Tetracyclines II

Separation and Determination by Column Chromatography

By PETER P. ASCIONE, JOHN B. ZAGAR, and GEORGE P. CHREKIAN

A column chromatographic method for the determination of demethylchlortetracycline (DMCTC), tetracycline (TC), and chlortetracycline (CTC) was developed. The chromatogram involved the separation of these tetracyclines from their related compounds on a column of acid-washed diatomaceous earth treated with a buffer consisting of 0.1 *M* ethylenediaminetetraacetic acid disodium salt (EDTA) at pH 6.0 and pH 7.0, glycerin, and polyethylene glycol 400 (PEG 400), followe dby a modified spectral determination of column eluates. Although only DMCTC, TC, CTC, and their related compounds were studied in the work reported here, it appears reasonable to assume that the technique described could be extended to the assay of other tetracyclines.

THE MOST widely used and official method of analysis of tetracyclines is the microbiological assay which is based on the ability of tetracyclines to inhibit growth of certain organisms. Inhibition of growth is used as a direct measure of activity and/or potency of these tetracyclines.

However, the microbiological assay is subject to inherent sources of error which limits its precision to ±15%. Specificity is also limited by the fact that several tetracycline-related compounds exhibit activity toward the test organism similar to the parent compound.

The methods currently in use for the determination of tetracyclines and related compounds are based on spectral behavior in the ultraviolet. The procedure of Woolford and Chiccarelli (1), which determined TC in CTC, is based upon the change in spectrophotometric absorption in acid and basic aqueous media. A second paper by Chiccarelli *et al.* (2) described an additional assay method for TC and CTC based on the formation of anhydro compounds, and the fact that CTC heated at pH 7.5 is cleaved to isochlortetracycline, whereas TC under the same conditions is relatively stable. Doskocil and Vondracek (3) determined TC and CTC with a method based on the different rates at which tetracyclines are inactivated in alkaline medium. A differential spectrophotometric method has been used in these laboratories for determination of related compounds.

Various chromatographic methods—paper, column, and thin-layer—are being used for the quantitation and investigation of tetracyclines. Selzer and Wright (4) described paper chromatography of tetracyclines and epimers using complex solvent systems and buffered paper. Kelly and Buyske (5) used sequestering agents in paper chromatography. Whatman modified cellulose phosphate cation-exchange paper has been used also (6). Hrdy and Vesely (7) described column chromatography using powdered cellulose impregnated with tartrate buffer. A column chromatographic method was developed by Kelly (8) for the determination of both anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC) in the presence of large quantities of TC. The use of Sephadex G25 polydextran in columns was reported by Griffiths (9). However, these procedures lack the necessary accuracy and/or specificity for proper evaluation of the true tetracycline content.

A thin-layer chromatographic (TLC) method was developed in these laboratories for the separation and examination of tetracyclines (10). The separation was carried out on acid-washed diatomaceous earth[1] impregnated with 0.1 *M* EDTA pH 7.0, glycerin, and PEG 400, with ethyl acetate as a solvent. With modification of both the thin-layer chromatography and spectrophotometric methods, a column method was developed.

Although the method, as described, is concerned only with crystalline samples, this technique has also made it possible to determine individual tetracyclines in fermentation-process control samples in which mixtures resulted from failure of the strain used to produce a pure tetracycline, in manufacturing processes in which potency was determined in finished products, and also in the study of stability of each individual tetracycline in pharmaceutical formulations, which will be reported later.

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[1] Marketed by Desaga/Brinkmann Co. under the trademark Kieselguhr G.

EXPERIMENTAL

Materials and Methods

Apparatus—Chromatographic column, 1.3 cm. × 40 cm. (Bellco VIP 50-ml. pipet, graduated in 0.2 ml., with the delivery tip cut off at the 48-ml. graduation). Glass tamping rod, with a diameter 1 mm. less than the internal diameter of the chromatographic column.

Preparation of Acid-Washed Diatomaceous Earth[2]—Slurry 200 Gm. of diatomaceous earth with 750 ml. of 6 *N* HCl for 10–15 min. Filter off diatomaceous earth and wash with distilled water until pH is neutral. Slurry the diatomaceous earth in 500 ml. of a solution of equal volumes of ethyl acetate and methanol for 10–15 min. Filter and vacuum dry at 60°.

Reagents—EDTA, pH 6.0 and 7.0: dissolve 37.2 Gm. of ethylenediaminetetraacetic acid disodium salt in approximately 800 ml. distilled water. Adjust the pH to 6.0 or 7.0 with ammonium hydroxide and dilute to 1 L. with distilled water.

Twenty per cent v/v polyethylene glycol 400 (PEG 400) in glycerin: to 80 ml. of glycerin, add sufficient PEG 400 to make 100 ml. Mix well.

Buffer, pH 6.0 and 7.0: to 95 ml. of EDTA pH 6.0 or 7.0 add sufficient 20% v/v PEG 400 in glycerin to make 100 ml. Shake well.

Benzene, chloroform, *n*-butanol, and methanol, reagent grade.

Alkaline methanol solution: dilute 5 ml. concentrated ammonium hydroxide with 95 ml. of methanol.

Determination of Demethylchlortetracycline[3] and Related Compounds

Procedure—*Column Support*—Mix 100 Gm. acid-washed diatomaceous earth with 50 ml. of buffer, pH 7.0, in a plastic bag or in a glass jar on rollers, until the diatomaceous earth is uniformly moist.

Column Preparation—Place a disk of filter paper (Whatman 541, approximately 1.2-cm. diameter) into the bottom of the column, as a plug. Weigh 8 ± 0.1 Gm. of column support and add to the column in three portions. After each addition settle the diatomaceous earth by firmly tapping the lower end of the column on a padded bench top from a height of 2 to 4 in. while maintaining the column in a perpendicular position. Then lightly tamp the surface with the glass tamping rod. The column height should be about 10.5 to 11.5 cm.

Sample Preparation—*Demethylchlortetracycline HCl*—Weigh accurately about 20 mg. DMCTC HCl, transfer to a 25-ml. volumetric flask with 4 ml. distilled water, and dissolve. Dilute to mark with buffer, pH 7.0. Prepare not more than 30 min. before assay. Pipet 1.0 ml. of sample into a 50-ml. beaker. Add 2 ± 0.1 Gm. of acid-washed diatomaceous earth, stir well, and pack on the column. "Rinse" the beaker with 0.5 ± 0.1 Gm. of column support, add to the column, and tamp well.

Demethylchlortetracycline Neutral—Accurately weigh 18 mg. of DMCTC neutral, transfer to a 25-ml. volumetric flask using 3 ml. of 0.1 *N* HCl, and dissolve. Add 15 ml. of buffer, pH 7.0, followed by 3 ml. of 0.1 *N* NaOH, swirl, and dilute to 25 ml. with buffer, pH 7.0. Prepare this solution not more than 30 min. before assay. Pipet 1.0 ml. of this solution into a 50-ml. beaker, add 2 ± 0.1 Gm. of acid-washed diatomaceous earth and transfer to the column as described above.

Development of Column—Use a 25-ml. graduate as the primary receiver under the column. Add 25 ml. of 40% benzene in chloroform to the column. When the solvent level reaches the top of the column packing, add 50 ml. of 30% benzene in chloroform. After collecting 22 ml. (cut *1*), remove the primary receiver and replace with 50-ml. volumetric flask. Collect the eluate in this flask until the level of the solvent in the column again reaches the top of the packing. Replace the 50-ml. volumetric flask (cut *2*) with a 10-ml. graduate, and add 30 ml. of chloroform to the column. When the volume of eluate in the graduate reaches 8 ml. (cut *3*), replace the graduate with a 50-ml. glass-stoppered graduate and continue collecting the eluate. When the chloroform reaches the top of the packing, add 40 ml. of 50% *n*-butanol in chloroform. When the 50-ml. glass-stoppered graduate contains 29.0 ml. (cut *4*), replace with another 50-ml. glass-stoppered graduate and collect all of the last solvent added to the column (cut *5*).

Assay of Column Eluates—To cut *2* (DMCTC), add cut *3*, and mix well. Add 2.0 ml. of alkaline methanol solution and dilute to volume with chloroform. Determine the absorbance of this solution in a 1-cm. cell against chloroform at a wavelength of 372 mμ on a suitable spectrophotometer within 10 min. after making the solution alkaline. To cut *4* demethyltetracycline (DMTC), add 1.0 ml. of alkaline methanol solution and mix. Determine the absorbance of this solution in a 5-cm. cell against chloroform at a wavelength of 366 mμ within 10 min. after making the solution alkaline. To cut *5* epidemethylchlortetracycline (EDMCTC) add 2.0 ml. alkaline methanol, adjust the volume to the nearest milliliter graduation with chloroform, mix, and record the volume. Determine the absorbance of this solution in a 5-cm. cell against chloroform at a wavelength of 372 mμ within 10 min. after making the solution alkaline.

Determination of Tetracycline[4] and Related Compounds

Procedure—The column support, column, and sample preparation are the same as for DMCTC.

Development of Column—Use a 25-ml. graduate as the primary receiver under the column. Add 20 ml. of benzene to the column. When the solvent level reaches the top of the column packing, add 60 ml. of chloroform. After collecting 25 ml. (cut *1*), remove the primary receiver and replace with a 50-ml. volumetric flask. Collect the eluate in this flask until the level of the solvent in the column again reaches the top of the packing. Replace the 50-ml. volumetric flask (cut *2*) with a 10-ml. graduate and add 40 ml. of 50% *n*-butanol in chloroform to the column. When the volume of eluate in the graduate reaches 8 ml., replace the 10-ml. graduate (cut *3*) with a 50-ml. glass-stoppered graduate and collect all of the last solvent added to the column (cut *4*).

[2] Marketed as Celite 545 by Johns-Manville Co.

[3] Marketed by Lederle Laboratories, Division American Cyanamid Co., under the trademark Declomycin.

[4] Marketed by Lederle Laboratories, Division American Cyanamid Co., under the trademark Achromycin.

Assay of Column Cuts—To cut *2* (TC), add cut *3*, and mix well. Add 2.0 ml. of alkaline methanol solution and dilute to volume with chloroform. Determine the absorbance of this solution in a 1-cm. cell against chloroform at a wavelength of 366 mμ on a suitable spectrophotometer within 10 min. after making the solution alkaline. To cut *4* epitetracycline (ETC), add 2.0 ml. of alkaline methanol, adjust the volume to the nearest milliliter graduation with chloroform, mix, and record the volume. Determine the absorbance of this solution in a 5-cm. cell against chloroform at a wavelength of 366 mμ within 10 min. after making the solution alkaline.

Determination of Chlortetracycline[5] and Related Compounds

Procedure—Column support, column, and sample preparation are the same as for DMCTC, but at pH 6.0.

Development of Column—Use a 25-ml. graduate as the primary receiver under the column, add 25 ml. of 80% benzene in chloroform to the column. When the solvent level reaches the top of the column packing, add 50 ml. of 40% benzene in chloroform. The development of this column to completion is described under *Procedure* for DMCTC continuing from and including cut *1*.

Assay of Column Cuts—The cuts are treated as directed under *Procedure* for DMCTC with cut *2* (CTC) and cut *5* epichlortetracycline (ECTC) read at 377 mμ, and cut *4* (TC) read at 366 mμ.

RESULTS AND DISCUSSION

Earlier investigation into the separation of tetracyclines by thin-layer chromatography suggested the development of this column chromatographic technique. Thin-layer chromatograms of these tetracyclines (10) showed the possible occurrence of related compounds in each tetracycline. In developing the column, various modifications were made to adapt the basic TLC system. The mobile phase was varied on the column from low to high polarity, starting with benzene–chloroform and ending with *n*-butanol–chloroform. The pH was varied from 6.0 to 7.0, but the ratio of PEG 400 to glycerin was maintained at 1:5.

The effectiveness of the column for separating each tetracycline from its related compounds was demonstrated by the use of TLC (10). Figures 1–3 show the chromatography of subsequent column eluates. According to the results of TLC, the tetracyclines were separated on a column of acid-washed diatomaceous earth treated with EDTA, PEG 400–glycerin at pH 6.0 or 7.0, using benzene, chloroform, and butanol as the developing solvents. In Fig. 1, DMCTC is separated from anhydrodemethylchlortetracycline (ADMCTC), demethyltetracycline (DMTC), and epidemethylchlortetracycline (EDMCTC). The column eluates were collected in fractions of 5 ml. and examined by TLC. The TLC shows the distribution of the DMCTC components isolated on the column and having R_f values similar to those of standard test references. In Fig. 2, TC is separated from anhydrotetracycline (ATC), CTC, and epitetracycline (ETC) on a column as described previously; in Fig. 3, CTC is separated from anhydrochlortetracycline (ACTC), TC, and epichlortetracycline (ECTC) on a column as described above but at a pH 6.0.

[5] Marketed by Lederle Laboratories, Division American Cyanamid Co., under the trademark Aureomycin.

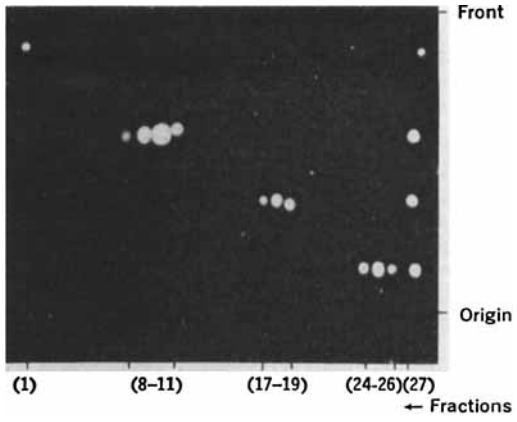

Fig. 1—Thin-layer chromatography of demethylchlortetracycline column fractions. Key: 1, ADMCTC; 8–11, DMCTC; 17–19, DMTC; 24–26, EDMCTC; 27, reference standard mixture.

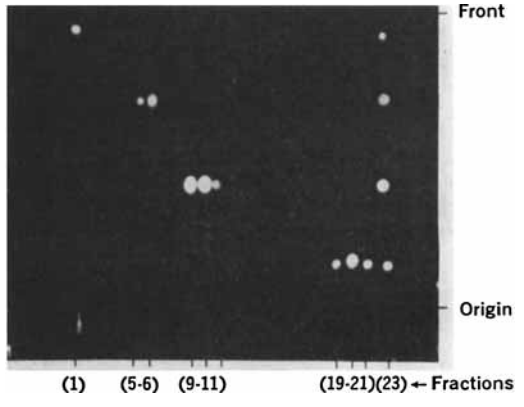

Fig. 2—Thin-layer chromatography of tetracycline column fractions. Key: 1, ATC; 5–6, CTC; 9–11, TC; 19–21, ETC; 23, reference standard mixture.

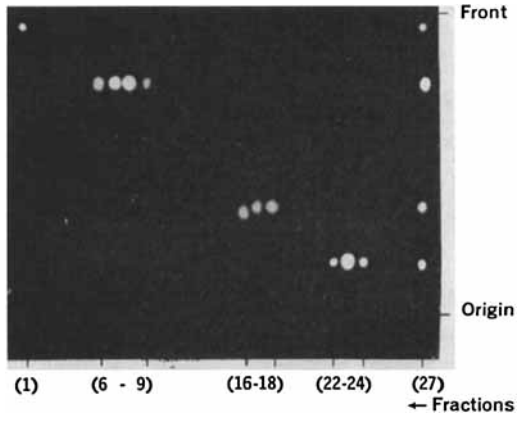

Fig. 3—Thin-layer chromatography of chlortetracycline column fractions. Key: 1, ACTC; 6–9, CTC; 16–18, TC; 22–24, ECTC; 27, reference standard mixture.

The effectiveness of the column and thin-layer chromatographic techniques for the determination of each tetracycline was corroborated by use of microbiological assays. The TLC-chromatogramed spots of tetracyclines were scraped and assayed by the pad-plate diffusion method while the column-chromatographed cuts were assayed by the turbidimetric method. In general, the microbiological results correlated well with the chromatographic data.

For convenience and ease of handling a 1.3-cm. × 40-cm. glass column was chosen. The pH of the buffer was adjusted to eliminate degradation during assay as well as to produce the desired chromatogram of the tetracycline. In the work reported, the sequestering agent (EDTA) was used in the development of TLC and the column. Kelly and Buyske (6) reported that EDTA showed strong chelating properties which improved the separation of tetracyclines. Benzene, chloroform, ethyl acetate, and *n*-butanol were tried as developing solvents, but none served well alone. The best separations were obtained when mixtures of the solvents were used.

Absorption Spectra—Absorption spectra in the region of 300 to 500 mμ were determined on a Cary model 11 recording spectrophotometer. Standard solutions of each tetracycline hydrochloride containing 0.8 mg./ml. were prepared by dissolving 80 mg. of standard in 10 ml. of methanol and then diluting to 100 ml. with chloroform. Two milliliters of the chloroform solution was transferred to a 50-ml. volumetric flask. Forty milliliters of chloroform and 2.0 ml. of alkaline methanol were added, and then diluted to 50 ml. with chloroform. Figures 4–6 show the effect of alkaline methanol, an auxochromic agent, on the absorption spectra of DMCTC, TC, and CTC. In each instance the alkaline methanol had a hypochromic as well as a hyperchromic effect. The wavelengths where maximum absorption occurred when alkaline methanol was introduced into the tetracycline chromaphoric system are as follows: DMCTC, 372 mμ; TC, 366 mμ; CTC, 377 mμ.

Standard calibration curves of DMCTC, TC, and CTC hydrochlorides were prepared with alkaline methanol reagent in chloroform solutions. Three standard curves are presented in Fig. 7. Typical linear Beer's law plot of absorbance *versus* tetracycline concentration range from 0.1 mg./50 ml. to 1.0 mg./50 ml. for all three tetracyclines.

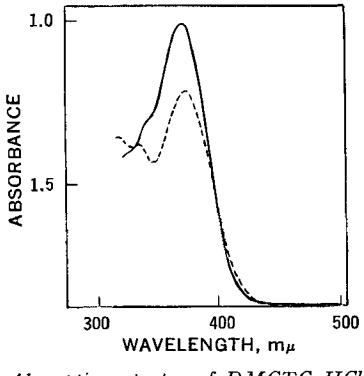

Fig. 4—Absorption spectra of DMCTC·HCl. Key: ----, CH_3OH–$CHCl_3$; ———, NH_4OH–CH_3OH–$CHCl_3$.

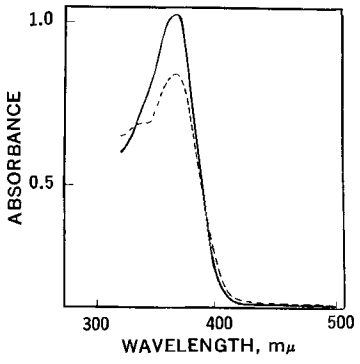

Fig. 5—Absorption spectra of TCH·Cl. Key: ----, CH_3OH–$CHCl_3$; ———, NH_4OH–CH_3OH–$CHCl_3$.

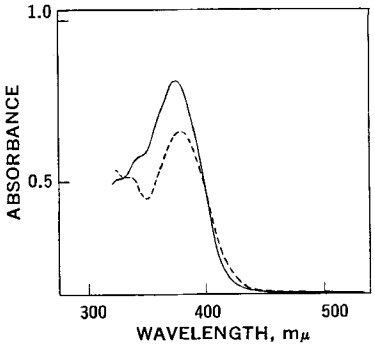

Fig. 6—Absorption spectra of CTC·HCl. Key: ----, CH_3OH–$CHCl_3$; ———, NH_4OH–CH_3OH–$CHCl_3$.

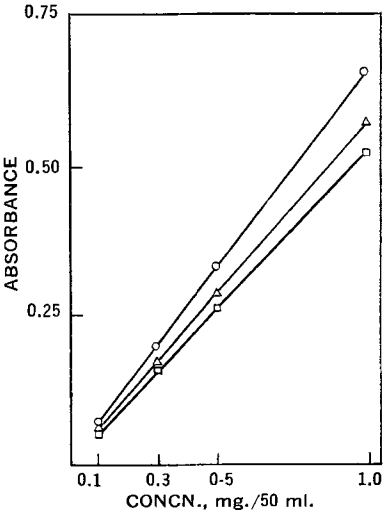

Fig. 7—Standard calibration curve. Key: ○, TC; △, DMCTC; □, CTC.

The absorptiometric system obtained a maximum absorbance value immediately upon the addition of the alkaline methanol reagent and this absorbance remained constant over a practical length of time. (Table I.) This should conservatively estimate the

span of time suitable for accurate quantitation. For example, data in Table I indicate at least 10 min. of stability in absorbance when CTC is being tested, and 30 min. stability for DMCTC and TC.

TABLE I—STABILITY OF TETRACYCLINES IN CHLOROFORM SOLUTION CONTAINING ALKALINE METHANOL

Time, min.	DMCTC, 372 mμ	TC, 366 mμ	CTC, 377 mμ
0	0.459	0.527	0.418
2	0.459	0.527	0.418
4	0.459	0.527	0.418
6	0.459	0.527	0.418
8	0.459	0.527	0.418
10	0.459	0.527	0.417
12	0.459	0.527	0.417
14	0.459	0.527	0.417
16	0.459	0.527	0.416
30	0.458	0.527	0.414

The concentrations of the three compounds tested were 0.80 mg./50 ml. for demethylchlortetracycline hydrochloride, tetracycline hydrochloride, and chlortetracycline hydrochloride, respectively.

The absorptivity for DMCTC, TC, and CTC was determined at the wavelength of maximum absorbance and found to be 2.89×10^{-2} for DMCTC, 3.071×10^{-2} for DMTC, 3.29×10^{-2} for TC, and 2.65×10^{-2} for CTC, as the hydrochlorides. The absorptivity is defined as the absorbance at the wavelength of each particular tetracycline for a solution containing 1 mcg. of tetracycline hydrochloride per milliliter, using a 1-cm. cell. Through examination of standards available in these laboratories, the absorptivity for each tetracycline and its epimer was found to be the same. The determination of absorbances of the column eluates after the addition of the auxochromic agent provided a quantitative spectrophotometric method for assaying tetracyclines in solvent solutions when compared with standards treated in like manner.

REFERENCES

(1) Woolford, M. H., Jr., and Chiccarelli, F. S., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 400(1956).
(2) Chiccarelli, F. S., Woolford, M. H., Jr., and Avery, M. E., *ibid.*, **48**, 263(1959).
(3) Doskocil, J., and Vondracek, M., *Chem. Listy*, **46**, 564(1952).
(4) Selzer, G. B., and Wright, W. W., *Antibiot. Chemotherapy*, **7**, 292(1957).
(5) Kelly, R. G., and Buyske, D. A., *ibid.*, **10**, 604(1960).
(6) Addison, E., and Clark, R. G., *J. Pharm. Pharmacol.*, **15**, 268(1963).
(7) Hrdy, O., and Vesely, P., *Cesk. Farm.*, **10**, 126(1961).
(8) Kelly, R. G., *J. Pharm. Sci.*, **53**, 1551(1964).
(9) Griffiths, B. W., *ibid.*, **55**, 353(1966).
(10) Ascione, P. P., Zagar, J. B., and Chrekian, G. P., *ibid.*, **56**, 1393(1967).

In Vivo Method for the Simultaneous Determination of Potassium and Sodium Depletion

By GORDON S. BORN, STANLEY M. SHAW, and JOHN E. CHRISTIAN

An *in vivo* radiotracer technique utilizing whole body liquid scintillation counting for the simultaneous detection of ^{42}K and ^{24}Na retention within the intact animal was developed. The technique allowed direct comparison of the retention of potassium and sodium in treated and control animals. The differences in retention indicated the kaliuretic and natriuretic effects of proven diuretic agents. Experimentation with standards was used to develop the simultaneous technique for ^{42}K and ^{24}Na determination. The method was tested *in vivo* in rats and swine, using furosemide and hydrochlorothiazide to show the effects of these diuretic agents in altering potassium and sodium metabolism, to illustrate the adaptability of the method in studies with various animal species, and to confirm the versatility of the method with a whole body liquid scintillation detector of the "human type." Results indicated the technique to be a sensitive method for measuring alterations in potassium and sodium metabolism in rats and swine caused by the diuretic agents.

RADIOTRACER techniques utilizing *in vivo* whole body liquid scintillation counting have been used in studying the effects of diuretics on either potassium or sodium metabolism. Using ^{22}Na, Rupe, Bousquet, and Christian (1) evaluated compounds for their natriuretic and diuretic activity in rats. Born *et al.* (2) and Seno (3), using ^{42}K, studied the kaliuretic properties of diuretics in the rat. Shaw, Kessler, and Christian (4) investigated the kaliuretic and natriuretic properties of a diuretic by using ^{42}K and ^{24}Na in separate studies in swine. The advantages of the whole body liquid scintillation counting technique over photometric methods of determining potassium and sodium elimination have been stated in the above references. However, an undesirable aspect of the whole body liquid scintillation counting technique was the inability to study the kaliuretic and natriuretic properties of a diuretic in the same group of animals in a single experiment.

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