RODENTICIDES

Problems Encountered in Physicochemical Determination of Warfarin

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Since the anticoagulant rodenticide, warfarin, was registered for general use (1950), commercial concentrates and finished baits have been assayed for warfarin content by physicochemical means. Concentrates and most finished baits presented no particular problem. However, two types of finished baits, one a coated grain and the other a pelletized grain mixture, yielded low results when assayed by the usual method employing a diethyl ether extraction. Substituting a weak alkaline solution for the diethyl ether allowed 90 to 100% recovery of warfarin in these baits. These findings were substantiated by biological assay.

ANTICOAGULANT RODENTICIDES had their inception with the isolation, identification, and synthesis of the active principle in spoiled sweet clover hay, which causes a hemorrhagic condition in livestock, particularly cattle, frequently resulting in death (2, 9, 13). This factor was found to be 3,3'-methylenebis (4-hydroxycoumarin), later designated as Dicumarol. Since its discovery, Dicumarol has played an important role in human therapy as a valuable agent in preventing coronary thrombosis and postoperative thrombi. Starting in 1940 Link and associates (9, 11) synthesized a large series of derivatives of 3,3'-methylenebis (4-hydroxycoumarin) and 3-substituted 4-hydroxycoumarins in an effort to find one with even better physiological and pharmaceutical properties than Dicumarol for therapeutic use. None proved to be superior to Dicumarol in the rabbit, used as the assay animal.

In 1948, the English worker, O'Conner (10), suggested the use of Dicumarol as a rodenticide. At the time of this suggestion, Scheel and Wu of Link's laboratory (12, 14) had re-evaluated a large number of the derivatives of Dicumarol and 3-substituted 4-hydroxycoumarins, employing the rat rather than the rabbit. Their research indicated that certain 3-substituted 4-hydroxycoumarins possessed greater killing powers than Dicumarol and related bis- compounds. The 3-substituted 4-hydroxycoumarin 3-(α-phenyl-β-acetylethyl)-4-hydroxycoumarin, now known as 3-(α-acetonylbenzyl)-4-hydroxycoumarin or by its generic name, warfarin, was found to be 40 to 50 times more potent in the rat than Dicumarol when given in divided doses. This finding prompted studies which demonstrated that this agent was an effective rodenticide (3–5).

The registration of warfarin for general use in July 1950 imposed upon manufacturers and regulatory officials the task of control, particularly on products in interstate commerce. Commercially, warfarin is available as a concentrate or as a finished bait. The concentrate consists primarily of cornstarch containing 0.5% warfarin. The finished baits are composed of single cereal grains or mixtures of such grains (either ground or cracked) containing 0.025% warfarin. The majority are prepared by the direct admixture of 1 part of the 0.5% concentrate with 19 parts of the bait material. Some baits also contain small percentages of fats, sugars, or other palatable ingredients.

Warfarin is a white crystalline solid with a melting point of 159° to 161° C. It is only sparingly soluble in water (4 mg. per 100 ml.) but more readily soluble in organic solvents—e.g., diethyl ether 1.93 grams per 100 ml.; benzene 0.4 gram per 100 ml.; Skellysolve B 1.25 mg. per 100 ml. As it is weakly acidic, it dissolves readily in sodium hydroxide solution with the formation of the sodium salt. Warfarin exhibits a characteristic absorption curve in the ultraviolet with a pronounced peak at 308 mμ. It has an extinction coefficient of 461 at 1% and 1 cm.

In the chemical determination of warfarin advantage is taken of the solubility of its acid form in organic solvents and its ready extractability therefrom into alkali in the form of its sodium salt. A quantitative estimation is made by determining the absorption of the alkaline solution at 308 mμ. This method is essentially the same as that described for Dicumarol (1).

The assay of warfarin in 0.5% concentrates has presented no particular problem. This method (originated by Jack Eble of Link's Laboratories, University of Wisconsin, Madison), referred to as Procedure I by LaClair (6), consists of a diethyl ether extraction of the concentrate, followed by a conversion of the acid form of warfarin to the sodium salt in aqueous sodium hydroxide. The latter solution is evaluated spectrophotometrically at 308 mμ against a sodium hydroxide blank.

Bait Formulations

Finished bait preparations have presented considerably more difficulties. Initially, Procedure III, a modification of Procedure I of LaClair (6), was employed. This method worked very well on most finished baits, yielding absolute values, providing an exact duplicate blank material was available for the necessary correction of the spectrophotometric reading at 308 mμ. Obviously it is impossible to obtain such blank materials

Table I. Determination of Warfarin on Coated Cracked Grains

| | % Warfarin | |
Sample	LaClair	Eble
1. Cracked corn + alcoholic warfarin solution	0.0110	0.0257
2. 1 + mineral oil spray	0.0106	0.0264
3. 2 + corn sirup spray	0.0024	0.0283
4. Blank	0.0007	0.0007

for over 150 bait formulations. In an effort to obviate the necessity of such blanks, correction values for various bait ingredients were determined, and correction blanks calculated from the bait composition were employed. In most instances the corrections were of very low order, but the wide variation in bait composition and the fact that blanks on the same component parts may vary, did not allow for complete assurance that

absolute values were obtained. In addition, certain additives to baits may also interfere at 308 mμ, thus further reducing the accuracy.

In the course of control work, two types of bait formulations have afforded considerable difficulty. One of these baits consists of partially cracked grains sprayed with an alcoholic solution of the acid form of warfarin. This initial spray is followed by subsequent sprayings of mineral oil and corn sirup, with final exposure to mild heat for drying purposes. The other bait is a pelletized one produced by the usual moist heat pelletizing process. Application of the LaClair (6) procedure yielded values markedly less than the theoretical.

In an effort to analyze these two types of baits, another spectrophotometric method was developed. This method (7) was also conceived by Eble and further modified by Coon and Richter (8). Briefly, it consists of extracting the bait material with a weak solution of sodium hydroxide or sodium pyrophosphate (rather than diethyl ether), thus converting the warfarin acid form to the sodium salt. The alkaline solution is acidified and extracted with a diethyl ether–Skellysolve B mixture. A weak alkaline solution is used to extract the warfarin from the organic solvents, again converting the warfarin to the sodium salt. The warfarin concentration is then evaluated spectrophotometrically against the appropriate blank.

Table II. Determination of Warfarin on Pelletized Baits

Size of Pellet	% Warfarin	
	LaClair	Eble
Small	0.013	. . .
Large	0.011	0.021
Small	0.011	0.025
Small	0.007	0.021
Small	0.013	0.025
Small	. . .	0.025

Corrected by means of calculated blanks.

In comparing Procedure III of La Clair with the modified Eble method, the main difference lies in the extracting medium—i.e., diethyl ether or a weak alkaline solution. Table I reveals the wide differences between the LaClair and modified Eble methods when applied to samples taken in the various stages of the coating process of the cracked grain(s). Apparently under these circumstances ether fails to be a good extracting agent. Recently LaClair (7) has substituted diethyl ether saturated with water for ether alone as the extracting agent. This change has resulted in substantially higher values which are in closer agreement with those obtained by the modified Eble procedure.

It is evident from Table II that a similar situation prevails with pelletized baits. The modified Eble method again gave substantially higher values than the LaClair method. This observation substantiates the fact that the method of extraction used in the LaClair procedure is not applicable to all types of finished baits. In the cases of pelletized baits, certain physical phenomena may take place which do not permit the diethyl ether extraction to remove the warfarin in its entirety.

In an effort to determine the effect of pelletizing on the warfarin content of a finished bait, the modified Eble method was applied to identical baits before and after pelletizing. The results indicated a loss of less than 10%. The ultraviolet absorption curves of the extracts were almost identical to that of pure warfarin. These data indicated that with the modified Eble method pelletized warfarin baits yield a potency value approaching the theoretical, based not only on a calculation obtained from the absorption at the wave length of 308 mμ, but also on a definition of the warfarin curve.

To substantiate further the advisability of employing an alkaline extraction procedure for the most complete removal of warfarin from pelletized baits, another approach was employed. In this series of experiments the residue remaining after the ether extraction (LaClair method) was subjected to assay by the modified Eble procedure. It is evident from Table III that a substantial quantity of warfarin remained in the residue after ether extraction. This was further attested to by the spectrophotometric absorption curve of the alkaline extract, which was almost identical to that of pure warfarin.

Biological Assay

In another series of experiments the physicochemical findings were confirmed by biological assay. The latter procedure consists of feeding suboptimal levels of warfarin to albino rats for 3 days, followed by an observation period of 7 days on an unsupplemented diet. The average score per level is obtained from the highest individual score of each rat during the 10-day period. Because death alone does not always indicate a complete evaluation, particularly when suboptimal amounts are fed, each rat is scored on the basis of the severity of the symptoms due to anticoagulant activity. The scoring was set up on an arbitrary basis as follows: no symptoms, 0; hemorrhage, 1; bleaching, 2; poor general conditions, 3; and death, 4. A series of levels of warfarin is used to establish a standard response curve. Simultaneously one or more levels of unknown are fed to another series of rats and the warfarin value is approximated from the response curve.

The results (Table IV) confirm the previous chemical findings and supplement them with biological assay data.

Thus it appears that a diethyl ether extraction (even after 24 hours) does not remove all the warfarin from pelletized baits. On the other hand, evidence is presented that the alkaline extraction of such a bait yields values approaching the theoretical.

Table III. Warfarin Values in Pelletized Baits and Residues

	Warfarin, %[a]
LaClair extract[b]	0.007
Eble extract of LaClair residue	0.017
Total	0.024
Eble value on original bait	0.0255
LaClair extract[b]	0.011
Eble extract of LaClair residue	0.015
Total	0.026

[a] Expressed as % warfarin in original bait (blank corrected).
[b] Ether extraction conducted for 24 hours rather than 30 minutes.

Difficulties have been encountered in the assay of certain warfarin baits as respects extraneous absorption of blank materials. Recently LaClair (7) reported on the introduction of a chromatography step in Procedure III to eliminate the absorption interference of materials other than warfarin at 308 mμ. Both silicic acid and Attapulgus clay when used as adsorbents yielded 100% recovery of warfarin. Using these adsorbing agents on a complex bait material, known to possess marked interference at 308 mμ, revealed values equivalent to the theoretical. Attapulgus clay apparently minimized the interference effect even more than silicic acid.

The application of the Attapulgus clay chromatographic step in the modified Eble procedure (8) proved to be of distinct advantage in this method as well. However, experience has demonstrated that all Attapulgus clays do not react in the same manner in allowing the warfarin to come through the column. In view of this, the clay to be employed should be tested prior to use in an assay. Evidence to date indicates that all interfering materials are not always removed by this chromatography step. However, this modification is a valuable adjunct to both procedures.

Some work has been done to devise a method of assay for warfarin in animal tissues. Certain difficulties are still extant in the absolute determination of warfarin in finished baits. The concentration present in a bait is many times more than that to be found in the tissues of animals poisoned by warfarin, and even more interfering materials may be present in tissues than in bait. Because of these complications, a usable method of assay for warfarin in animal tissues is not available.

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Table IV. Biological and Chemical Values of Pelletized Baits and Residues

	Total Dose of Sample, Mg./Rat	Av. Score per Rat	Total Dose of Warfarin, Mg./Rat		No. of Rats per Group
			Eble	Biological	
Warfarin	0.0625	0.25	...	...	8
	0.1250	0.75	...	...	8
	0.1875	1.37	...	...	8
	0.2500	2.37	...	...	8
LaClair residue					
A[a]	0.83	0.75	0.125	0.125	8
	1.25	2.37	0.1875	0.25	8
2B[a]	2.00	2.50	0.18	0.25[b]	4
4B[a]	3.60	2.50	0.18	0.25[b]	2
Eble residue	10.6	0.0	0.0	0.0	4
Pelletized bait	0.5	0.62	0.125	0.115	8
	0.75	2.00	0.1875	0.230	8

[a] Ether extraction conducted for 24 hours rather than 30 minutes.
[b] Values only approximations because of small number of animals used.

MILK SOURING

Effect of 2-Methyl-1,4-naphthoquinone on the Rate of Souring of Milk

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As 2-methyl-1,4-naphthoquinone (menadione) is known to be bacteriostatic in very low concentrations, the effect of relatively low doses administered orally to lactating cows was investigated. Menadione, added at a concentration of 0.1γ per ml. of milk, significantly retarded the rate of souring of milk at 37° C. Milk from cows not fed menadione generally soured in about 12 hours when incubated at 37° C. Milk from cows that received 25 mg. of menadione per day remained sweet for 18 to 24 hours. During one period the feeding of menadione or its addition to milk did not retard the rate of souring. More research will be required to determine the cause for such variations; the type of pasture and fodder may be a factor. The nature of the compound secreted with the milk after feeding menadione has not been established. The milk from cows which received these relatively low doses of menadione had no detectable "off-flavor"; when it finally became sour, no hard curds were formed and the milk had a clean sour odor. Pasteurized milk from menadione-treated cows remained sweet about 20% longer. When milk was stored at 20°, 10°, or 5° C., the effect of menadione was more pronounced. The results of these preliminary experiments should stimulate research on an economical and practical method of reducing the spoilage of milk by feeding of menadione to milk cows.

2-METHYL - 1,4 - NAPHTHOQUINONE (MENADIONE), the easily available precursor of vitamin K, was reported in 1943 by Armstrong and coworkers (6) to be bacteriostatic against several Gram-positive pathogenic cocci. Earlier reports indicate the importance of the quinone structure to bacteriostatic and bactericidal properties of certain compounds (5, 9, 11, 12). Later some of these observations were confirmed and extended (7, 8).

Because the menadione is bacteriostatic in very low concentrations, and vitamin K is secreted in small amounts in milk (7), it seemed worth while to investigate whether relatively low doses of menadione administered orally