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NEW POTENTIAL DIAGNOSTIC METHODS FOR IDENTIFYING ANTICOAGULANT RODENTICIDE POISONING IN DOGS AND OTHER NONTARGET ANIMALS

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Analytical methods for anticoagulants are complex and encumbered with highly sophisticated instrumentation for determination of anticoagulant concentrations. This greatly limits the application of anticoagulant measurement in biological samples as a diagnostic criteria to determine anticoagulant rodenticide poisoning. In the field of veterinary medicine, clinical laboratory measurements of the coagulation status in viable patients have been used historically as the means to make a diagnosis of anticoagulant poisoning. Nontarget animals (companion animals, e.g., dogs and cats; livestock; or wildlife), which are found dead in the context of an anticoagulant application area, are a tremendous diagnostic challenge in spite of postmortem evidence of a hemorrhagic diathesis common to death from such poisons. The recent understanding in the veterinary field (Mount and Feldman 1983, Mount et al. 1986) of the short versus long-acting anticoagulants has created a diagnostic dilemma in relation of vitamin Id therapy. It is therefore evident that analytical assays which possess high specificity, sensitivity, and economic feasibility are needed to better clarify these issues.

Assays which possess those features are immunoassays. These have been applied to hormones and steroids which are found at very low concentrations in biological fluids. They have also found application in drug monitoring and drugs-of-abuse identification in the medical field. The goal of this paper is to give an understanding as to how immunoassays are developed and how applied to anticoagulants. The development of such systems is timely and costly, but, once established, the cost per assay is minimal and their applicability is adaptable to most laboratory settings. The enzyme-linked immunosorbent assay (ELISA) is the immunoassay to be discussed. Using this system, assays can be run within an 8-hour day and sensitivity is in the ng/mL range, which is parts per billion (ppb) of biological material.

The primary goal in developing an immunoassay is to produce antibodies in a living animal which recognizes the substance of interest. When people are vaccinated with an infectious agent to protect them from a specific disease, they are injected with proteins from a bacteria or virus. These materials are antigenic, allowing their bodies' immune systems to be activated so as to process these foreign proteins, resulting in clones of lymphocytes which produce antibodies capable of recognizing these newly introduced foreign proteins. Proteins are large molecular weight complexes (greater than ~ 40,000 g/mole). Steroids, therapeutic drugs, as well as anticoagulants, are low molecular weight compounds (less than ~ 1000 g/mole) and are not antigenic. However, if these low molecular compounds are covalently coupled to proteins, they are made antigenic since antibodies produced against the coupled proteins will recognize these low molecular compounds when present in a solution uncoupled to protein. Thus these low molecular weight compounds can be defined as haptens; that is, by themselves these compounds will induce antibody production. But, when coupled to proteins, antibodies are produced which recognize the low molecular weight compounds when present in a solution containing the antibodies.

In order to produce antibodies against anticoagulants, which can be referred to as haptens, they must be coupled to protein. Therefore, the first objective in the development of an immunoassay is to covalently couple the anticoagulant to a protein. To do this, the anticoagulant (hapten) must have chemical groups allowing for direct coupling to protein or allowing for attachment of a chemical bridge which introduces a functional group allowing for covalent linkage to protein. This is summarized in Figure 1. The resulting product is referred to as the immunogen and used to vaccinate rabbits. The

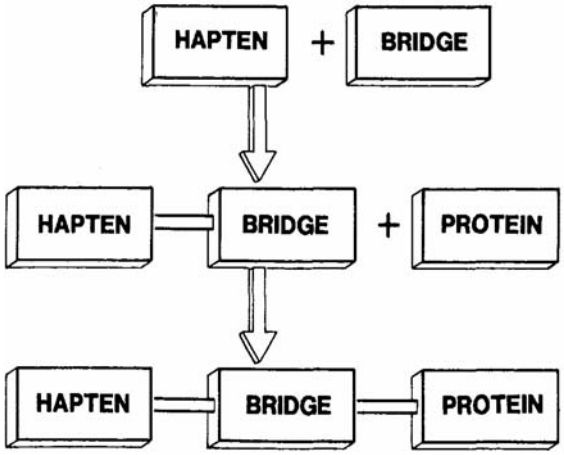
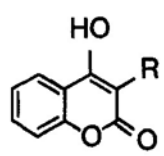


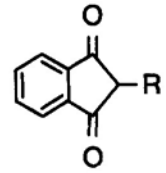
Figure 1. Basic steps required to convert a small molecular weight compound (Hapten) into an antigenic derivative. The bridge represents a chemical unit which is capable of covalently binding to the hapten so that the free end of the bridge contains a functional group which is able to covalently link to protein. Thus the HAPTEN is functionalized by linkage with the BRIDGE allowing for covalent binding with the PROTEIN. The protein-bound hapten is now antigenic.

1986, Proceedings Twelfth Vertebrate Pest Conference
(T.P. Salmon, Ed.). Printed at Univ. of California, Davis, Calif.

site of coupling is important in order to maintain high specificity of antibodies to the anticoagulant of interest. Figure 2 demonstrates the two basic chemical structures of anticoagulants used in the field



4-HYDROXY-COUMARIN NUCLEUS

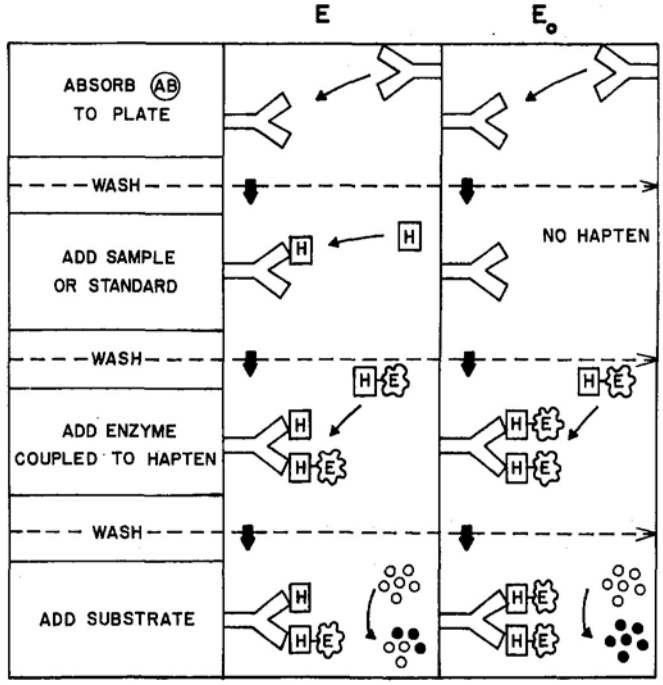


INDANDIONE NUCLEUS

Figure 2. All coumarin anticoagulants have the 4-hydroxy-coumarin nucleus and all indandione anticoagulants have the indandione nucleus. The "R" groups differ between the differing anticoagulants.

today. The --R group is the portion of the molecule which differentiates one coumarin (or indandione) from the other. Thus the goal of our work has been to couple via the coumarin or indandione nucleus thus exposing the --R group for antibody recognition.

Antibodies raised in rabbits following several months of immunogen vaccinations, are collected and applied to the ELISA. A competitive ELISA has been developed for diphacinone and is outlined in Figure 3.



COMPETITIVE ELISA

Figure 3. The competitive enzyme-linked immunosorbent assay (ELISA) is illustrated. 1) Antibody specific for the anticoagulant is adsorbed on to a solid support of a microtiter plate. 2) Samples, standards, and control reagents are added which occupy antibody binding sites depending on the amount of anticoagulant in those samples. 3) The enzyme-conjugate of anticoagulant covalently bound to enzyme is added and more or less binds to the remaining available antibody binding sites depending on the amount of anticoagulant in Step 2. 4) Substrate is added which is converted to a colored product dependent on the amount of enzyme present. Thus an accurate measure of the amount of anticoagulant and specificity for an anticoagulant can be determined.

The anticoagulant is coupled to the enzyme used in the assay. Antibody is a adsorbed onto a solid surface (Step 1). Samples which may or may not contain the anticoagulant (the hapten) and analytical standard concentrations of anticoagulant which are added to the solid surface will result in the "hapten" occupying the available antibody binding sites (Step 2). Thus when the anticoagulant-coupled-to-enzyme is added, more or less will occupy the antibody binding sites, depending upon how much anticoagulant was present in the sample or standard (Step 3). Following this, the substrate is added (Step 4) and allowed to develop, producing a color which can be measured with a spectrophotometer. The result is that a standard curve is generated which allows for accurate determination of the anticoagulant (hapten) in the biological sample.

Currently, diphacinone is readily detected in sera of dogs exposed to the anticoagulant diphacinone. Chlorophacinone, which is identical to diphacinone except for one chlorine on the --R group, does cross-react with the antibody, but it requires approximately 10 times the concentration of chlorophacinone to give the same effect as diphacinone. Sensitivity of the assay is 1 ppb. Since this compound is a long-acting anticoagulant, dogs or cats presented to clinicians with a coagulopathy syndrome and responsive to vitamin K_1, can now easily be positively identified as poisoned with diphacinone. This allows clinicians to be aware of potential relapse of clinical signs if taken off of vitamin K_1 therapy too early.

Currently a coupling method has been developed allowing for covalent linkage of the coumarin nucleus to protein. Warfarin, brodifacoum, and bromadiolone are soon to be tested for antibody production.

In conclusion, the application of ELISA to anticoagulant analysis offers tremendous advantages over conventional approaches. The diphacinone ELISA allows quick, sensitive analysis of sera and urines of animals thought exposed to diphacinone. Once identified, therapeutic recommendations can be assuredly made. The recent successful coupling of warfarin to protein opens the way to couple all coumarin anticoagulants with anticipation of being able to raise antibodies which are highly specific for each individual coumarin anticoagulant. With antibody in hand, ELISA development would follow, leading to rapid identification of the coumarin anticoagulant responsible for poisoning. Technological developments are currently available to perform rapid qualitative assays within 5 minutes using specially designed field kits. Such a kit for anticoagulants (once developed) would offer a revolutionary change in anticoagulant analysis and provide tremendous advantages to the veterinarian as well as to researchers and commercial operators to perform field monitoring.

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