The relationship of chlorophyll content and carbon dioxide uptake was examined. Total chlorophyll and chlorophyll a were determined for both the aqueous and deuterated algae. As shown in Table I, the ratio of chlorophyll content for the deuterated to that of the aqueous algae approximates the carbon dioxide uptake relationships shown by the uptake curves. Greater differences are to be noted between the *Chlorella* cultures than between the *Scenedesmus* organisms, which is consistent with the results from the uptake studies.

The procedure described in this report for studying carbon dioxide uptake is a simple one and reproducible results within 5% were obtained when duplicate runs were conducted. Further work is currently in progress in this laboratory to extend these studies to include the effect of variation in light intensity and temperature on the photosynthesis of deuterated algae.

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Determination of Barbiturates in Urine Containing Salicylate

By DONALD M. STOKES, WALTER J. R. CAMP, and ERNST R. KIRCH

A procedure is described in which urine pigments, salicylate, and other chromogenic materials which preclude spectrophotometric evaluation of barbiturate by virtue of their strong absorption in the ultraviolet region are removed from a chloroform solution by selective adsorption.

The extraction from urine specimens of salicylate and other substances together with barbiturates has been a problem frequently encountered during toxicologic analysis. At the present time, the quantitative determination of barbiturates in biological material is best accomplished by means of ultraviolet spectrophotometry (1, 2). Analytical procedures commonly employed involve preliminary extraction of the malonylurea derivative from the specimen. by an immiscible solvent such as ether or chloroform. The spectral characteristics of the malonylurea molecule are most definitive in alkaline solution, which may be obtained by re-extraction of the barbiturate from an immiscible solvent into aqueous alkali. Normal physiological constituents which absorb strongly in the ultraviolet region and other drugs including salicylates are often extracted along with the barbiturate. The presence of these nonbarbiturate chromogens in the final alkaline extract precludes definitive spectrophotometric evaluation of the malonylurea compound unless steps are taken to remove the interference.

The attempts by various investigators to eliminate interfering substances by use of lengthy, complicated extraction procedures which resulted in loss of sensitivity, low recoveries, and failure to eliminate nonbarbiturate chromogens have been criticized (3). Differential spectrophotometric techniques (3–5) in which determination of barbiturate is based on the differences in the ultraviolet absorption spectra of strongly alkaline and pH 10–10.5 buffered solutions represent a significant improvement over older methods employing conventional spectrophotometry. It has been reported (3) that ultraviolet-absorbing substances including the drugs

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dicoumarol, diphenylhydantoin, and sulfadiazine, which appear in the final alkaline extract, do not interfere with barbiturate determination by differential spectrophotometry because they have the same absorption in strong alkali and in pH 10.5 solution. But salicylate, like barbiturate, exhibits spectral differences (6) dependent on pH and must be removed from a sample before quantitative analysis of the malonylurea derivative can be made.

Although it has been claimed (3, 4, 6) that separation of barbiturate from salicylate can be accomplished by extraction from phosphate-buffered aqueous solution at pH 7.4, the present authors have been unable to secure complete separation of the two compounds by this method. Furthermore, extraction with ether or chloroform of many urine specimens at various pH levels ranging from less than 1 to 7.4 has been found to result in subsequent alkaline extracts which were often highly colored and contained such large amounts of nonspecific chromogenic material that differential spectrophotometric evaluation was not possible.

In this investigation, a reasonably short, simple procedure was employed in which salicylate as well as urinary pigments and chromogens were removed from a chloroform solution by means of a selective adsorption column and barbiturates were eluted quantitatively from the column in sufficient purity to permit determination by automatic differential ultraviolet spectrophotometry.

Spectrophotometric Determination of Barbiturates.—The ultraviolet absorption spectrum of 5,5-disubstituted malonylurea derivatives in strong alkali such as 0.45 N sodium hydroxide exhibits a maximum at 252–255 mμ and a minimum at 234–237 mμ. In a buffer solution at pH 10–10.5 there occurs a higher maximum at 238–240 mμ with no minimum (3, 4). The difference between the absorbance in strong alkali and that in the buffered solution is greatest at 260 mμ, decreasing through zero at 247–250 mμ to a maximum negative at 235–240 mμ, and increasing through zero at 227–230 mμ to a positive difference at lower wavelengths. The absorbance difference at 260 mμ has been employed to measure the concentration of barbiturate solutions by differential spectrophotometry (3, 4).

Recently, the differential spectrophotometric technique has been adapted to utilize the automatic ratio-recording features of the Beckman model DK-2 spectrophotometer by Williams and coworkers, who have published procedures for determination of both barbiturates (5) and salicylates (6). The method is based on the fact that in alkaline solution salicylate, like barbiturate, can exist in two different ionic states which give rise to two different ultraviolet spectra, depending on the pH of the solution. The instrument automatically substracts one spectrum from the other and draws a composite curve.

The composite curves shown in Fig. 1 are representative of the differential absorption spectra of 5,5-disubstituted malonylurea derivatives and were obtained using various standard concentrations of secobarbital. In each case the 0.45 N sodium hydroxide solution was scanned against the pH 10–10.5 solution as reference, using the differential absorbance range (-0.3 to $+0.7$) of the Beckman model DK-2 spectrophotometer as described by Williams and Zak (5). The maximum and minimum absorption peaks, occurring at 260 and 238 mμ, respectively, represent the corresponding absorbance differences between strongly alkaline and pH 10–10.5 spectra.

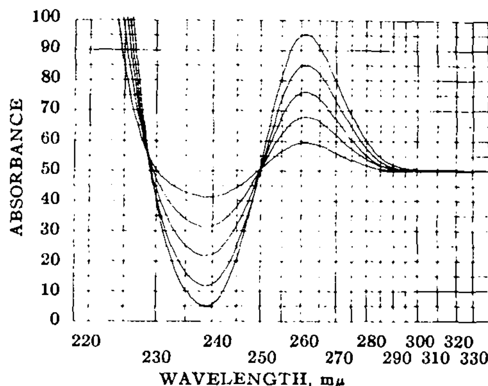

Fig. 1.—Differential absorption spectra of standard solutions of secobarbital in concentrations of 4.994, 9.988, 14.98, 19.98, and 24.97 mcg. per ml.

Salicylate in 0.45 N sodium hydroxide, when read against 0.45 N sodium hydroxide as a reference blank, exhibits a maximum at 300 and a minimum at 267 mμ. In a buffered solution at pH 10–10.5 there occurs a maximum at 295 and a minimum at 257 mμ. This spectral shift with change in pH gives rise to the differential spectrum. The composite spectrum of salicylate exhibits maxima at 318 and 246 mμ and minima at 283 and 233 mμ (6).

When both barbiturate and salicylate are present in the solutions scanned in the spectrophotometer the differential spectrum exhibits the 318 mμ peak characteristic of salicylate, but the shorter wavelength peaks for barbiturate and salicylate are replaced by a single absorption maximum occurring at about 253 mμ (6). The effect of the presence of salicylate on the differential absorption spectrum of barbiturate is demonstrated in Fig. 2. The composite curve illustrated here was obtained using a solution of salicylate and pentobarbital, each being present in a concentration of 20 mcg. per ml. The composite barbiturate-salicylate spectrum is inadequate for definitive determination of the malonylurea derivative, although quite accurate estimation of the salicylate concentration is possible using the 318 mμ peak (6). Thus it is imperative that salicylate be removed before definitive determination of the barbiturate content of a sample can be made.

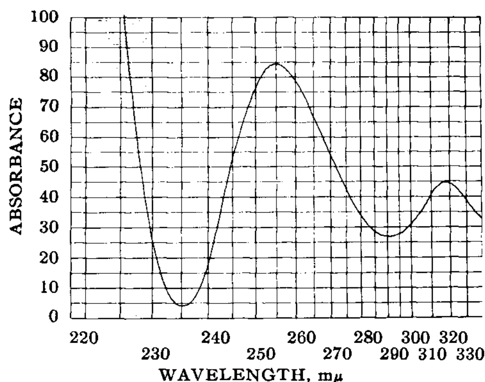

Fig. 2.—Differential absorption spectrum obtained from a solution containing both salicylate and pentobarbital in concentrations of 20 mcg. per ml.

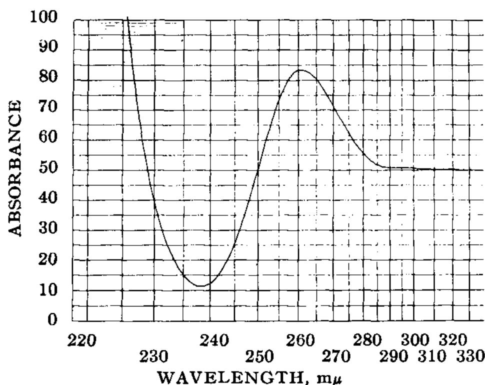

Fig. 3.—Differential absorption spectrum of secobarbital recovered from a Florisil column. A mixture of 10.0 ml. of standard secobarbital solution, 19.98 mcg. per ml., and 10 ml. of urine containing 20 mg. of salicylate was extracted.

A typical differential spectrum obtained on barbiturate recovered from a Florisil[1] adsorption column by the procedure described in this investigation is illustrated in Fig. 3. An extract of a mixture of 10.0 ml. of standard secobarbital solution, 19.98 mcg. per ml., and 10 ml. of urine containing 20 mg. of salicylic acid was purified by passage through the column followed by elution of the barbiturate. The quantitative recovery of secobarbital as well as the effectiveness of the method in removing salicylate and urinary chromogenic material is indicated by comparison of Fig. 3 with the curve obtained on the corresponding standard secobarbital solution shown in Fig. 1.

EXPERIMENTAL

Reagents.—Florisil, 60/100 mesh; diethyl ether, anhydrous reagent; chloroform, reagent grade, redistilled; methanol, anhydrous reagent; hydrochloric acid (A.R.) solution, approximately 6 *N*; sodium hydroxide (A.R.) solution, 0.45 *N*; ammonium chloride (A.R.) solution, 16%.

[1] Florisil is a granular magnesia-silica gel adsorbent supplied by the Floridin Co., Tallahassee, Fla.

Standardization of Barbiturate Solutions.—Standard solutions of the representative 5,5-disubstituted malonylurea derivatives amobarbital, pentobarbital, phenobarbital were prepared as follows: a pure sample of each barbiturate was dried for at least 1 week under vacuum over phosphorus pentoxide. A portion close to 0.25 Gm. was weighed accurately and dissolved in 0.45 *N* sodium hydroxide to make 500.0 ml. of stock solution. Standard solutions ranging in barbiturate concentration from 5 to 30 mcg. per ml. in 0.45 *N* sodium hydroxide were immediately prepared from the stock solution and subjected to differential spectrophotometry. To 3.0 ml. of the standard solution, 0.5 ml. of 0.45 *N* sodium hydroxide was added. A second 3.0-ml. portion of the standard was mixed with 0.5 ml. of 16% ammonium chloride to obtain a buffered solution of pH 10–10.5. The strongly alkaline solution was scanned as sample against the buffered solution as reference, using the differential absorbance range of the Beckman model DK-2 spectrophotometer as described by Williams and Zak (5).

The differential absorbance spectra of various standard concentrations of secobarbital are shown in Fig. 1. Similar curves were obtained on standard solutions of amobarbital and pentobarbital. Although the value of the differential absorbance maximum at 260 mμ was found to be nearly proportional to the concentration, much better linearity resulted when the differences in absorbance between the maximum at 260 mμ and the minimum at about 238 mμ were plotted as a function of the concentration. Calibration curves (Fig. 4) in which the absorbance differences ($A_{max} - A_{min}$) are graphed against concentration demonstrate adherence to the Beer-Lambert law found for each of the barbiturates tested.

Preparation of Adsorption Column.—A small piece of glass wool was fitted into the bottom of a glass tube having the approximate dimensions 65 cm. in height and 1 cm. in inside diameter. Five grams of Florisil was packed fairly loosely to a height of about 10 cm. in the tube by gentle tapping. Another small piece of glass wool was placed on top

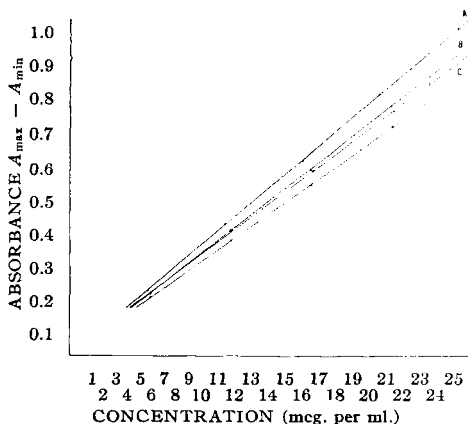

Fig. 4.—Absorbance *vs.* concentration curves for standard solutions of four common barbiturates. *A*—Amobarbital, *B*—phenobarbital, *C*—pentobarbital, *D*—secobarbital.

Table I.—Recovery of Pentobarbital from Pure Solutions by Use of Florisil Columns

Concn., mcg. per ml.	Absorbance: Standard A_{max}	Standard A_{min}	Standard $A_{max} - A_{min}$	Eluate A_{max}	Eluate A_{min}	Eluate $A_{max} - A_{min}$	Recovery, %
5.024	0.609	0.412	0.197	0.609	0.415	0.194	98.5
10.05	0.700	0.305	0.395	0.701	0.310	0.391	99.0
20.10	0.891	0.115	0.776	0.887	0.110	0.777	100.1

of the adsorbent and lightly pressed into place with a glass rod. The column so prepared was ready for use.

Isolation of Barbiturate from Urine.—A 10.0-ml. sample of urine was acidified to pH 1 or below by addition of 3–4 ml. of 6 *N* hydrochloric acid and extracted with ether in three successive portions of about 40 ml. each. The ether phases were carefully separated, combined, and evaporated to dryness on a 70° water bath with the aid of a gentle stream of air. Approximately 10 ml. of redistilled chloroform was added to the dry residue thus obtained and the mixture was warmed on the water bath to facilitate solution of the barbiturate. The Florisil column was treated with 20 ml. of chloroform to initiate solvent flow and wash out any loose particles of adsorbent. The chloroform extract was then placed on the column and followed by two additional 10-ml. portions of chloroform similarly employed to insure complete transfer of barbiturate to the adsorption column. At no time was the liquid level in the column allowed to fall below the upper surface of the adsorbent.

As the developing solvent flowed through the column, a yellow color band was formed within the uppermost part of the adsorbent and a clear, colorless effluent was obtained. The rate of flow varied between 0.5 and 3 ml. per minute, depending on the height of the liquid in the column. When the level of the chloroform was 1 or 2 cm. above the surface of the Florisil, 25 ml. of 10% (by volume) methanol in chloroform was carefully added to the column to elute the barbiturate. The resulting colorless eluate was collected in the receiver containing the chloroform effluent. Little if any diffusion of the urine pigment in the colored band was discernible during elution and salicylate, if present, was retained on the adsorption column.

After the eluant had ceased to flow, the methanol-chloroform solution was evaporated to dryness on a 70° water bath with the aid of a gentle stream of air. The resulting dry residue was subsequently taken up in 10.0 ml. of 0.45 *N* sodium hydroxide and stirred to dissolve the barbiturate completely. The clear, colorless, or nearly colorless solution so obtained was suitable for spectrophotometric determination of quantities of barbiturate less than 0.25 mg. Further dilution to an accurately measured volume was necessary when a larger amount was present in the eluate residue in order to secure a final concentration of 0.025 mg. per ml. or less.

Spectrophotometric determination of the barbiturate concentration of the 0.45 *N* sodium hydroxide solution obtained from the eluate was carried out in exactly the same manner as described for the standardization, using the differential technique described by Williams and Zak (5). The quantity of barbiturate present was then calculated by comparison of the spectrum so obtained with that of a standard solution of the barbiturate.

In order to evaluate the recovery of barbiturate from a Florisil column, the procedure was carried out using 10.0-ml. aliquots of the standard solutions. When the eluate residue was subsequently dissolved in 0.45 *N* sodium hydroxide to make 10.0 ml. of solution, the percentage of barbiturate recovered could then be calculated from the relationship

$$\frac{(A_{max} - A_{min})\text{ eluate}}{(A_{max} - A_{min})\text{ standard}} \times 100 = \text{per cent recovery}$$

The recovery of pentobarbital from pure aqueous solution (Table I) and from urine (Table II) is representative of the results obtained for all of the barbiturates studies. The actual absorbances as read from the differential curves, together with the percentage recovered using 10.0 ml. of the indicated concentrations of standard solutions are shown in Table I. A 10.0-ml. portion of each of the standard solutions was added to 10 ml. of drug-free urine to obtain the results listed in Table II. Duplicate determinations were made as an indication of the reliability of the method.

The separation of barbiturate from salicylate using the Florisil column technique was demonstrated by addition of an aliquot of a solution containing 10 mg. of salicylic acid to 10.0 ml. of standard barbiturate solution before extraction and passage of the chloroform extract through the adsorption column. The results of duplicate analyses carried out on phenobarbital are shown in Table III.

Table IV lists the average recoveries obtained by duplicate determinations on each of the four barbiturates investigated. In each case 10.0 ml. of a standard barbiturate solution and 20 mg. of salicylic acid were added to 10 ml. of drug-free urine prior to extraction and separation on a Florisil column.

Table II.—Recovery of Pentobarbital from Urine by Use of Florisil Columns

Concn. Standard, mcg. per ml.	Added to 10 ml. Urine, mg.	Absorbance, $A_{max} - A_{min}$ Standard	Eluate	Recovered, mg.	Recovery, %
5.024	0.0502	0.197	0.199	0.0508	101.0
5.024	0.0502	0.197	0.196	0.0500	99.5
10.05	0.1005	0.395	0.390	0.0992	98.7
10.05	0.1005	0.395	0.392	0.0997	99.2
20.10	0.2010	0.776	0.767	0.1987	98.8
20.10	0.2010	0.776	0.771	0.1997	99.4

Table III.—Recovery of Phenobarbital from Aqueous Solutions Containing 10 mg. of Salicylic Acid by Use of Florisil Columns

Concn. of Standard, mcg. per ml.	Added, mg.	Absorbance, $A_{max} - A_{min}$ Standard	Eluate	Recovered, mg.	Recovery, %
4.980	0.0498	0.194	0.193	0.0495	99.5
4.980	0.0498	0.194	0.192	0.0493	99.0
9.960	0.0996	0.394	0.397	0.1004	100.8
9.960	0.0996	0.394	0.394	0.0996	100.0
19.92	0.1992	0.793	0.789	0.1982	99.5
19.92	0.1992	0.793	0.799	0.2007	100.8

TABLE IV.—RECOVERY OF BARBITURATES FROM URINE CONTAINING 20 MG. OF SALICYLIC ACID BY USE OF FLORISIL COLUMNS

Barbiturate	Added, mg.	Recovered, mg.	Recovery, %
Amobarbital	0.0480	0.0473	98.5
	0.0960	0.0946	98.6
	0.1920	0.1913	99.7
	4.800	4.798	99.9
Pentobarbital	0.0502	0.0504	100.3
	0.1005	0.0995	99.0
	0.2010	0.1992	99.1
	5.024	5.008	99.7
Phenobarbital	0.0498	0.0501	100.5
	0.0996	0.0977	98.1
	0.1992	0.1977	99.3
	4.980	5.033	101.0
Secobarbital	0.0499	0.0499	100.0
	0.0999	0.1000	100.1
	0.1998	0.1978	99.0
	4.994	4.970	99.5

As a further test of the Florisil separation technique, the column procedure was applied to actual toxicological specimens. In a limited number of such cases in which sufficient urine containing barbiturate was available for experimental purposes, a 10.0-ml. sample of the urine was extracted and subjected to the Florisil column procedure in order to determine the concentration of barbiturate present. To a second 10.0-ml. portion of the specimen an aliquot of a standard barbiturate solution was added and the procedure was again carried out. The results of these analyses are shown in Table V. The barbiturate concentration before and after addition of a known quantity of a specific malonylurea derivative was then calculated from the standard spectrophotometric calibration curve for that particular compound. Thus, when phenobarbital was added, the amount of barbiturate originally present in the specimen was calculated as phenobarbital. In this way the amount of phenobarbital recovered could be determined by subtracting the number of milligrams originally present from the total number of milligrams recovered after addition of a known quantity of phenobarbital.

A ferric chloride test was performed to indicate the presence or absence of salicylate in each specimen. Urine samples B and C were large enough to permit separate determination of salicylate by the method of Williams and co-workers (6). In these samples the salicylate concentration was so high that the absorbance peak at 318 mμ could be used to estimate the salicylate even though barbiturate was also present.

DISCUSSION

A procedure for determination of barbiturates in urine specimens containing salicylate is described which involves the separation of barbiturate from salicylate by selective adsorption on Florisil. A chloroform extract obtained from urine is passed through a Florisil column. Upon subsequent elution with a 10% solution of anhydrous methanol in chloroform, salicylate as well as other chromogenic material normally present in urine extracts remains adsorbed, while barbiturate is recovered quantitatively in the eluate. Spectrophotometric determination of the barbiturate can then be carried out without interference from salicylate and urinary constituents which exhibit absorption in the ultraviolet region.

Selection of Adsorbent.—Of the several adsorbents investigated, only Florisil was found to give consistent and reproducible results. This magnesia-silica gel preparation has been successfully used for the chromatography of a wide variety of compounds which have otherwise proven difficult to separate. The 60/100 mesh grade may be easily and uniformly packed in a column which permits the solvent to flow at a fairly rapid rate. The material activated at 1200° F. or 500° F. was found to give equally satisfactory results. The complete retention of at least 20 mg. of salicylic acid on the adsorption column (Table IV), together with the purity of the barbiturate eluate obtained attest to the efficiency of Florisil as used in the procedure described in this study.

Selection of Solvents.—In order to facilitate extraction of barbiturate from urine samples acidified with hydrochloric acid, ether rather than chloroform was used. Troublesome emulsions usually encountered with chloroform were thus avoided, and the ether as well as the small amounts of dissolved water and hydrochloric acid present in the extract could be readily evaporated on a 70° water bath. Application of an ether extract containing dissolved water and hydrochloric acid to a Florisil column resulted in incomplete retention of salicylate and urinary chromogenic material but barbiturate was apparently not adsorbed and could be at least partially removed from the column by washing with ether. The removal of some water-soluble material from the Florisil which produced a yellow color in 0.45 *N* sodium hydroxide was also found to be due to the presence of water and hydrochloric acid. When an ether extract was evaporated to dryness to remove all traces of water and hydrochloric acid and a dry ether solution of the residue was applied to a Florisil column, barbiturate, salicylate, urinary pigments, and chromogens were completely adsorbed.

A number of different solvents were investigated in order to determine optimum conditions for retention of salicylate and urinary chromogens during elution of barbiturate from a Florisil column. A close parallel was found between the action of these solvents and their polarity. Although barbiturate was completely adsorbed from ether solution, the somewhat more polar solvents, chloroform and ethyl acetate, permitted small amounts of barbiturate to pass through the column when 50 ml. of either solvent were applied, while pigments and salicylate were retained. On application of acetone, urinary chromogenic material was removed. Increasing the polarity still further by use of ethanol or methanol resulted in partial elution of salicylate, which was accompanied by removal of most of the urinary pigments as well. It therefore became necessary to select a suitable solvent for elution of barbiturate which would not elute salicylate or urinary chromogenic material under practical analytical conditions. A 10% solution of anhydrous methanol in redistilled chloroform proved effective for this purpose. The addition of methanol to chloroform in this proportion produced a solvent of

TABLE V.—RECOVERY OF BARBITURATES ADDED TO TOXICOLOGICAL SPECIMENS OF URINE CONTAINING BARBITURATE BY USE OF FLORISIL COLUMNS

Urine Specimen →	A	B	C	D
Salicylate present, mg. %	0	9.2	10.8	[a]
Barbiturate present[b] in 10.0 ml. of urine, mg.	0.1141	0.1908	0.7071	0.1330
Barbiturate added to 10.0 ml. of urine, mg.	Phenobarbital 0.09960	Phenobarbital 0.1992	Phenobarbital 0.1992	Secobarbital 0.0999
Total barbiturate[b] recovered, mg.	0.2176	0.3927	0.9108	0.2354
Recovery of added barbiturate, %	103.9	101.3	102.3	102.5

[a] The presence of salicylate in urine specimen D was assumed on the basis of a strongly positive reaction to ferric chloride.
[b] In each case the amount of barbiturate was calculated in terms of the particular compound added as a recovery standard.

sufficient polarity to elute as much as 5 mg. of barbiturate (corresponding to a urinary concentration of 50 mg. % based on a 10-ml. sample) using only 25 ml. of eluant (Table IV).

The capacity of the adsorption column for retention of salicylate under the condition used for elution of barbiturate was determined by extracting aliquots of standard salicylic acid solutions and subjecting the extracts to the Florisil column procedure. Although the quantity of salicylate added to urine samples was limited to 20 mg. for recovery experiments on barbiturates (Table IV), it was found that as much as 25.0 mg. of salicylic acid could be retained successfully on the column provided that no more than 25.0 ml. of 10% methanol in chloroform was added in the elution step.

While urinary pigments as well as salicylate were not removed from a Florisil column by washing with chloroform, it was found that barbiturate was not completely retained on the column from chloroform solution, a small amount appearing in the effluent. For this reason the eluate obtained by treatment of the column with 10% methanol in chloroform was combined with the chloroform effluent. By use of these particular solvents, salicylate and interfering substances present in urine extracts were completely eliminated from the barbiturate eluates. The degree of purity of the eluates thus obtained made possible the quantitative recovery of as little as 0.05 mg. of barbiturate (Table IV), corresponding to a level of 0.5 mg. % in urine using a 10-ml. sample.

The technique herein described has been applied successfully in the isolation of barbiturates from a number of specimens of urine for toxicological purposes. That recovery of barbiturate by this method is quantitative from such specimens is evident from the data in Table V. It is to be noted that accurate determination of the relatively low barbiturate content of these urine samples was made possible by first removing the salicylate from the extract on a Florisil column. Chloroform extracts from blood and gastric contents have also been subjected to the Florisil purification process, resulting in solutions free of nonbarbiturate chromogenic substances and thus making possible the accurate determination of barbiturates by automatic differential ultraviolet spectrophotometry.

SUMMARY

A procedure for separation of barbiturates from urine specimens containing salicylate is described which is based on selective adsorption. Isolation of the barbiturate present in a chloroform extract is accomplished by means of an adsorption column from which the malonylurea derivative may be eluted conveniently while interfering substances are retained.

Salicylate, urine pigments, and other chromogenic materials which preclude spectrophotometric determination of barbiturate by virtue of their strong absorption in the ultraviolet region are removed from chloroform solution by Florisil. Elution of the barbiturate with a small volume of 10% methanol in chloroform results in quantitative recovery of the drug in sufficient purity to permit spectrophotometric analysis.

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