using 6 M alone but it would have come off so rapidly as to appear totally in the first fraction (figure 17).

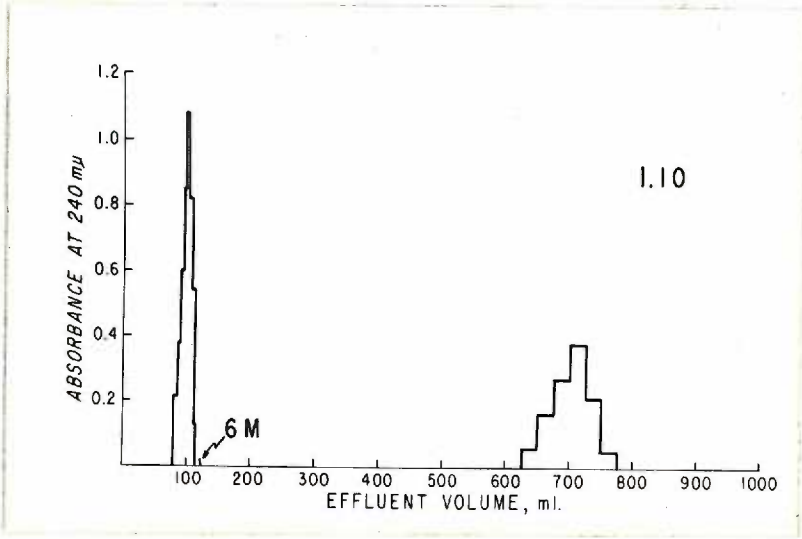


Figure 17. - Barbital-Phenobarbital, 10 cm. column with 2 M and 6 M eluent.

5. Barbital-Pentobarbital - Barbital was also run with pentobarbital individually using 10 cm. columns with 2 M and 3 M eluent (figure 18).

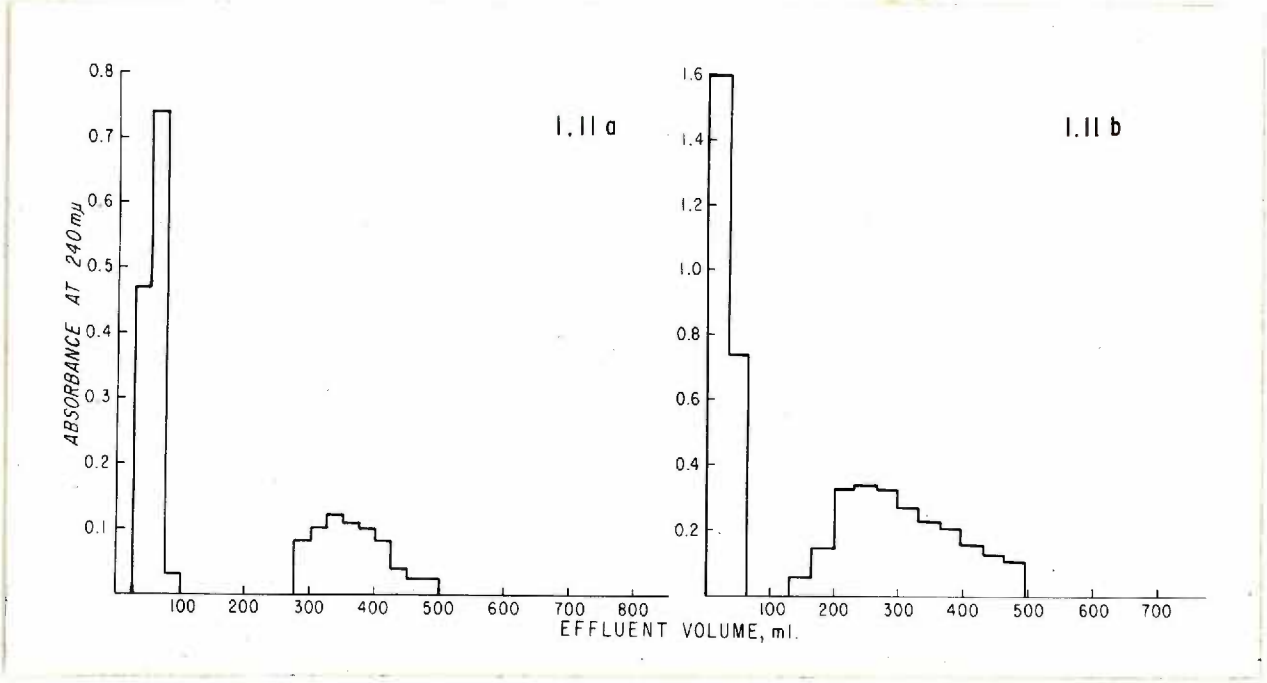


Figure 18. - Barbital-Pentobarbital, 10 cm. column with 2 M eluent in 1.11-a and 3 M eluent in 1.11-b.

6. Barbital-Phenobarbital-Pentobarbital - A combination of these three drugs was selected for separation. This choice of a combination of barbiturates was made since barbital is easy to remove and phenobarbital difficult to remove while the elution of pentobarbital is somewhere in between. A 10 cm. column was used. A 3 M eluent was used initially rather than 2 M since this concentration will remove pentobarbital more readily. Either molar concentration will remove barbital easily. After the first two fractions were removed, the concentration of eluent was increased to 6 M (figure 19).

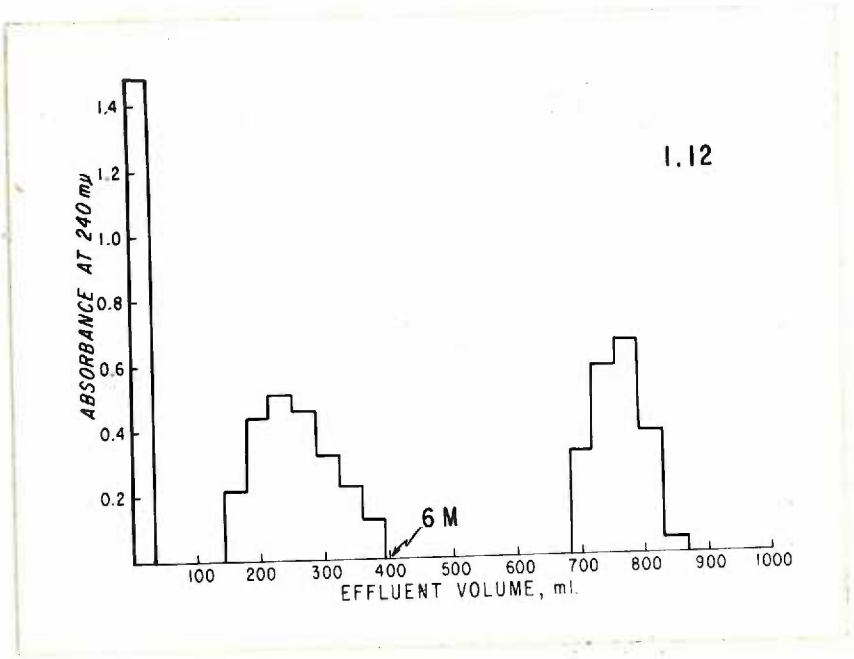


Figure 19. - Barbital-Phenobarbital-Pentobarbital, 10 cm. column with 3 M and 6 M eluent.

7. Barbital-Pentobarbital-Secobarbital-Phenobarbital - As a final analysis of this phase of the project 0.5 mg. each of barbital, pentobarbital, secobarbital and phenobarbital were run on a 10 cm. column. Amobarbital was excluded since previous runs showed that it could not be separated from pentobarbital. The column was eluted with 3 M eluent. Only two drug fractions were obtained in the effluent within 500 cc. However, the second fraction was considerably larger than expected suggesting that both pentobarbital and secobarbital had been eluted as one fraction. After the concentration of eluent was increased to 6 M only one more fraction was obtained. This third fraction was phenobarbital. Repeated trials failed to yield more than three fractions. Increasing the eluent concentration to 4 M and column length to 20 cm. was without benefit (figure 20).

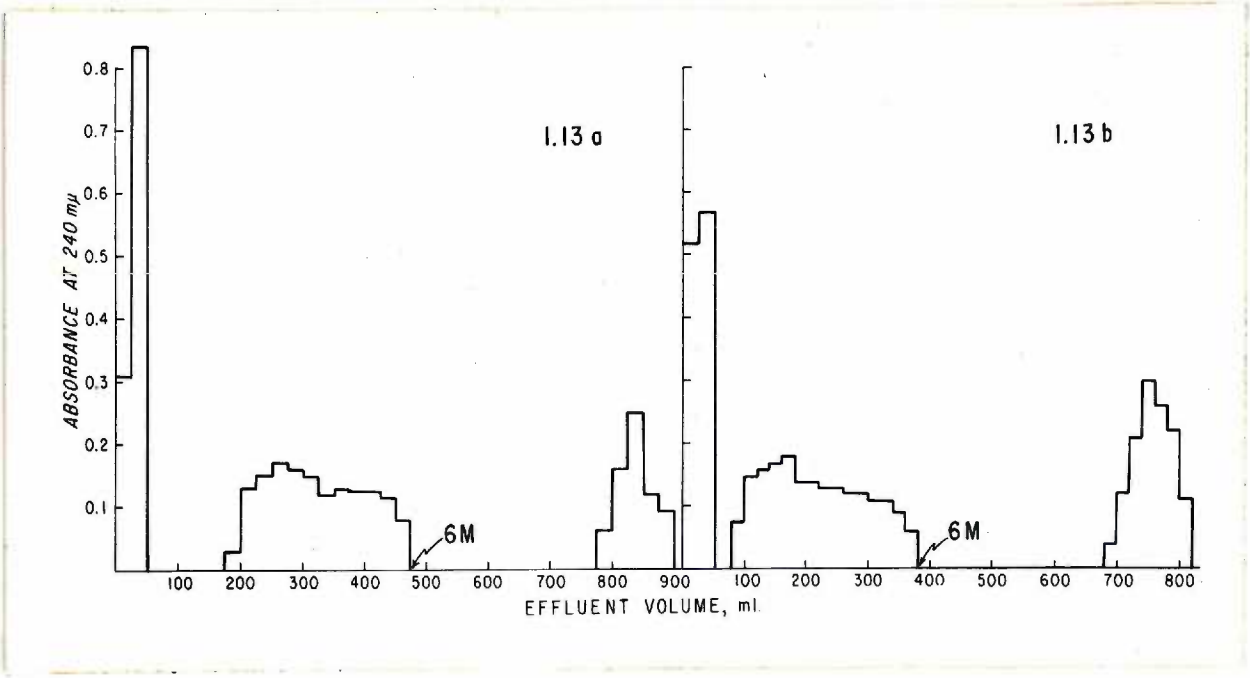


Figure 20. - Barbital-Pentobarbital-Secobarbital-Phenobarbital, 10 cm. column with 3 M and 6 M eluent in 1.13-a and 4 M and 6 M eluent in 1.13-b.

Since the preliminary work with this method of ion-exchange yields only fair results, emphasis was shifted to the second basic concept being studied in this project. It is realized at this point in this study that only a few of the variables have been explored to any extent in this first method, and that more extensive manipulations of these variables might provide satisfactory data.

II. Method B Ion-Exchange System.

The second method used to separate and identify the barbiturates was adapted from Moore and Stein's procedure (26). The approach to this method was similar to that of Method A, namely, to analyze each of the 5 barbiturates individually and then in various combination. The columns were prepared and buffered in the manner described previously using 50 cm. lengths. This longer length was chosen at the suggestion of Dr. David Jackson, Assistant Professor of Biochemistry in this school, who has had considerable experience with Dowex-50, as used in ion-exchange studies.

The columns were initially buffered with 0.1 N phosphate buffer at pH 7.40 which is slightly under the lowest pK of the drugs being used. (see table). One milligram of each drug was run individually. The columns

Table of pK. Values For Several Barbiturates

Phenobarbital	7.41
Barbital	7.91
Amobarbital	7.94
Secobarbital	8.08
Pentobarbital	8.11

were eluted with the initial buffering solution and 10 cc. fractions were collected. Since the effluent pH was also 7.40, 1 cc. of 4 M NH_4OH-NH_4Cl buffer at pH 9.9 was added to increase the effluent pH to 9.9 for maximum ultraviolet absorption of the barbiturates. This procedure eluted the barbiturates listed above rather rapidly and the elution curves were quite similar; consequently, the pH of the eluent buffer was decreased to 6.8. It was hoped this would slow the rate of exchange and allow greater differences in point of elution of each of the drugs. The elution curves of both pH's are shown in figure 21.

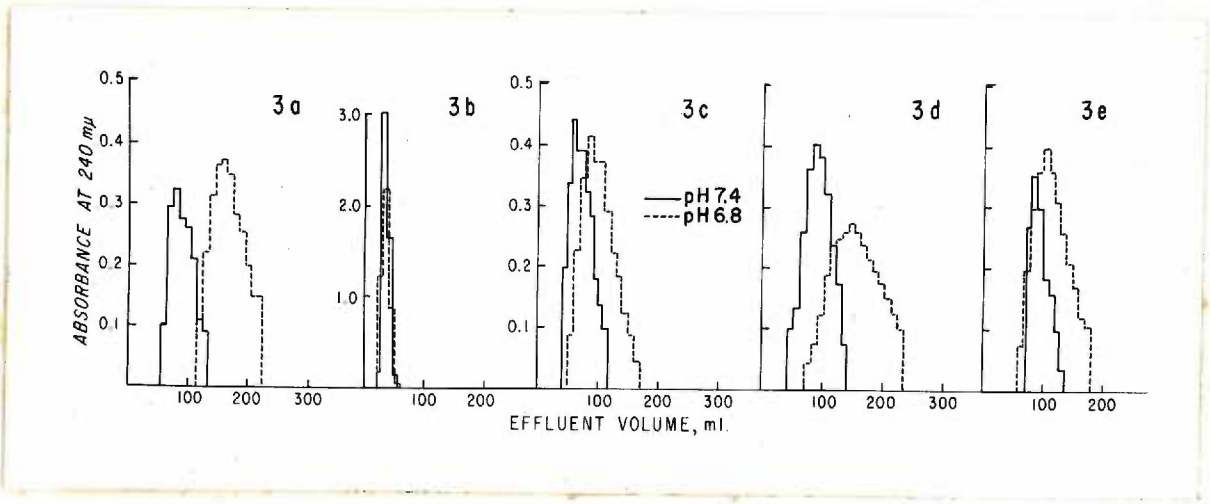


Figure 21. - 50 cm. columns, 3a. Phenobarbital, 3b. Barbital, 3c. Amobarbital, 3d. Secobarbital, 3e. Pentobarbital.

Since decreasing pH did not improve the resolution of the drugs, the column length was increased to 100 cm. keeping all other factors the same. The elution curves were very similar to those obtained with the shorter column except that the time required for elution was increased about two-fold.

Rather than to continue using the drugs singly, 0.5 mg. of barbital and 0.5 mg. pentobarbital were combined. These two barbiturates were selected since they showed the greatest variation in rate of travel when run

individually. Then, these two drugs were tried on 50 and 100 cm. columns using an eluent buffer pH of 6.8, 7.0, 7.2, 7.4, 7.6, and 7.8. In every case only a single fraction was obtained. The same procedure was evaluated using 0.5 mg. barbital and 0.5 mg. phenobarbital, again with no indication of separation. At this point it was felt that further investigation with this resin under these circumstances was not profitable and that possibly cross-linkage variation might prove beneficial. The results of this phase of the study could not be included in the main text of this thesis because of limitations in time.

DISCUSSION

It is apparent from the results described in the preceeding section that neither of the two basic methods of ion-exchange separation used in this investigation even come close to meeting the criterion of an ideal method. In the method using Dowex-1, four of the five drugs used were eluted at different points when examined individually. Only pentobarbital and amobarbital yielded overlaping elution curves. When, however, the drugs were analyzed in combination only three fractions were identified. On the basis of data from the individual experiments it was felt that the first and most easily removed fraction was barbital. The second fraction was large and felt to be a combination of secobarbital and pentobarbital. The third and last fraction was presumed to be phenobarbital since 6 M eluent was required to remove it. By variations in molarity of eluent and column length it was not possible to bring about adequate resolution of the five drugs. None of the drugs were removed in small concentrated fractions; instead, broad and poorly differentiated zones were obtained. As expected, all of the drugs reacted to variations in column length and molarity in that elution was delayed with increased column length and decreased with greater eluent molarity. When a 4 per cent cross-linked resin was substituted for the cross-linked 8 per cent resin smaller and more concentrated fractions were obtained. With the two drugs tested, pentobarbital and secobarbital, elution occurred so rapidly that it was felt that satisfactory resolution could not occur when combinations were used. However, in retrospect it appears that more work should have been done utilizing variations in cross-linkage of resins since the molarity and

column length changes were unprofitable; at least in the ranges studied. Moore and Stein found considerable differences in regard to resolution of closely related compounds by changing cross-linkage only.

Certainly the results indicate that variations in the interaction of the drug with the resin do occur but that not to a sufficient degree to allow separation under the circumstances employed. The only variable which was explored in some detail was molarity of the eluent.

Since only two column lengths were used, further variation in this respect might prove useful. However, no significant differences in resolution could be ascertained on 10 and 20 cm. columns. If further work were to be done with this resin we feel that alterations in pH might be useful since electrochemical attraction certainly must play some part in the drug-resin interaction. Such factors as flow rate, temperature, and cross-linkage variations would have to be examined to make evaluation of the method more complete. In Method B using Dowex-50, the resin which Moore and Stein used for separation of the amino acids, results were also disappointing. When the drugs were analyzed individually each drug had a slightly different elution curve and point of maximum elution, however, the differences were small. As expected, decreasing the pH of the eluent from 7.4 to 6.8 delayed slightly the elution of the drug. This did not provide greater differences in point of elution than did use of an eluent at pH 7.4. When the drugs in combination were eluted from the column, only single fractions were obtained. At no time was there any indication of separation. Serial decreases in eluent pH and increasing column length did not alter the situation. In theory, increased column length should provide more opportunity for the resin to differentiate each drug and

variations in flow rate should be magnified. This, however, could not be demonstrated, suggesting that the variations noted on the shorter columns may not have been the result of the effects of pK. If the differences in point of maximum elution noted are real, then the data indicates that there are slight variations in the rate of flow of the five drugs examined in combination. It is interesting to note that barbital is removed more easily than phenobarbital which has a lower pK confirming the finding of Moore and Stein. Apparently factors other than pK affect the exchange rate and the order of removal from the column cannot be predicted on the basis of pK alone.

Even though results using this method were even less impressive than with Method A, it is felt that Method B offers a better approach from a theoretical standpoint and that continued manipulation of the variables already used and those not explored might provide suitable results. Unfortunately, time limited continued work on this phase of the study. It would have been interesting to further explore the use of resins of other than 8 per cent cross-linking and eluent solutions of other than 0.1N strength. It might also be advisable to use a basically similar resin of different commercial source. The problem becomes more distressing when one remembers that even though we have picked five commonly used barbiturates there are at least ten to fifteen more which are manufactured in this country with pK's which fall within the range of the five used.

This work has been based on the theoretical consideration discussed in a previous section. It is realized, however, that other factors such as differential solubility, non-ionic interaction, and molecular resonance may play an important role in the resolution of the barbiturates by ion-exchange. These, however, are not within the realm of the authors discussion.

SUMMARY

The basic aim of this project was to develop a method for barbiturate separation which had a high degree of specificity and yet retained sensitivity. The need for such a method is based on the continued frequent use of the barbiturates in clinical medicine and the increasing use of barbiturates as a means of poisoning and suicide. At present there is no method or combination of methods which provides adequate separation and identification of the individual 5-5 substituted barbiturates.

A review of the literature failed to reveal the use of ion-exchange chromatography for such a procedure. In an attempt to employ this form of chromatography for barbiturate separation two theoretically different methods of ion-exchange chromatography were set up. One method utilizing Dowex-1 (an ion-exchange resin) was adapted from a single report by Hayaishi and Kornberg who separated barbituric acid and 1-methyl barbituric acid. The second method was adapted from the theory of Moore and Stein which they used for the separation of the amino acids. Neither of the two methods were successfully developed in the present study. However, only several of the variables known to effect ion-exchange chromatography were manipulated with each method. This work can be considered only as a very incomplete evaluation of ion-exchange chromatography as a method for barbiturate separation. Certainly no positive conclusions can be drawn from the results, however, the negative data may be useful in future exploration into this problem.

BIBLIOGRAPHY

1. Algeri, E.J. & McBay, A.J. Paper chromatography of urinary metabolites of pentobarbital. Am. J. Clin. Path., 1953, 23(7): 654-660.

2. Algeri, E.J. & Walker, J.T. Paper chromatography for identification of the common barbiturates. Am. J. Clin. Path., 1952, 22: 37-40.

3. Allgen, L.G. Separation and identification of barbiturates in biological material by paper chromatography. Scan. J. Clin. Lab. Invest., 1955, 7(Supp. 20): 124-126.

4. Bradford, L.W. & Brackett, J.W. Ultraviolet absorption spectrophotometry in the solution of criminalistic problems pertaining to the identification of pills, capsules, narcotics, drugs, and poisons. Personal communication.

5. Canback, T. Infra-red spectra of barbiturates. Pharm. Wbl., Amst., 1955, 90(4): 116-118.

6. Cassidy, H.G. Fundamentals of chromatography. New York: Interscience Publishers, Inc., 1957.

7. Cohen, E.L. The determination of barbiturates in toxicological and clinical investigation. Am. J. Pharm., 1946, 118: 40-62.

8. Dybing, G. Estimation of barbiturates in clinical chemistry. Scan. J. Clin. Lab. Invest., 1955, 7(Supp. 20).

9. Eastoe, J.E. The chemical composition of gelatin-II. The amino acid composition of gelatin, collagen and reticulin. Research Report Series A 13. The British Gelatin and Glue Research Association, London, 1954.

10. Goldbaum, L.R. Ultraviolet procedure for determination of barbiturates. J. Pharm. & Exp. Ther., 1948, 94: 68-75.

11. Goldstein, S.W. Barbiturates: A blessing and a menace. J. Am. Pharm. Asso., 1947, 36: 5-14.

12. Goodman, L.S., & Gilman, A. The pharmacologic basis of therapeutics. New York: Macmillan Company, 1956.

13. Gradwohl, R.B.H. Legal medicine. St. Louis: The C.V. Mosby Company, 1954.

14. Hambourger, W.E. Promiscuous use of barbiturates; analysis of hospital data. J. Am. Med. Asso., 1940, 114: 2015-2019.

15. Hayaishi, O., & Kornberg, A. Enzymatic formation of barbituric acid from uracil and of 5-methyl barbituric acid from thymine. J. Am. Chem. Soc., 1951, 73: 2975-76.

16. Huang, T.Y., & Jerslev, B. On the use of X-ray powder patterns for identification of barbiturates in forensic chemistry. Acta. Pharm. Tox., Kbh., 1951, 7(3): 227-230.

17. Iverson, M. On the serviceability of Koppanyi's method for quantitive determination of barbituric acid derivatives in urine. Acta. Pharmacol., 1945, 1: 255-262.

18. Koppanyi, T., & Murphy, W.S., & Krop, S. Colorimetric detection of barbital and its applications. Proc. Soc. Exp. Bio. Med., 1933, 30: 542-544.

19. Krahl, M.E. Effect of variation in ionic strength and temperature on apparent dissociation constants of thirty substituted barbituric acids. J. Phy. Chem. 1940, 44: 449-463.

20. Krause, R.F., & Riley, R.F. The reliability of the cobalt-isopropylamine color reaction for Amytal. J. Pharm. and Exp. Ther., 1941, 71: 287-292.

21. Lange, N.A. Handbook of Chemistry. Sandusky, Ohio: Handbook Publishers, Inc., 1952.

22. Lous, Per. Quantitative determination of barbiturates. Acta. Pharmacol., 1950, 6: 227-234.

23. Lowther, C.P. Barbiturate poisoning. Scot. Med. J., April, 1959, 4(4): 163-76.

24. Marshall, R. Rapid method for detection of barbiturates in urine and stomach contents. Brit. Med. J., 1953, 4832: 379-380.

25. McBay, A.J., & Algeri, E.J. Application of densitometry to paper chromatography of the barbiturates. Am. J. Clin. Path., 1954, 24(10): 1139-1143.

26. Moore, S., & Stein, W.H. J. Biol. Chem., 1951, 192, 663-681.

27. Pinker, R.H. X-ray diffraction used in identification of drugs. Ann. West. M. & S., 1952, 6(9): 595-599.

28. Porter, G.A. A correlative study of pentobarbital distribution in dogs utilizing selected tissues and body fluids. Unpublished Master's Thesis. Univ. of Oregon Med. Sch., 1957.

29. Price, W.C., Bradley, J.E.S., Frazer, R.D.B., & Quilliam, J.P. The relationship between the infra-red absorption spectra of some 5-5 substituted barbituric acids and their pharmacological activity. J. of Pharmacy and Pharm., 1954, 6: 522-528.

30. Raventos, J. The distribution in the body and metabolic fate of barbiturates. J. Pharm. & Pharmacol., 1954, 6: 217-235.

31. Riley, R.F., Krause, R.F., Stedman, L.T., Hunter, F.E., & Hodge, H.C. Color reaction of barbiturates. Proc. Soc. Exp. Biol. Med., 1940, 45: 424-427.

32. Ryan, J.C., Tanowski, L.K., & Pifer, C.W. Analysis of barbiturates: comparison of aqueous and non-aqueous methods. J. Am. Pharm. Ass., 1954, (sc. ed.), 43(11): 656-659.

33. Samuelson, Olof. Ion exchange in analytical chemistry. New York: John Wiley & Sons, 1953.

34. Selwyn, J.G., & Dark, F.A. The identification of barbiturate drugs in gastric contents and urine. J. Clin. Path., 1950, 3(2): 152-156.

35. Vespe, V., & Fritz, J.S. Titration of barbiturates and sulfa drugs on non-aqueous solution. J. Am. Pharm. Ass., 1952, (sc. ed.), 41(4): 197-199.

36. Walker, J.T., Fischer, R.S., & McHugh, J.J. Quantitative estimation of barbiturates in blood by ultraviolet spectrophotometry. Am. J. of Clin. Path., 1948, 18: 451-61.

37. Webster, R.W. Legal Medicine and Toxicology. Philadelphia: W.B. Saunders Co., 1930.

38. Wickstrom, A., & Salvesen, B. The separation and identification of some barbiturates by paper partition chromatography. J. Pharm. & Pharmacol., 1952, Lond., 4(2): 98-102.

39. Williams, L.A., & Zak, B. Determination of barbiturates by automatic differential spectrophotometry. Clin. Chem. Acta., Amst., March, 1959, 4(2), 170-4.

40. Wright, J.T. A rapid method for identifying particular barbiturate derivatives in small samples of blood by paper chromatography. J. Clin. Path., 1954, Lond., 7(1): 61-65.