Quantitative Chromatographic Analysis of Methallibure in Animal Feed Mixtures

G. J. KROL, J. F. CARNEY, and B. T. KHO

Abstract □ Methallibure [1-methyl-6-(1-methylallyl)-2,5-dithiobiurea] was separated from the constituents of feed extracts by partition column chromatography. The separation utilized polydextran gel as a stationary phase and a composite organic solvent as a mobile phase. The method requires no special treatment of the solid support and the column can be used for an indeterminate number of elutions. The observed interference of metal ions in the extraction and chromatography step was eliminated by the addition of dithizone to the extracting solvent. The procedure is quantitative and applicable to routine analysis of different commercial field samples. The chromatographic separation was tested for specificity with a thiadiazole derivative, which was prepared from methallibure, and complete separation between the two related structures was observed. The chromatographic column, used in the analysis, yielded approximately 900 theoretical plates.

Keyphrases □ Methallibure—determination □ Animal feed mixtures—methallibure determination □ Column chromatography—separation □ Dithizone—metal-ion interference elimination □ UV spectrophotometry—analysis

A partition chromatographic method for analysis of methallibure, an oestrus regulator for veterinary applications, in the presence of certain animal feed mixtures was reported by Hudson and Pearson (1). The method used diatomaceous earth[1] as a solid support and chloroform, formamide, and *n*-hexane as a two-phase solvent system. However, this procedure is relatively time consuming, since it requires preparation of a new column for every elution and involves relatively large volumes of eluent.

In view of these limitations, an alternative chromatographic method was developed. The method is based on an alkylated polydextran gel[2] solid support and an eluent composed of a mixed organic solvent. The preferential solvation of the gel by the more polar components of the solvent mixture yields a partition effect similar to that observed by Nystrom and Sjovall (2). In contrast to the diatomaceous earth method (1), the liquid partition effect obtained in this method requires no special treatment of the solid support, since the solvation of the gel and elution are carried out with the same mixed solvent system. Furthermore, once the column is packed, it can be used for an indeterminate number of elutions.

During the development phase of this method, a complication was encountered since some feed samples contained significant amounts of metal salts and the dithiobiurea structure of methallibure is a relatively efficient metal-ion complexing group (3, 4). In a number of instances, this situation led to a significant apparent loss in the percent recovery of the methallibure, since a significant fraction of methallibure was complexed by metal ions and eluted in different volume. To avoid this complication, the extraction was carried out in the presence of an excess amount of diphenylthiocarbazone (dithizone), which competes effectively for metal ions with methallibure. Initially, 8-hydroxyquinoline was utilized for this purpose; however, it was subsequently observed that some metal-ion complexes of 8-hydroxyquinoline were eluted in the same volume as methallibure. A complete separation between the methallibure, dithizone, and dithizone metal-ion complexes was obtained.

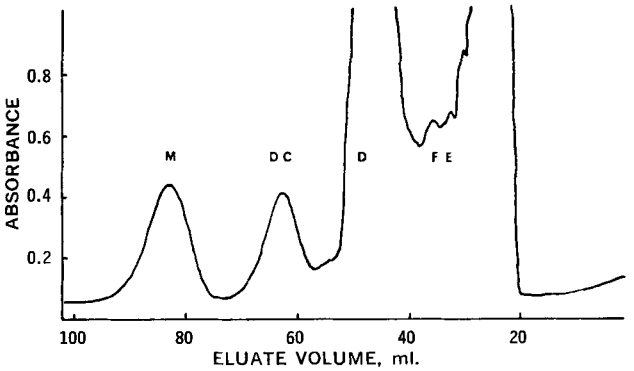


Figure 1—*Separation of methallibure from diphenylthiocarbazone and Feed Mixture B extract (Drago) on polydextran gel (1.2 × 44-cm. column) with isopropanol–chloroform–cyclohexane–acetic acid–water (100:100:130:8:2) solvent. Key: M, methallibure; D, diphenylthiocarbazone; DC, diphenylthiocarbazone complex; and FE, feed extractives.*

EXPERIMENTAL

Equipment—Kontes glass chromaflex columns (or equivalent), 50 × 1.2 cm. (i.d.) and 50 × 5 cm., were used (Kontes, Vineland, N. J.). To prevent introduction of UV-absorbing extractives from the O-ring which is provided with the above columns, the O-rings have been replaced with O-rings improvised from 0.32-cm. (0.125-in.) (o.d.) organic solvent resistant tubing.[3] The sintered-glass disk, which is present in the column, was covered with a nylon mesh (Pharmacia Fine Chemicals Inc., New Market, N. J.). The smaller column was fitted with a 500-ml. separator, the larger with a 2-l. funnel. Each separator had an airtight connection to the column. A Cary model 14 was used for the UV determinations, while Beckman DB was used in the method development to determine the optimum chromatographic solvent and the elution volumes. UV flow cell (1-cm. light path) was obtained from A. H. Thomas Co.

Solvents and Chemicals—Redistilled isopropanol (analytical reagent grade, Mallinckrodt), chloroform, glacial acetic acid, and spectro grade cyclohexane were used. The chromatographic solvent consisted of isopropanol, chloroform, cyclohexane, glacial acetic acid, and water (1.0:1.0:1.3:0.08:0.02 by volume, respectively).

[1] Celite, Johns-Manville, New York, N. Y.
[2] Sephadex LH-20, Pharmacia Fine Chemicals, Inc., New Market, NJ 08854
[3] Acidflex, Technicon Inc., Chauncey, N. Y.

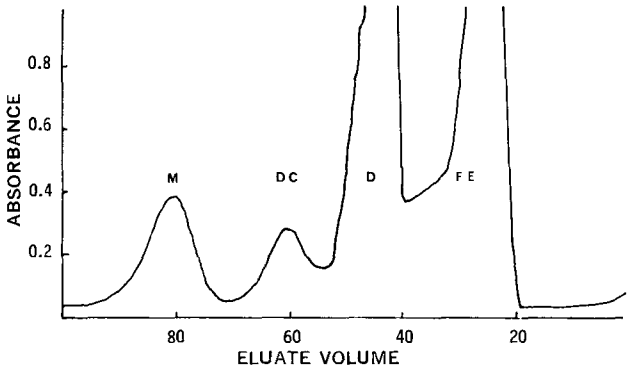


Figure 2—*Separation of methallibure from diphenylthiocarbazone and Feed Mixture C (Kent) on polydextran gel (1.2 × 44-cm. column) with isopropanol–chloroform–cyclohexane–acetic acid–water (100:100:130:8:2) solvent. Key: M, methallibure; D, diphenylthiocarbazone; DC, diphenylthiocarbazone complex; and FE, feed extractives.*

Methallibure was obtained from I.C.I., Pharmaceutical Division, Macclesfield, Cheshire, England.

Since the commercially available dithizone (obtained from either Eastman Organic Chemicals or Fisher Scientific Co.) was found to be insufficiently pure for purposes of this study, it was purified by the following procedure: 250 mg. of dithizone was dissolved in 50 ml. of the chromatographic solvent. The solution was filtered, applied to the larger column (23-cm. bed height), and eluted with 460 ml. of the chromatographic solvent. The preparation of the large column is analogous to the procedure described for the preparation of 50 × 1.2-cm. columns. However, the solvent used for both preparation and elution may be composed of undistilled reagent grade solvents. The purified dithizone fraction, which was contained in the major blue band, was eluted in the 350- to 460-ml. fraction and used without further treatment. The solution prepared previously was stored in the dark at 4° and may be used for several days. The chromatographic column may be used repeatedly for the preparation of more dithizone after washing the column with additional 300 ml. of the solvent between sample applications.

Procedure—During the development phase and the subsequent testing of the method with different commercial feeds, the authors have utilized a Beckman DB flow cell–log recorder system. The spectrophotometer was set at the wavelength of the absorption maximum of methallibure (249 mμ), and the area of the methallibure peak on the recorder scan was found to be proportional to the amount of methallibure. Representative elution patterns are illustrated by Figs. 1–3. However, since the volume which contained the methallibure fraction was found to be quite reproducible, the procedure was modified to a more suitable routine analysis which does not require a spectrophotometer–flow cell system. The following description outlines the procedure for routine analysis.

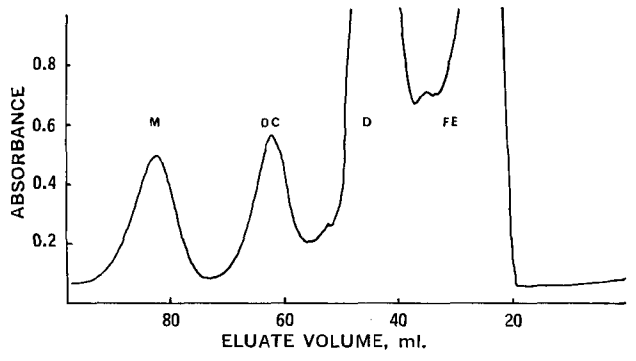


Figure 3—*Separation of methallibure from diphenylthiocarbazone and Feed Mixture A extract (Felco) on polydextran gel (1.2 × 44-cm. column) with isopropanol–chloroform–cyclohexane–acetic acid–water (100:100:130:8:2) solvent. Key: M, methallibure; D, diphenylthiocarbazone; DC, diphenylthiocarbazone complex; and FE, feed extractives.*

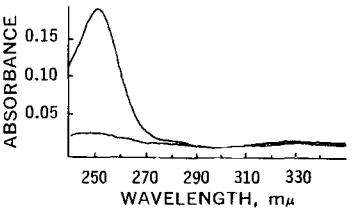


Figure 4—*UV scan of the 25-ml. methallibure and the 4-ml. blank fraction. Origin of the fraction: Feed Mixture B extract (Drago).*

Preparation of Column—Polydextran gel was suspended in the chromatographic solvent (200 ml. of solvent/10 g. of polydextran gel) and the slurry equilibrated by shaking for 2 hr. The gel was allowed to settle, excess solvent decanted, and a fresh portion of solvent added. This procedure was repeated three times. The final slurry was transferred to the column by gravity feed. The column height was 44 cm.

Extraction of Methallibure from Feed—A 40-g. feed sample, containing approximately 2.2 mg. of methallibure, was transferred into a 250-ml. bottle and extracted with 10 ml. of the dithizone solution and 150 ml. of the chromatographic solvent. The extraction was aided by continuous shaking for 5 min. This procedure is applicable to a methallibure premix, such as polyethylene glycol (PEG), which is readily soluble in the chromatographic solvent. If methallibure is present in a methylcellulose premix, which is not soluble in the chromatographic solvent, the extraction could be carried out with a high speed homogenizer (for 15 min. at 40,000 r.p.m.). A VirTis "45" homogenizer is suitable for this purpose. Alternatively, the extraction could also be carried out by shaking with the more polar components of chromatographic solvent (omit cyclohexane). A portion of the extracted feed slurry was filtered through a fine- or medium-porosity sintered-glass funnel to provide a 4-ml. aliquot of clear filtrate. In order to avoid evaporation, the filtration was carried out under positive pressure with the aid of a

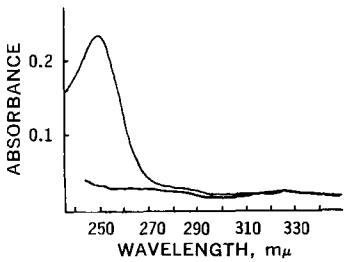


Figure 5—*UV scan of the 25-ml. methallibure and the 4-ml. blank fraction. Origin of the fractions: Feed Mixture A extract (Felco).*

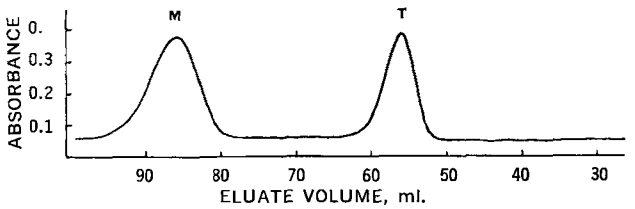
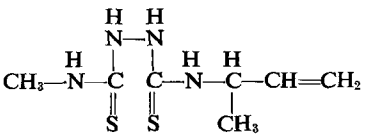
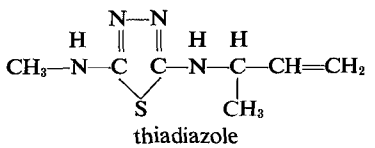


Figure 6—*Separation of methallibure (M) from thiadiazole (T) on polydextran (1.2 × 44-cm. column) with isopropanol–chloroform–cyclohexane–acetic acid–water (100:100:130:8:2) solvent.*



methallibure



thiadiazole

Table I—Observed Recoveries of Methallibure from Feed Samples Spiked with Methylcellulose and PEG Premixes

Feed Mixture	Premix Calculated, mcg.	Premix Observed, mcg.	Premix Recovery, %
	Methylcellulose		
A[a]	29.9	29.8	99.6
		29.6	98.9
	35.0	36.8	105.0
		38.3	109.3
	33.8	32.0	94.7
		33.0	97.7
B[b]	32.2	33.8	104.8
		33.8	104.8
	33.2	33.0	99.4
	35.4	33.5	94.6
		33.0	93.3
C[c]	34.4	34.4	100.0
		34.6	100.6
	32.1	34.8	108.3
		33.0	102.8
	34.4	35.2	102.2
		34.6	100.6
	PEG		
A[a]	35.7	35.4	99.2
		33.3	93.2
	30.5	29.8	97.8
		30.6	100.3
	31.4	32.8	104.4
		31.0	98.8
B[b]	34.2	35.3	103.1
		32.8	95.9
	29.3	28.3	96.6
		29.4	100.3
	31.6	32.4	102.4
		33.8	107.0
C[c]	30.3	30.3	100.0
	33.7	32.4	96.2
		35.1	104.1
	32.5	31.0	95.5
		30.5	93.8

[a] Felco Sow Chunks. [b] Drago, 3 parts ground corn and 1 part Sow Concentrate 853. [c] Kent Hand Feed Sow Mix.

rubber bulb. A 4-ml. aliquot of the filtrate was transferred into a 5-ml. volumetric flask and diluted to 5 ml. with 1 ml. of dithizone solution.

Chromatography—A 3-ml. aliquot of the prepared solution was applied quantitatively to the column. The column was eluted with 102 ml. of the chromatographic solvent at a flow rate of approximately 0.5 ± 0.1 ml./min. (A pressure head was provided by a 300-ml. solvent reservoir in the separator above the column.)

The first 73-ml. fraction, which contained the feed extractives and dithizone (free and chelated by metal ions), was discarded and the next two fractions containing 25 and 4 ml. were collected and retained for quantitation by UV absorption. Both fractions were scanned in the 350–240-mμ range against the chromatographic solvent. Figures 4 and 5 illustrate typical UV scans of the 25- and 4-ml. fractions.

Quantitation—If the UV absorption of the 25-ml. fraction matched (within ±0.005) the UV absorption of the 4-ml. fraction in the 350–290-mμ range, the absorbance (at 249 mμ) of the 4-ml. fraction was subtracted from the absorbance of the 25-ml. fraction and the difference in absorption $(A)_c$ used to calculate the amount of methallibure in the sample. The following formula was used to calculate the amount of methallibure in the 25-ml. fraction.

$$\text{mcg. (of methallibure recovered after chromatography)} = \frac{(A)_c \times \text{vol.}_f}{0.129} \quad \text{(Eq. 1)}$$

$$\text{mcg. (of methallibure per g. of feed)} = (A)_c \times 194 \times {}^5/_3 \quad \text{(Eq. 2)}$$

where $(A)_c$ is $(A_{25} - A_4)$, absorbance of the 25-ml. fraction which contains methallibure corrected for background absorbance as reflected by the 4-ml. fraction; vol._f is the volume of the fraction which contains the methallibure; 0.129 is the extinction factor of methallibure in the chromatographic solvent expressed in ml./mcg. units; and ${}^5/_3$ is the dilution factor of the feed extract.

Table II—Accuracy and Precision of the Methallibure Assay

Feed Mixture	Premix Mean Recovery, %	Premix *SD*	Premix *CV*
	Methylcellulose		
A[a]	100.9	5.3	5.3
B[b]	99.4	5.4	5.5
C[c]	102.4	3.1	3.0
	PEG		
A[a]	99.0	3.6	3.7
B[b]	100.9	4.2	4.2
C[c]	97.9	4.1	4.2

[a] Felco Sow Chunks. [b] Drago, 3 parts ground corn and 1 part Sow Concentrate 853. [c] Kent Hand Feed Sow Mix.

If the absorbance of the 25-ml. fraction in the 350–290-mμ range does not match the absorbance of the 4-ml. fraction, one may determine the amount of methallibure in the 25-ml. fraction by the curvature inversion technique (5, 6). This procedure involves scanning the methallibure fraction (25 ml.) against several reference solutions of methallibure at different concentrations but in the concentration range of the unknown sample. From the curvature at the inflection point (249 mμ, absorption maximum of methallibure), one can determine the concentration of the unknown sample. However, essentially all feed mixtures the authors encountered were analyzed without resorting to the curvature inversion technique. Usually, the magnitude of background absorbance was predictable and equivalent to 10–15% of the total absorbance of the methallibure fraction. The estimation of the background absorbance did not introduce more than a 3% error in calculation of the amount of methallibure.

RESULTS AND DISCUSSION

Three different commercial feed mixtures were spiked with known amounts of PEG and methylcellulose premixes. The samples were analyzed by direct UV determination without the aid of the curvature inversion technique. Tables I and II present the observed recoveries and the statistical analysis of the observed data. It is apparent from Tables I and II that although the standard deviations are relatively large, the observed recovery is essentially complete and independent of the nature of the feed mixture.

The chromatographic separation utilized in this study has also been tested for specificity. A complete separation between methallibure and its oxidation product, thiadiazole, has been observed (Fig. 6). On the basis of the elution volume and peak width, the chromatographic column used in this analysis yielded approximately 900 theoretical plates. This is equivalent to 20 theoretical plates/cm.

A similar partition chromatographic system was applied to the separation of the coccidiostat, nequinate (oxyquinoline structure), from feed mixtures (7). Although the chemical structure of methallibure is unrelated to the structure of nequinate, the chromatographic system is specific for both compounds; each compound was separated from closely related structures. This chromatographic approach was also extended to the quantitative separation of structurally related steroid compounds (8).

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(6) J. B. Wilkie, *ibid.*, **36**, 896(1964).

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Improved Colorimetric Determination of Primary Aromatic Amines with 9-Chloroacridine: Application to Some Local Anesthetics

J. T. STEWART and D. M. LOTTI

Abstract □ Improvement in the stability of the 9-chloroacridine stock solution has been made in the colorimetric method for primary aromatic amines based on the reaction between the acridine and an amine. The improved procedure has been applied to some local anesthetics and local anesthetic mixtures. It has been found to be comparable in sensitivity to other local anesthetic determinations, particularly the popular diazotization-coupling procedures. Quantitative data from several systems reveal that use of this procedure permits the determination of local anesthetics in the presence of various drugs and other local anesthetics. Comparative analyses were performed with the method of Bratton and Marshall on procaine, metabutethamine, and nesacaine hydrochlorides.

Keyphrases □ Amines, primary aromatic—improved analysis □ 9-Chloroacridine solution—stabilization □ Tetrahydrofuran—9-chloroacridine solvent □ Colorimetric analysis—spectrophotometry

A colorimetric method for the determination of small quantities of primary aromatic amines with 9-chloroacridine and its use in the analysis of some sulfonamides have been previously reported by this laboratory (1, 2). Data presented in these papers showed that the sensitivity of the method rivals that of the commonly used diazotization-coupling procedures for primary aromatic amines and sulfonamides. It is necessary for the ethanolic 9-chloroacridine stock solution to be prepared immediately before use since the acridine undergoes rapid ethanolysis in ethanol (3). This fresh solution is permissible to use for approximately 0.5 hr. after preparation.

The objective of this paper is to report an improvement in the existing method by stabilization of the 9-chloroacridine stock solution, and application of the method to the analysis of several local anesthetics and local anesthetic mixtures. A comparative study of this improved technique was made with the procedure of Bratton and Marshall (4).

EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with spectrophotometers (Perkin-Elmer, model 202, and Beckman, model DU). Matched cells with a 1-cm. optical path were used.

Reagents and Chemicals—9-Chloroacridine[1] was used as the chromogenic reagent. Powdered samples of propoxycaine hydrochloride,[2] butacaine sulfate,[3] butesin,[3] butethamine hydrochloride,[4] metabutethamine hydrochloride,[4] nesacaine hydrochloride,[5] benzocaine,[1] and procaine hydrochloride[6] were used in the analytical procedure for preparation of standard curves. Piperocaine,[7] lidocaine,[8] and tetracaine[9] hydrochlorides were also used in the analysis. All other chemicals used were the highest grade of the commercially available materials.

Solutions (4×10^{-4} *M*) were prepared by dissolving weighed amounts of benzocaine and butesin in ethanol and the remaining local anesthetics in water. The reagent solution of 9-chloroacridine (4×10^{-4} *M*) was prepared by dissolving a weighed amount in tetrahydrofuran[10] and storing in a light-resistant volumetric flask.[11]

Procedure—One milliliter of an ethanolic or aqueous solution of a local anesthetic (4×10^{-4} *M*) was placed in a 10-ml. volumetric flask. To this was added 1 ml. of a tetrahydrofuran solution of 9-chloroacridine (4×10^{-4} *M*). Then the pH was adjusted to approximately 4 with 10% v/v aqueous hydrochloric acid. The solution was shaken and allowed to sit for 15 min. at room temperature, followed by the addition of ethanol to volume, and absorbance was measured at 435 mμ. Absorbance measurements were corrected for reagent blanks in the procedure.

RESULTS AND DISCUSSION

The colorimetric method for primary aromatic amines using 9-chloroacridine has been improved by using tetrahydrofuran in place of ethanol as solvent to make the acridine stock solution. Data shown in Table I for some local anesthetics reveal that the addition of small amounts of tetrahydrofuran to the analytical procedure does not cause any significant change in the sensitivity. The ethanolic solution was only useful for about 0.5 hr. due to reaction between the acridine and ethanol, and it was a disadvantage to prepare new acridine stock solutions that often. There was a need for a solvent in which dissolution, but no reaction between the acridine and solvent, would occur. Miscellaneous solvents were investigated, but only tetrahydrofuran proved successful in meeting these requirements.

[1] Eastman Chemical Co.
[2] Sterling Drug Co., Rensselaer, N. Y.
[3] Abbott Laboratories, North Chicago, Ill.
[4] Novocol Chemical Manufacturing Co., Inc., Brooklyn, N. Y.
[5] Strasenburgh Laboratories, Rochester, N. Y.
[6] Purocaine Chemical Co., New York, N. Y.
[7] Eli Lilly and Co., Indianapolis, Ind.
[8] Astra Pharmaceutical Products, Worcester, Mass.
[9] Winthrop Laboratories, New York, N. Y.
[10] Mallinckrodt, analytical reagent grade.
[11] Low actinic volumetric flask (Corning No. 55640).