

SECTION II

**ANALYTICAL METHODOLOGIES FOR
ANIMAL DRUGS: TISSUE RESIDUES**

Chairperson

Dr. Steven A. Barker

Cochairpersons

Dr. Richard L. Ellis

Dr. Robert Livingston

Analytical Detection Systems: Present and Future

Richard L. Ellis, PhD

*U. S. Department of Agriculture, Food and Safety Inspection Service,
Science, Chemistry Division, Washington, DC 20250.*

Introduction

It is a pleasure to address this Symposium sponsored by the American Academy of Veterinary Pharmacology and Therapeutics on analytical detection systems—past, present, and future. We rely heavily on residue analytical methods within USDA, Food Safety and Inspection Service to support the Meat and Poultry Inspection program. In 1986, we analyzed over 330,000 samples. These analyses included a broad spectrum of topic areas including antibiotic residues, food chemistry, chemical residues, food microbiology, species identification, non-residue pathology samples, and serological tests.

I believe it is particularly appropriate to review this topic with you because, in genealogical terms, our National Residue Program (NRP) is approaching adulthood. We have raised it through its infancy and adolescent growing pains, albeit not without close oversight by FSIS, the regulated industry, consumer groups, Congress, congressional oversight, and our peers. We are pleased in its growth and direction. There is, in many respects, a parallelism between the NRP development and the growth and development of our analytical detection systems over the past 20-25 years. Chromatography systems to separate individual components of a mixture were entering a period of rapid development that is still going on. It has been aided by integrating attributes of computer science, electronics, materials sciences, and the physical sciences.

Before getting ahead of ourselves, there is a touch of irony that played an important role in our NRP. Biotechnology is a very popular, if not magical, topic in the physical, biological, and agricultural sciences, among others. However, it is not new to the NRP or regulatory analysis. One of the earliest analytical methods used for pesticides was a bioassay. The difference between then and now was that we used the whole system as the detector not a highly refined, specific entity. In the age of acronyms, this one was F-L-Y. Meat samples were placed in an enclosed environment with a number of flies. If the flies died, pesticides were present. Granted, it was not sophisticated, highly sensitive or definitive for structure, but it was easy to read the analytical response. In a similar manner, test for antimicrobial and antibiotic residues commonly have been based on inhibition of microbial growth.

Twenty years ago, chromatographic sciences were emerging with the development of gas chromatography instruments. Their focus was on materials that could be converted into a vapor or gaseous state without thermal degradation. Many pesticides satisfied these needs. Detectors were developed beginning with the Thermal Conductivity Detector. Although it had by today's standards, low-level sensitivity (in the sub-milligram to microgram range), it was an important beginning. From here evolved the Flame Ionization Detector for carbonaceous materials and later for nitrogen and phosphorous compounds, the Electron Capture Detector for halogen containing compounds; the Flame Photometric Detectors, for phosphorus and sulfur containing compounds; the Hall Detector; for electron withdrawing groups; the Thermal Energy Analyzer, for nitrogen and oxygen functional groups and, near the close of the 1970s, the Thermionic Detector. During this period, sensitivity had improved to where sub-microgram to nanogram quantities of analyte could be detected. These analytical systems required a well equipped laboratory with trained analysts to achieve desired results.

Similarly, during this time-frame methods based on microbial inhibition were being developed and employed for laboratory use. Though they were highly capable of detecting microbial inhibition, they did not provide much structure identity. Using a battery of such assays however, permitted a finger-printing-like approach to tentatively identify a specific analyte. Where possible, efforts were placed on coordinating microbiological assays with chemical assays such as thin-layer chromatography methods to provide collaborative evidence of structure and quantity.

Thin-layer chromatography development was taking place during this same time period as was development of color-generating reagent systems to identify analytes. These color generating systems emerged from relatively nonspecific to relatively specific capabilities, giving color reactions dependent on functional groups in the molecules of interest.

Improvements in chromatographic adsorbents and concepts of improving flow rates comparable to gas chromatographic systems led to the emergence of High Performance Liquid Chromatography. This broadened the range of analysis that could be separated and quantified to include nonvolatile and thermally labile materials.

A new set of detection systems was needed. Again, early

models such as the refractive index detectors were relatively insensitive and nonspecific. Rather quickly, ultraviolet fixed wavelength detectors were developed followed by variable wavelength ultraviolet detectors, fluorescence detectors, and photo diode array detectors. Sensitivity now achieved nanogram quantities of analyte. To be sure, development of new chromogenic and fluorescent generating materials contributed to this dramatic improvement. Today, a growing percentage of our analytical methods depend on liquid chromatography technology. Again, however, these methods require a well-equipped laboratory to achieve desired performance.

Throughout this period as well, developments were made in methods to provide unambiguous structure of analyte. Mass spectrometry emerged as a mature technology going through similar improvements in design, and detection systems. When coupled with computer capability their power became evident. Again, new detectors were developed and one, the mass selective detector, filled a cost-effective need for residue analysis. However, again we are wed to well-equipped laboratories and highly trained analysts.

Clearly, the developments of the past have shaped the present. But during the decade of the 1980s, heightened awareness for food safety required more analytical capability and capacity. Higher levels of sampling were called for to provide greater assurance of a safe food supply for consumers. This can be described by the proposed National Residue Program for 1989 through 1993. Emphasis focused on developing and using rapid testing systems. This concept was facilitated by the biologically based sciences. Today, several immuno-based assay systems are being developed, such as enzyme labeled immunosorbent assays (ELISA).

Regulatory Method Attributes

For present and future needs, I envision regulatory programs employing methods with attributes of presumptive presence, quantitation and positive identification. To accomplish this goal, methods with these different attributes must perform in concert with each other for highly effective residue control in our meat and poultry supply, regardless of regulatory mandates.

Though the focus will be on screening methods, a brief description of the method types helps to understand their interrelationship.

Level I type methods incorporate the ability to quantify the amount of specific analyte or class of analytes and positively identify their presence in a single analytical procedure. These assays are unequivocal at the level of interest. They may be single procedures that determine both the concentration and identity of the analyte, or combinations of methods for determining and confirming a residue for definitive identification. Several mass spectrometry methods coupled with gas or liquid chromatography systems fit into this category.

Level II methods are not unequivocal. They are used to determine the concentration of an analyte at the level of interest and to provide some structural information. For example,

these methods may employ structure, functional group or immunochemical properties as the basis for the analytical scheme. They may be used to corroborate the presence of a compound or class of compounds. Thus, two Level II methods may provide information suitable for Level I attributes providing they employ different chemical technologies. The majority of laboratory methods presently available and used by regulatory control agencies are Level II methods.

Level III methods are those that generate imperfect though useful information. These procedures detect the presence or absence of a compound or class of compounds at some designated level of interest and often are based on non-instrumental techniques for analytical determination. Results on a given sample are not as reliable as Level I or II methods without corroborating information. Level III methods are not poorly described or sloppy methods, rather, they must have defined operating characteristics of reliable performance. Many microbiological procedures and immunoassay test systems may fall into this category. The advantages of Level III methods include convenience, potential suitability to non-laboratory environments, analytical speed, sample efficiency through batch analysis, portability to nonlaboratory environments, sensitivity, and the ability to detect classes of compounds. The hallmark of Level III methods is that action based on individual positive results require substantiation using Level I or II methods as required by the uncertainty of an individual results.

The use of Level III methods should be measured in part by their performance characteristics, as well as their ability to handle relatively large numbers of samples within a given time-frame. Two key characteristics requiring definition include their percent false positives (reporting a positive response when no analyte is present) and percent false negatives (reporting a negative response when the analyte is present) when measured against a validated quantitative assay in a statistically designed protocol. The percent false negatives must be quite low at the levels of interest while slightly more flexibility may be acceptable for false positives. A minimum level of residue detection can be described based on a balance between these two parameters.

Attributes of Screening Methods

To ensure analytical reliability, performance characteristics of a method must be determined by multilaboratory evaluation. By consensus, the principle attributes of analytical methods are specificity, precision, systematic error, and sensitivity. Other attributes relevant to screening methods will be described as well. Because of time restrictions, I will be brief in my comments on attributes.

Specificity is the ability of a method to respond only to the substance being measured. Methods should be able to qualitatively differentiate the analyte from analogues or metabolic products of the compound(s) of interest under the experimental conditions employed.

Precision is a measure of the variability of results when

separate portions of a homogeneous sample are analyzed. Precision is usually expressed as a standard deviation. Another useful term is the relative standard deviation, because it is usually constant over a considerable concentration range, ideally covering the level of interest.

Systematic error is analytical method bias, the difference of the measured value from the true, assigned, or accepted value (mean value). It is commonly expressed as the percent recovery of added analyte to a sample blank. Regardless of what average recoveries are observed, low variability is a desirable feature.

Accuracy refers to the closeness of agreement between the true value and the mean result. The accuracy requirements of different types of methods will vary with the use being made of the results. For screening methods, characteristics of false positive results and false negatives define a methods operating range.

The sensitivity of a method is a measure of the ability to discriminate between small differences in analyte concentration.

Beyond these method characteristics are a number of collateral criteria particularly suitable for screening methods for regulatory control programs. Methods should be rugged or robust, cost-effective, relatively uncomplicated, portable and capable of handling a set of samples simultaneously in a time effective manner. Ruggedness of a method refers to its capability to be relatively unaffected by small deviations from the established use of reagents, quantities of reagents, time factors for extractions or reaction or temperature. This does not, however, provide latitude for carelessness or haphazard techniques. Cost-effectiveness refers to use of relatively common reagents, efficient use of resources and using instrumentation common for trace environmental analyses. A method being relatively uncomplicated refers to use of simple, straightforward mechanical or operational procedures throughout the method. Portability is the characteristic of the method that enables it to be transferred from one location to another without loss of established performance characteristics. The capability to analyze a set of samples simultaneously reduces the analytical time requirements of sample analysis. This is particularly important for screening methods where large numbers of samples are to be analyzed in short or fixed time-frames.

The capability to analyze a relatively large number of samples in a given unit of time, and their robust nature allows latitude for using rapid test methods in nonlaboratory surroundings. In these instances, methods may often be used by individuals not necessarily experienced in analytical chemistry techniques. This places a constraint on certain types of methodology. It limits use of certain types of equipment, instruments and reagents. Further, methods need simple, unambiguous test instructions that will enable a tester to correctly prepare the test material, conduct the analysis, interpret and report the test findings. Process controls defining critical steps in the test procedure are very relevant

to the success of such a testing program.

In the analysis of regulatory samples, a principle objective for us becomes one of assuring we have a well planned and executed quality assurance program. Its importance cannot be overemphasized no matter whether in reference to laboratory based methods or rapid test methods that may be used in nonlaboratory environments. Proper use of methods and evaluation of results are of paramount importance because they may influence the disposition of product from a producer, an establishment or a country. Integrity of our program must not be compromised.

Integrating a Residue Monitoring Program

Integrating rapid test methods for residue programs can depend on residue violation rates and public health food safety issues.

With a low level violation incidence from statistical based random sampling programs, screening methods are particularly attractive because they allow for methods to test large numbers of food products. Residue monitoring data indicates that the large majority of samples contain nondetectable and low level concentrations of residues. Having a screening test provides programs, with the opportunity to pass product with nondetectable residues or detectable below a tolerance and to retain suspect positives for more definitive laboratory analysis. Screening tests allow for more effective use of expensive laboratory resources and also the significant costs involved in sample collection and shipping all samples to a designated laboratory.

A noteworthy application of a rapid test method is the Sulfa Onsite (SOS) test for sulfamethazine residues in hogs. SOS provides FSIS and its inspectors with the capability to rapidly test animal urine for sulfamethazine. The test is being used to clear hogs with sulfamethazine below a level of interest and to retain suspect carcasses for quantitative analysis in the laboratory.

For other situations where there is a known or high residue violation incidence, quantitative immunochemical, enzyme inhibition assays and thin-layer chromatographic systems designed for rapid testing in laboratory environments become very attractive. They provide data to take regulatory action on violative product.

In detecting residues of unapproved pesticides and veterinary drugs or drugs used in an unapproved manner, residue screening tests are very attractive. In these situations detection of any amount of residue in meat and poultry is a residue violation. Screening test results normally require support by a confirmatory procedure. In this scenario, quantitation is not a specific requirement.

Perceived constraints with screening tests are that they are not specific and they consume too large a portion of valuable resources to identify the residue of interest. A possible resolution to this would be to develop a hierarchy of test methods based on current and emerging technology focusing on the concept of simplicity in design and application, auto-

mation technologies and commercially available systems and equipment with potential for broad application. Before going back to the future with biotechnology based methods mentioned earlier, it is worthwhile to note what is currently available offering potential for regulatory control programs.

Thin-layer chromatographic procedures satisfy a significant number of desired attributes for screening methods. With these procedures there is a wide variety of absorbants, chromatographic solvents and reagents facilitating residue detection. In addition, residue detection is a static process rather than a dynamic one, making quality assurance easier because control samples and reference standards can be analyzed simultaneously with the test samples.

In addition to the SOS test for sulfamethazine, one that has been reduced to practice for a regulatory program consists of thin-layer chromatography for separation of organophosphate pesticides using cholinesterase enzyme inhibition for residue identification.

Although these are limited applications for regulatory programs, this technique offers promise for the future as new reagents improve sensitivity and thin-layer chromatography systems provide new approaches for effective sample purification and analysis.

The detection limits of most color-producing or fluorescent-generating reagents provide sensitivity at low microgram per gram (ug/g, ppm) concentrations. Reagents using enzyme inhibition allow detection limits in some systems at low nanogram per gram (ng/g, ppb) concentrations. For example, many herbicides employing photo-synthesis inhibition as a mechanism of action have been detected at picogram (10^{-12} g) levels using plant chloroplasts and a reduction-oxidation chemical indicator. Classes of herbicides adaptable to this detection system include triazines, phenylureas, phenylcarbamates, uracils and acyl anilides. This suggests the possibility of broad spectrum screening tests suitable to nonlaboratory use.

Immunological methods for many antibiotic and antimicrobial agents including chloramphenicol, several sulfonamides, neomycin, and gentamycin are being evaluated for regulatory use. Similarly, test systems are available for the aflatoxins.

Immunochemical assays are also available for the chlorinated triazines, paraquat, chlordane (heptachlor, dieldrin, endrin, aldrin, and endosulfan are detected via cross reactivity), pentachlorophenol and polychlorinated biphenyls (PCB's) at levels of interest. FSIS has method development contracts for developing immunochemical assays for heptachlor related organochlorine pesticides, ivermectin, synthetic pyrethroids (permethrin, cypermethrin, and deltamethrin) and nitroimidazoles in meat and poultry tissue. These methods are expected to provide improved laboratory analytical capability for these analytes. Development of qualitative screening assays are possible.

A commercial pesticide detection system based on cholinesterase enzyme inhibition has been developed. The enzyme ticket system detects common insecticides used in the United States at concentrations in the low ng/g (ppb) range in water systems. The design of the system allows for a two-tier analytical scheme that will allow differentiation of organic sulfur containing organophosphate insecticides from their oxygen analogs. This advantage reduces some of the needs of further analysis to quantify and confirm these analytes. Research is being done to enable analytes from an organic extract to be analyzed with the test system. Quantitative and confirmatory analysis using other analytical technologies is needed to support these qualitative methods.

Implementing Immunological Methods

Adopting qualitative or quantitative immunochemical assays is likely to take time before confidence and recognized legal status for such methods is attained. It may require considerable experience and familiarization with the technology by regulatory agencies to use test systems containing unknown reagents ("black box" test systems) to assure ourselves that public health protection is not compromised.

Occasionally in the development and design of ready-to-use products such as these tests, reliability and consistent performance of the assay can vary from lot to lot. Quality control for production will likely improve with experience. Nevertheless, users of these systems must employ good quality control/quality assurance protocols to ensure method performance. Developing data packages for such systems would be a step in the right direction to facilitate their acceptance by regulatory agencies planning to use these methods.

Some of these assays are more sensitive than the quantitative and confirmatory assays now available, so that these qualitative results may not be confirmed at the same level of sensitivity. This may limit further regulatory use or hopefully, drive technology to develop new quantitative and confirmatory methods to match the sensitivity levels. Regulatory agencies need to confirm what they have the capability to detect.

We have to address the heavy reliance on using aqueous media for performing these assays. For certain food types this may be of little consequence; for others, e.g., meat and poultry tissue, it may be a measurable deterrent. For example, most chemical-based assays rely on use of organic solvents to release the analyte of interest from the test sample matrix. This requires developing solvent systems providing sufficient transfer from the organic extraction solvent to the test system while not denaturing or deactivating the immunochemical reagents. Progress is being made in this area. One company has developed an assay for chlorinated triazines (Res-I-Mun ©) that allows low ng/g (ppb) detection using select aqueous organic solvent systems. This system is currently being evaluated by FSIS for meat products.

Future Development

As the past has helped shape the present, so does the present begin to shape the future. Future regulatory programs will be influenced by increasing requirements for more analytical testing for assurance of food safety, by facilities, equipment and personnel, and by continuing development in biotechnology, computer science, materials science, physical sciences, and electronics. Futures forecasting is influenced by the information available to the forecaster. With that in mind, here are some of my perceptions and avenues for future regulatory analytical developments.

Sample preparation considerations are becoming a rate-determining step in the application of rapid testing methods. With immuno-based assays, we can presently analyze hundreds of prepared samples daily. Developing rapid, and hopefully, universal extraction procedures may emerge from applications of supercritical fluid chemistry either alone or in combination with ultrasound or other physical aids, and use of solid phase extraction media containing detergent-like surfaces.

Pharmacokinetic and drug metabolism data of veterinary drugs and pesticides will provide new approaches for residue analysis. For example, residue concentration relationships in tissue and biological fluids will provide new opportunities for field or plant inspectors to predict potential residue concentrations in animal carcasses. One can also imagine that mechanism-of-action data will provide potential enzyme reactions as models for residue analysis. Such would open opportunities for bio probes, basically, electrodes coupled with biological membranes, that generate a very small electrochemical reaction at the cellular level that can be electronically amplified to produce a measurable response. Systems similar to this concept exist now for monitoring glucose and insulin levels.

Analytical detectors and readout systems, based on concepts from process analytical chemistry, computers and vid-

eo recording systems offer potential for overcoming the labor intensive sample cleanup that is common with today's procedures. Portable video cameras coupled with computer microchip technology may allow analysis of multicomponent samples by selectively scanning specific regions of light from the ultraviolet to the infrared and displaying resultant individual components. Results may be stored in memory and later transcribed to a laboratory data base for analysis and evaluation. These concepts are part of the development now taking place with process analytical chemistry and expert system analysis.

Technology will likely assist us in developing structural identity procedures that do not rely on mass spectrometry. Advances in techniques such as Fourier Transform-Infrared spectroscopy may make this technology economically feasible. Other possibilities include multidimensional chromatography with video scanning, as previously mentioned, or with existing diode array detectors. It is also within reach to perform mass spectrometry structure identification of analytes from thin-layer chromatographic systems. This concept is now being developed. Further away perhaps, is the potential use of piezo electric crystals that generate electrical signals in response to changes in pressure such as vibrational stimuli.

On the chemicals side, we can expect to identify more sensitive and specific color forming reagents and fluorescent generating reagents and fluorescent probes, selective biochemical-color forming reagent systems that match the sensitivity, specificity performance characteristics needed for regulatory methods.

Clearly, these are speculative concepts. We will need keen intellect and an alert eye to separate the science from the fiction. I am optimistic however, that many of these advances will find useful places in our future. Perhaps in another 20 years, we can look back on these techniques to focus again on the future.

Analytical Methodologies for Establishing Residue Control

M. A. Norcross, VMD, PhD; A. R. Post, PhD and
W. R. Miller, DVM, PhD

*U. S. Department of Agriculture, Food Safety and Inspection Service,
300 12th Street, SW, Washington, DC 20250.*

Introduction

No single entity has full responsibility for monitoring and controlling the use of animal drugs and agricultural chemicals in the food production sector. The control of residues is a complex and difficult task shared by government, academia and industry and to be successful there must be continuous and complete coordination and cooperation between those responsible for residue control. Symposia such as this one greatly enhance our efforts to improve mechanisms for cooperation between residue control entities.

The past 4 decades have seen a remarkable scientific revolution in agriculture in which chemical technology has played a crucial role. Pharmaceutical and other chemicals have been used to fertilize the soil, destroy pests, enrich feed, control disease and promote the growth of livestock and poultry. Few will question the advances conferred on today's agriculture by chemical technology. The use of these chemical agents has assured an abundant wholesome food supply and in many ways contributed to the health and welfare of our nation. While these chemical entities have contributed positively to each of these goals, in some instances they have had undesirable side effects, including polluting the environment, jeopardizing human health, or, in the longer term, intensifying the productivity problem the chemical was to solve.

The exposure of food-producing animals to environmental contaminants, or the use of pesticides or animal drugs in a way that does not conform with approved uses, can leave unacceptable levels of chemical residues in edible tissues. Advances in analytical technology have made possible the detection of increasingly lower levels of these chemical residues, causing new concerns about their potential toxicological significance.

It is a fact that most agricultural chemicals have some toxic properties, and while the scope and degree of toxicity vary widely among the compounds, virtually none can be considered harmless. For most toxic effects there is a threshold dose. Agricultural chemical use is regulated so that the threshold dose of toxicity is not exceeded and that safety is assured when these chemicals are properly used. It is not surprising, therefore, that the general public is aroused when

instances of careless or unnecessary use of agricultural chemicals are brought to their attention.

National Residue Program

Since 1967, the Food Safety and Inspection Service (FSIS), United States Department of Agriculture (USDA), has conducted a National Residue Program (NRP) to help prevent the marketing of animals containing illegal residues of drugs and other chemicals. FSIS is responsible for enforcing the Federal Meat Inspection Act and the Poultry Products Inspection Act (1). Under these laws, the Agency ensures that meat and poultry products in interstate commerce and for import are wholesome, not adulterated, and properly labeled and packaged. The meat and poultry inspection laws provide for the retention or condemnation of carcasses suspected of containing or found to contain illegal drug and chemical residues. To accomplish this, about 7,000 Federal inspectors and veterinarians carry out inspection in some 7,200 meat and poultry plants throughout the country. This is the largest inspection force in the Federal government, both in absolute numbers and in the ratio of inspectors to regulated facilities. In 1987, USDA inspected 121 million head of livestock, nearly 5 billion birds, and 127 billion pounds of processed meat and poultry products. As part of this responsibility, samples of meat and poultry are collected at slaughtering establishments. Each year, over 2 million laboratory analyses and in-plant tests are performed on over 450,000 samples to support our residue and microbiological monitoring and surveillance programs. Each chemical residue sample costs an average of \$140 to process—including collection, shipping, testing, overhead for laboratories and scientific support.

FSIS carries out its residue control program in cooperation with other Federal agencies. For the most part, these efforts are coordinated among FSIS, the U.S. Food and Drug Administration (FDA) and the U.S. Environmental Protection Agency (EPA). Both FDA and EPA establish tolerances for compounds to which livestock and poultry are exposed. They also determine the approved methods of use of compounds on specific crops or animals to ensure that tolerances will not be exceeded. EPA establishes tolerances for pesti-

cides, while FDA sets tolerances for animal drugs and other nonpesticide contamination in food. Where formal tolerances are not established, FDA and EPA recommend, upon request, action levels to FSIS for unavoidable contaminants.

Under the provisions of the Food, Drug and Cosmetic Act (FDCA), FDA has authority over the approval and regulation of all human and animal drugs (2). The approval procedure for these drugs involves, among other considerations, the efficacy of the compound proposed for use, safety to the target species, human food safety, environmental safety and methods of analysis. When animal drugs are approved, use restrictions, withdrawal periods, and tolerances are established.

EPA enforces the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). Under the law, the Agency has the responsibility for regulation and premarketing registration of pesticides, including the establishment of tolerances (3). EPA is also responsible for the regulation of industrial chemicals under the Toxic Substances Control Act (TOSCA). In enforcing residue limits in meat and poultry products, FSIS relies on information and tolerances established by FDA and EPA. Presently, USDA has no regulatory authority to establish criteria and standards for controlling the use of agricultural chemicals on the farm and in livestock and poultry production.

Criteria for Compound Evaluation and Selection

It is not feasible to monitor for residues of all chemicals in meat and poultry. Further, this is not necessary to adequately protect public health. It is, however, important to assess the likelihood that animals exposed to chemicals may contain residues at levels of concern and to conduct monitoring, where test methods are available, for those chemicals that are most likely to present the greatest potential risk. A hierarchic compound assessment scheme is used for this purpose.

Each compound is evaluated on a number of factors to judge the potential for animal exposure and significance for human health. These factors include:

- Amount of actual or probable use;
- Conditions of use as related to residues at slaughter;
- Potential for misuse to result in harmful residues;
- Metabolic patterns of the chemical in animals, plants and the environment, including the bioavailability and persistence of residues; and
- Toxicity of the residue.

Compounds are selected for monitoring and included in a plan for the calendar year based on several factors, including:

- Compound ranking assigned;
- Whether a practical test method is available and is suitable for regulatory use;
- Whether the compound is measurable in a multi-residue method where many compounds, even though all may not be assigned a high ranking, can be tested for at a relatively low cost; and

- Monitoring or other experience that shows whether adulterating residues are present in meat and poultry.

FSIS works from a list of about 400 compounds that includes certain environmental contaminants in addition to animal drugs and pesticides. At present, FSIS has suitable regulatory methods of analysis for 145 of these compounds. Some compounds are routinely included in monitoring on a cyclical basis to confirm periodically that a potential residue problem does not exist. Cycling of compounds in monitoring allows the Agency to include more compounds in the program than would otherwise be possible within its resources. Compounds rotated out of the program for a specific year are not disregarded; if the need arises, they can be added during that year. Over the last 10 years, virtually all the residues for which a suitable method was available have been monitored, except when a compound had an especially low ranking.

The process of compound evaluation and ranking is a dynamic one. Additional compounds must be considered as they are introduced, agricultural use practices change, and additional research on a compound's toxicity and its potential for leaving harmful residues may affect previous rankings. The Agency uses an advisory board of scientists from EPA, FDA, and USDA (FSIS and the Agricultural Marketing Service) to identify significant new information that may affect a compound ranking or indicate an urgent need for monitoring.

In 1985, FSIS announced the implementation of a new prototype Compound Evaluation System (CES). The CES is designed to provide the Agency with a more systematic approach to the categorization of compounds and to the analysis of their likelihood of occurrence in meat and poultry and their potential impact on public health.

Briefly, the CES addresses the risk of residues in meat and poultry as a function of two major elements. They are hazard (adverse effects of a given compound) and exposure (residue level and amount of product consumed containing residue of concern). The proposed system is a two-value, hierarchic compound ranking scheme that classifies a given pesticide, animal drug or contaminant in any one of 16 categories. Compounds of greatest concern carry a designation of A-1 (high health hazard potential; high likelihood of residue occurrence). Those compounds of least concern are designated D-4 (negligible health hazard potential; negligible likelihood of residue occurrence). Care is taken to avoid the use of exact numerical rankings that might suggest a high degree of sophistication which is possibly not justifiable because of data limitations or the assumptions inherent in the ranking process (4, 5).

FSIS believes that the Compound Evaluation System is sufficiently flexible to permit rapid response to new information that may affect previous rankings and to allow for the use of scientific or expert judgment. However, it must be emphasized that the CES was neither designed nor intended for use in the development of formal quantitative esti-

mates of risk from meat-borne residues (6). Rather, it provides a rational basis for changes in compound emphasis within the National Residue Program and encourages development of new analytical methods for important compounds for which no methods exist. As such, the CES serves as a useful guide in the planning and allocation of FSIS program resources for those residues considered to represent the greatest potential effect on public health. The CES is updated as appropriate to provide the FSIS with a constant, informative, and sound approach to dealing with residues in meat and poultry.

Residue Enforcement Programs

The National Residue Program (7-10) has three major aspects:

1. **Monitoring** is conducted to obtain information on the frequency and levels of residues occurring nationwide over time. (Area monitoring may be conducted where a localized potential problem appears.) Monitoring information is obtained through the statistically-based selection of random samples from healthy appearing animals under inspection. (Probability of 95 percent that a residue problem will be detected if the problem occurs in 1 percent or more of a major species or production group.) The samples are then analyzed for a predetermined number of residues based on an annual plan. The data are used to evaluate residue trends and to identify problems within the industry where educational or other efforts may be employed to correct problems. The information is also used to identify producers marketing animals with violative levels. When such producers subsequently offer animals for slaughter, the animals are subjected to testing until compliance is demonstrated. Thus, monitoring not only gathers information, but also deters practices that lead to violative residues.

2. **Surveillance** is designed to investigate and control the movement of potentially adulterated products. The sampling is biased and is directed at particular carcasses or products in response to information from monitoring or other sources (e.g., industry members or a State agency), or from observations during ante- or post-mortem inspection indicating that adulterating levels of residue may be present.

3. The **exploratory** phase is conducted for a variety of reasons such as increasing information about specific chemical compounds and their use or establishing data on the transfer of naturally occurring toxins from feed to livestock, e.g., mycotoxins in corn or peanuts.

In addition, FSIS is implementing a nationwide data base, the Residue Violation Information System (RVIS), to receive, store, cross-reference, and manage all residue data obtained by FSIS, FDA, or other agencies from residue violation cases. This will include names and addresses of seller and producer, dates, and results of investigation. USDA will also implement a swine identification program to trace hogs back to their farm of origin. A number of industry and consumer groups have urged the Department to broaden its iden-

tification requirements. A national swine identification program will facilitate not only residue control efforts, but also activities to control the spread of swine diseases.

The National Residue Program is described each year in a plan, published at the beginning of the calendar year in a document entitled *Compound Evaluation and Analytical Capability/National Residue Program Plan* (11).

Overall, the number of products containing violative residues is quite low—about 1 percent of samples tested. Violative residues are found less frequently in poultry than in livestock. More occur in swine than in other species; the fewest are in fed cattle and broilers. Yearly summations (1983-1987) of our domestic residue monitoring data are published in a document entitled *Domestic Residue Data Book, National Residue Program* (12).

Analytical Methods

The FSIS residue program uses recognized methods—both official and unofficial—for the analysis of samples from slaughtering establishments. Official methods include methods approved by the Association of Official Analytical Chemists (AOAC), validated methods, Federal Register methods, and historical official methods. AOAC methods are those that have been subjected to extensive interlaboratory study involving at least five laboratories before official acceptance by AOAC. Validated methods have been subjected to interlaboratory study by three or more laboratories and reviewed by a peer group of government scientists. Federal Register methods are those that have been published in the Federal Register and later incorporated into the Code of Federal Regulations. Historical official methods are those that have been considered to be the best available at the time of initial acceptance and continued in use over an extended period of time in the absence of a more effective method (11).

Unofficial methods are those requiring additional product analysis before regulatory action is taken. These methods are used to determine the need for official methods in product testing and as a preliminary phase of official methods development. There are three types of unofficial methods—the pilot study, the screening, and the published method.

The pilot study method is developed in a single laboratory, where statistics on methods performance are generated. The method is used to determine the need for additional testing or the development of official methods. The screening method is semiquantitative and is used to determine the presence or absence of compounds. A screening method permits rapid processing of large numbers of samples in less time than would be required by an official method. Positive results at or above specific levels require further analysis by an official method. A published method is one that has been published and subjected to a ruggedness test in an FSIS laboratory but has not been thoroughly evaluated outside the originating laboratory. It may be used in nonrecurring analyses and requires the development of a rigorous protocol for sample analysis. Before acceptance of analytical results for

regulatory action, it is necessary to repeat the entire series of analyses through an official method.

Methods Development

Methods development by FSIS proceeds in response to information indicating the need to detect a specific residue. To prove the efficacy of a given test method, FSIS generally evaluates the performance of the method by analyzing blank tissue, spiked tissue, and tissue with incurred residue at various levels of interest and using tissues from the various species of interest. The method is subjected to a statistically designed ruggedness test to identify the weak points in the method and to determine the limits that these weak points can be subjected to without having an effect on the analytical finding.

To provide proof of effectiveness in producing accurate and reproducible data, the method is subjected to a validation study involving three or more laboratories and perhaps an evaluation by the AOAC, using at least five laboratories.

A quality assurance plan, a systematic scheme to provide documentation of the quality of a determination, is devised as part of the development of the new method. Quality Assurance plans are also developed for existing methods (13). As part of the plan, each analyst using the new method in FSIS laboratories is required to successfully analyze a series of validation samples before the analyst is qualified to analyze official samples. The methods used in our laboratories vary considerably in time, complexity, and expense. The Agency's goal in recent years has been to develop a reliable test for laboratory use, and then to devise a simple, convenient form for screening, first in the laboratory and then at the slaughter plant or dockside.

Many of the standard methods of testing are laborious, expensive, and slow. Although standard methods have the great advantage of being definitive and providing state-of-the-art results for regulatory use, quick, inexpensive, relatively sensitive screening tests can inform regulators and producers of specific problems and, furthermore, can provide much valuable information within a short span of time. Positive results will still need to be confirmed by chemical detection. Conversely a negative finding demonstrates that none of the compounds included in that particular class are present at detectable levels. The approach can be defined essentially as a QQC sequence: qualitative, quantitative, confirmative.

The Emergence of Rapid Tests

Recent advances in science and technology have made possible rapid, sensitive and inexpensive biotechnological procedures based on immunological and recombinant DNA principles and chemical procedures. Some of these procedures can be applied at the processing facility or farm level for screening residues in meat or poultry tissue. Currently, FSIS uses 3 major categories of rapid tests to screen for the presence of residues: microbiological, chemical and immunological assays. These techniques lend themselves to regu-

latory residue control programs. Adaptation of these techniques for regulatory use relies on their development and refinement (14-16).

For example, the calf antibiotic and sulfa test (CAST) was designed to detect violative levels of antibiotics and sulfonamides in bob veal calf kidney tissues. The CAST is a microbiological assay. Under the CAST regulatory program, begun in 1984, producers and dealers could certify that their calves do not contain violative residues, in which case the healthy appearing, certified calves could be processed without delay. In contrast, each lot of noncertified calves had to be held overnight until a statistically-based number of calves were tested and passed. All calves showing signs of disease or treatment or that were from previously identified violative sources had to be tested. The CAST program has been very successful in reducing violations from a high of 5.5 percent of calves tested during the summer of 1984 to about 1.5 percent during May of 1988.

Recent changes in treatment and marketing practices led FSIS to make changes in the CAST program; these changes were published in the Federal Register on January 20, 1987. At first, sulfonamides had accounted for a large share of the violations, but we now find a greater variety of antibiotics, some of which cannot be identified with present methods. Because of marketing changes, some establishments can no longer purchase certified calves, thus burdening FSIS with testing responsibilities regardless of the establishment's compliance history. We are also concerned that certified calves have been found to contain violative residues. Under the new CAST interim regulations, the number of healthy-appearing calves tested will be based on a percentage of the estimated day's slaughter. Initially the rate in all establishments will be 5 percent for certified calves and 10 percent for noncertified, but the rate may increase or decrease at each establishment according to its compliance history. Among other changes in the interim rule, explicit authority is given to reduce line speeds to accommodate testing in those plants that require a high level of testing because of noncompliance.

Sulfonamide residues rank highest among all compounds in prevalence of detectable residues found. Ten years ago, more than 13 percent of slaughtered hogs had violative residues of sulfonamides, mostly from sulfamethazine. USDA and industry cooperated in an educational campaign aimed at informing producers and their feed suppliers about the problem and about prevention measures. The violation rate dropped significantly, but has persisted at an unacceptably high level. In 1987, approximately 3.6 percent of market hogs tested contained residues above 0.1 parts per million in liver, indicating that producers of those hogs did not employ a proper withdrawal period before slaughter.

Recently, the National Center for Toxicological Research conducted studies on the chronic toxicity and potential carcinogenicity of sulfamethazine in rats and mice. The data show that sulfamethazine is associated with dose-related

thyroid gland adenomas. In February, FSIS announced an intensified monitoring program to reduce violations as rapidly as possible and in March FSIS announced the details of the plan. The plan includes the development and publication of a proposed rule to use rapid sulfamethazine screening tests in hog slaughtering plants, programs for lot testing, and voluntary preslaughter testing of live hogs. Until recently, laboratory testing of swine tissue (liver and muscle) has been performed only under the National Residue Program. Under the NRP, inspectors collect samples of liver and muscle tissue from animals as directed and submit these samples to an FSIS laboratory or an FSIS accredited laboratory. The samples are then analyzed using the Sulfa Thin Layer Chromatography Fluorescence (STLC-F) analysis (an AOAC approved method of analysis for the presence of sulfamethazine residues). The liver sample is tested first, and if the results indicate violative levels of sulfamethazine (above the tolerance of 0.1 ppm), the muscle tissue is analyzed to determine whether the muscle tissue also contains residues above the tolerance. The residue level in the liver is approximately 3 times the residue level in muscle tissue. Therefore, even though the liver is above tolerance, the muscle tissue may not be. If the level of residue in the muscle tissue falls between 0.11 and 0.15 ppm, i.e., slightly above the tolerance of 0.1 ppm, FSIS policy has been to further confirm the test result through the use of Gas Chromatography/Mass Spectrometry (GC/MS) analysis (an AOAC approved method of confirmation analysis for sulfonamide residues). The GC/MS confirmation will no longer be performed. This decision was based on the high degree of STLC-F correlation versus subsequent confirmation using the more costly and time consuming GC/MS procedure. Thus, the use of the more cost effective STLC-F procedure provides the same degree of consumer protection and reliability as the GC/MS test.

Samples collected under the intensified monitoring program for sulfas announced in February of this year will test only muscle to determine whether violative levels of sulfamethazine are present in the carcasses. From the approximately 1,600 muscle samples tested under the intensified monitoring program, there have been 29 violations. It must be understood that this is an inspectional program and one cannot extrapolate these figures to a nationwide incidence.

As part of the announced intensified monitoring program, FSIS scientists developed a rapid screening test known as the Sulfa-on-Site (SOS) Test to be used by Agency inspectors in Federal establishments to estimate the level of sulfamethazine residues in swine tissues in a matter of a few hours. The laboratory method of analysis is inappropriate for on-site use because it must be done in FSIS laboratories or FSIS accredited laboratories, and test results generally are not available until 3 to 4 days after the sample was taken (17, 18).

The SOS Test involves the use of thin layer chromatography coupled with fluorescent detection. Samples of urine are spotted on chromatography plates along with two stan-

dards containing known amounts of sulfamethazine. The plates are developed and viewed under ultraviolet light. The test is calibrated so that test readings predict liver or muscle tolerance violations corresponding to the 0.1 ppm tolerance for sulfamethazine.

FSIS began phasing in the SOS test in April and it is now being used in 100 of the largest swine slaughtering establishments. FSIS plans initially to test 6 hogs each day in each of these establishments. Carcasses of pigs selected for testing will be retained pending results of the SOS screening tests.

To date, the results of the in-plant testing program using the SOS test show 60 confirmed violations from approximately 11,400 SOS tests conducted. Like the muscle testing program described above, the results of this program cannot be extrapolated to national incidence figures. This program also is an inspectional program and is not designed to obtain national incidence data.

Where results of the screening test indicate that carcass muscle tissue may contain levels of sulfamethazine or other sulfonamide residues above tolerance, the carcass will be retained until the results are confirmed by quantitative analysis at an FSIS-accredited laboratory.

Additionally, FSIS is coordinating the joint efforts of the Extension Service and veterinary medical associations to expedite the development of a preslaughter testing program for detecting violative hogs.

A technology of growing importance to the Agency is the immunological assay, particularly in the matter of chloramphenicol (CAM). Chloramphenicol is used effectively against a wide range of microorganisms, e.g., *Salmonella typhi*, other salmonellae, *Clostridia*, *Listeria*, etc. Because of definite evidence of serious bone marrow effects, the medical community reserves CAM use for those patients with infections where it has been shown to be clearly advantageous and for infections that have failed to respond to other therapy.

Chloramphenicol is not approved for use in food animals in the USA. The only approved use in this country at this time is for medicating dogs and cats. The drug is readily available to the veterinary community and is illegally used on occasions to treat meat animals. Some nations exporting meat to the USA also legally allow CAM use in medicating food animals. The potential, therefore, exists for CAM residues in the meat supply. In response to FSIS/FDA concerns that CAM was being used illegally in bob veal calves sent to slaughter, FSIS conducted an exploratory survey at several slaughter plants. The CAM card test, an enzyme-linked immunosorbent assay (ELISA) specific for CAM at low parts per billion concentrations and rapidly performed on site, was used in plants to test slaughtered calves' urines for CAM residues. More than 300 calves were tested over a 2-month period. Urines from several calves were CAM positive by the card test which could not be confirmed in tissue by chemistry methodology at that time. To resolve this

problem, FSIS chemists developed more sensitive GC/MS methods for CAM in urine and muscle. The CAM calf project was conducted to verify that the new GC/MS methods would indeed confirm CAM card positive urine findings. Data derived from the CAM calf study confirm that now all positive in-plant CAM card urine tests will be confirmed by the new GC/MS methods. The CAM card urine test is scheduled for implementation at selected bob veal slaughter plants in FY 1989.

Federal meat inspection regulations require that prior to condemning edible meat for biological residues the residue must be identified and quantified to confirm the violation. FDA regulations require that before prosecuting a producer for misuse of antibiotics, etc., the samples must be analyzed and confirmed violative by validated definitive methodology. The CAM card test is acceptable for screening samples for CAM and confirming CAM residue, but cannot be used for definitive quantitation, only estimation.

Conclusion

FSIS is making a determined effort to reduce chemical residues in the meat and poultry supply. The Agency is providing resources such as educational materials and inexpensive screening tests to industry for preventing residues in meat and poultry before marketing. Rapid screens such as CAST and SOS are currently being used in the plant by inspectors to screen animals at slaughter.

FSIS will continue to improve testing capability to detect more residues more efficiently. The Agency will focus on rapid, cost-effective screening methods; multi-residue procedures for major compound classes; testing procedures for metabolites that are more significant than their parent chemicals and improved sensitivity of methods to provide better information on residue frequency distribution for assessment purposes. Further, we will continue the development of forensic chemical biotechnology techniques and epidemiological capabilities for identifying and evaluating presently unknown contaminants that appear frequently at low levels in meat and poultry when multi-residue tests are performed.

The future will be characterized generally by the maximum use of residue avoidance and cooperative programs directed toward prevention of problems through appropriate controls at all critical stages of the production cycle. There will be closer coordination with industry in evaluating new compounds with respect to existing priorities and testing the effectiveness of management control systems. We will work diligently with other areas of government, academia and industry to ensure that our meat and poultry supply remains the safest in the world.

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Multiresidue Methods: Chemical Assays

C. Greg Guyer, BS

*Division of Chemistry, Center for Veterinary Medicine,
Food and Drug Administration, Rockville, MD 20857.*

Introduction

As an integral part of the drug approval process, the sponsor of a new animal drug for use in food-producing animals has the responsibility to develop analytical methods that are capable of quantitating the amount of residue that remains in the animal after the administration of their product. Then, a procedure must also be developed by the sponsor that can confirm the presence of that residue. These procedures have varied from mouse uterine assays to microbiological assays to chemical assays. The one common theme that these procedures have is that they are relatively cumbersome and tedious to perform. In other words, they are not very amenable to short analysis times. Under FDA's current SOM guidelines, the drug sponsor is not held accountable for developing methods with short analysis times, but rather methods with reasonable analysis times (usually no more than 48 hours).

As defined by Dr. Ellis and Dr. Norcross of FSIS in their presentations given earlier today, the challenge of monitoring for drug residues in animal tissues is a great one. With the large number of approved veterinary drugs and the billions of food-producing animals slaughtered every year in the U.S., I think you can get an idea of the monumental task that USDA has. Over the last several years, FDA and USDA have been working together both through our contracts and through our own research to develop strategies to make the task more manageable. The two primary schemes of thought in these discussions have been that we either have to develop methods that can detect the residues utilizing shorter analysis times or develop methods that can concomitantly detect more than one residue. Although both avenues are being explored in great detail, my discussion will concentrate on the development of chemical methods that can detect more than one residue at a time. These methods are referred to as multiresidue methods.

Discussion

The idea of multiresidue procedures is not a new one, but a fascinating one, and one that we feel is well worth the research effort. Although multiresidue methods have been applied to analysis of pesticide residues in food, very little research has been conducted in applying multiresidue procedures to veterinary drug analysis. First, we must consider that there are some innate problems that are encoun-

tered in trying to develop multiresidue procedures.

Some of the considerations are:

1) **Target Tissue.** This is usually designated as the tissue that contains the residue of interest for the longest period after drug administration. The problem lies in the fact that the target tissue can differ from drug to drug and even from species to species for the same drug as is the case with ivermectin. This problem is reduced somewhat when we only consider drugs in the same class, because they tend to have the same relative distribution in the same tissue.

2) **Sample Matrix.** The endogenous compounds that are coextracted from the sample matrix can cause severe interference and background problems. Anyone who has tried to develop a method in tissues, milk, plasma, or urine realizes the difficulty in trying to resolve just one component from the matrix, so the problem is really exacerbated when a number of compounds are tried to be resolved at one time.

3) **Metabolites.** This problem is similar to the one you encounter with the sample matrix components. In a chromatographic system, when you are monitoring for the parent compound, you also see spurious peaks in your chromatogram which are due to the metabolites that are present. Therefore, the complexity arises when metabolites interfere or coelute with the determination of other parent compounds of interest. When you combine this problem with the matrix interference problem, I think you can really get an idea of the complexity of the situation.

4) **Targeted Residue Level.** This problem is due to the difference in tolerances for veterinary drugs. We have methods ranging in sensitivity from the high parts per trillion for Chloramphenicol to the mid parts per million for pyrantel tartrate. It is hard to develop a method to cover these four orders in magnitude in sensitivity difference.

With all these innate problems, we decided to begin by trying to develop multiresidue methods for individual classes of compounds. Then, we will ultimately apply the information we gain from this endeavor to producing a universal method for monitoring all veterinary drugs. In order to eventually develop this universal method for residue analysis, we must eliminate as many of the obstacles discussed above as possible.

As part of the multiresidue program, we have tried to alleviate some of these obstacles by looking at the current methodology and determining which steps in the process

produce the most problems. In designing this strategy we had to look at the 4 primary steps of sample analysis:

1. collection
2. purification
3. detection (determination)
4. identification (confirmation)

When considering these 4 steps, it seems that step 2 is the major time-limiting factor since steps 3 and 4 can be automated. Since the classical extraction techniques involve extraction/back extraction and solvent transfer methods, which are labor and materials intensive, we had to explore alternatives. With the advent of technologies such as supercritical fluid (SFE) and solid-phase (SPE) extraction, the time involved to conduct the extraction part of the procedures can be greatly reduced. CVM currently has four cooperative agreements with experts from academia and industry researching these areas of extraction.

As you will see in Dr. Steve Barker's presentation, which describes his solid phase extraction approach, these techniques show great promise for future method development by greatly reducing analysis times. Also, both CVM and FSIS are currently researching the use of supercritical fluid extraction as another universal extraction technique and it also shows wide utility, but problems with current commercial instrumentation is a major limiting factor.

Also with the recent advances in detection systems such as the photodiode array detector, which is a simultaneous multiple UV wavelength detector, several residues may be determined from a single injection. This technology has been

successfully applied in the development of a multiresidue method for the Beta-Lactam antibiotics by the Research Triangle Institute and the North Carolina State Veterinary School.

Both CVM and FSIS are currently evaluating these technologies in our laboratories and contracts to discern if these techniques are applicable and practical. We know that ultimately these techniques must be evaluated in terms of complexity, cost, and analysis time. The techniques listed above show an improvement in all three of these areas.

Summary

The need for multiresidue methodology is evident due to the enormous number of samples that must be monitored every year in the U.S. The Center for Veterinary Medicine obviously will continue to develop, evaluate and validate the use of multiresidue methodology in the analytical laboratory. With the continuing advances in detection systems, analytical instrumentation in general, and laboratory computer software, our ultimate objective of developing a universal methodology for veterinary drugs or at least classes of drugs can be achieved. The approaches that I have defined have not been solely the work of analytical chemists in the laboratory; on the contrary, many of the ideas have come from veterinarians, pharmacologists and others who are closely involved with veterinary drug analysis. The continued support by these scientists is essential for this project to become a reality.

Biochemical Assays for Residue Analysis

John J. O'Rangers, PhD

*Division of Chemistry, Center for Veterinary Medicine,
Food and Drug Administration, Rockville, MD 20857.*

Introduction

The growth in applications of biochemical methods of analysis began in 1959 with the introduction of radioimmunoassay by Berson and Yalow in the United States and Roger Ekins in England. Since its introduction in 1959, radioimmunoassay has evolved from an esoteric research technique to a widely used tool in laboratory medicine. Al-

though radioimmunoassay will continue to be extensively used, the rate of growth of the RIA market appears to be slowing with the so-called "non-isotopic" or more accurately, "non-nuclidic" immunoassays coming into wide use. The commercial outlook for these "non-nuclidic" procedures is quite rosy. It is expected that the immunoassay and microbiology portions of the total clinical products market will

grow to 15%-23% of the total market by 1990.

In the veterinary diagnostic area, it is estimated that the market for antibody based veterinary diagnostic tests will rise from the current 1.5 million per year to over 60 million by 1990. It is particularly interesting that of 105 new biotechnology companies that have been formed, 40% are in the hybridoma, monoclonal area, with the biggest commitment being in the pharmaceutical area. It is quite likely that not only will new pharmaceutical products be forthcoming but the associated immunochemical methods of analysis will also be available.

I think it is evident that biochemical methods of analysis, especially ligand assay techniques, offer many potential advantages for the screening and monitoring of veterinary drugs and residues. However, the reliable application of rapid biochemical tests can be compromised if the test methods exhibit inconsistent performance. For example, a residue screening test that has an excessive false positive rate would result in unnecessary withdrawal or retention of marketable product. Typically, samples testing positive would be submitted to further laboratory analysis for confirmation of screening test results and a false positive rate would result in the unnecessary expenditure of analytical resources. In many cases, residue screening methods will be used in field environment by persons not specifically trained in chemical analysis and associated quality assurance procedures. It is essential that tests used in this milieu be analytically rugged and not exhibit reagent instability.

These are two examples that indicate that rapid screening tests for residues should meet rigorous performance criteria. Indeed, given the intended use of screening tests, I believe it can be argued that screening test performance criteria be among the most rigorous applied to analytical methods.

Discussion

The balance of this talk will deal with a set of technical considerations that are generally applicable to the development and evaluation of biochemical screening methods. These technical points are not specific evaluation criteria since the evaluation of a specific test depends on the intended use of the data developed by the test. The technical points that follow exemplify the test performance information that should be available for all screening tests. This information should be of great help to a potential test user in deciding whether a specific test will be suitable for a defined need.

TECHNICAL CONSIDERATIONS IN THE DEVELOPMENT AND EVALUATION OF BIOCHEMICAL ANALYTICAL METHODS

1. The Intended Use of the Method

- A. Limitations of the method
- B. Appropriate applications of the method
- C. Interpretation of data

At the very outset, the rationale for the development of the method should be specified. The type of analytical information that will be provided by the method should be available. In answering this question, a comparison with existing methods should be made. This comparison will give some assessment of the unique contribution of the proposed analytical method.

The intended use of the method should be set forth in detail. This discussion should also include a listing of the limitations of the method and inappropriate applications of the technique or interpretations of data.

2. Description of the Scientific Principles of the Method and the Critical Reagents or Instruments Used in the Method

The design of validation tests will depend on this information. For example, if a uniquely derivitized solid phase HPLC support was used in the method, this would be classified as a modification to existing technology. As such, the developer of the method should show that the technique of preparing the HPLC media is well understood and can be reproduced yielding batch-to-batch uniformity.

This point is especially important in evaluating analytical methods that depend on biological reagents, such as enzymes, antibodies, receptors, etc. Although modifications to technology may not be involved, critical reagents are used that in themselves are subject to variability. It must be shown that these reagents can be consistently produced and are available. It serves no purpose to collaboratively study a method which uses unique reagents of limited availability.

The information that can be deduced from the analytical response should be discussed. For example, a GC-MS method, can give both quantitative and qualitative (identity) information about the test analyte. Note that the fragmentation of a molecule in mass spectrometry is ultimately dependent on the chemical structure of the molecule.

In contradistinction to mass spectrometry a method based on ligand binding, such as immunoassay, actually measures the response of an analytical system to the effects of the test analyte. In the case of immunoassay, this system response typically is the displacement of another molecule from the antibody binding site. The effectiveness of the displacement mechanism can be generally traced to some common structural domain shared by the competing molecules. The displacement process may not give complete certainty of the structure of the test analyte. Where the competing molecules must share 100% of structural features for method performance, then absolute specificity would be obtained in this case. If the competing molecules share less than 100% functional identity (as is generally the case), then relative specificity occurs. This effect can also be called "non-specificity," although this is a less accurate term for the analytical performance. The point to keep in mind is that biological tests are, in general, refinements of a bioassay. Bioassays measure *function* directly and *structure* by inference.

3. Method of Synthesis and Characterization of the Critical Reagents

- A. Specifications for evaluation of reagents
- B. Evidence of consistent manufacture of test systems
- C. Specifications of quality assurance tests

Quality assurance specifications should be defined for all reagents especially biological reagents. As outlined above, it must be shown that biological reagents can be consistently produced on a batch-to-batch basis. Performance specifications or specification range should be established for critical biological reagents. These performance specifications can be derived from the overall method performance specifications that are to be established. For example, in an immunoassay, the specificity, sensitivity and freedom from matrix interferences of the final test can be used to establish procedures and criteria for titer determination, specificity assessment, and the buffer composition for the antibody.

The stability of all reagents should be shown. This stability should deal with the reagents in their manufactured or storage form and at analytical dilution. The stability of the reagents is judged by the rate of deviation of the reagents from the quality assurance limits.

This data should specify the characteristics of the reagent or device used to generate the analytical signal. At a minimum, these characteristics should test for linearity, specificity, selectivity and interferences.

The algorithms used to assess the above elements should be defined since they are important in evaluating method performance. For example, logarithmic plots are quite useful, but when they are used they tend to suppress observed real variability in data, and may limit the useful range of the "linearized" data.

An assessment of baseline variability should be presented. This is especially important with biological methods where incubation times, temperature, radiation measurement, etc., are usually critical to the analytical response.

In certain biochemically based test methods, the detector or indicating instrument is the analyst himself. Many rapid tests, for example, depend on a visual color interpretation by the analyst. This means that the method should show that it is invariant or minimally sensitive to variations in the analyst's interpretation.

4. Test System Logistics

- A. What is the long term availability of reagents?

This item deals with the manufacturing aspects of the test method or kit. Data should be available demonstrating that the methods of manufacture are well understood and under control. It is important to know that the test characteristics can be maintained from batch-to-batch of critical reagent.

5. Stability Data On The Critical Reagents

- A. Storage and analytical forms

Test kit reagents and other components are typically lyo-

philized when feasible. Properly dried biochemical reagents are usually quite stable for extended periods, typically six months to one year or longer. However, when reagents are prepared for use by the test user, the useful life-time of the reagents user can be drastically reduced. This stability information should be available to the test user so as to preclude test malfunction due to degraded reagents.

6. Stability Data On The Analyte(s)

- A. For example: pH, heat, light, cold, freeze/thaw enzymatic activity are effects to be measured
- B. Stability in the natural matrix
- C. Stability in partially purified extracts
- D. Stability to enzymatic action

This data is especially important in drugs or chemicals occurring in biological matrices. Metabolism of the test analyte can continue at the cellular level or in homogenates.

7. Determination Of Assay Sensitivity With Replication And Statistics

Sensitivity can be defined as the ability of a test to discriminate between adjacent levels or concentrations of test analyte. There are other definitions of sensitivity, but the one I have specified is sufficiently general to serve several needs in residue analysis. For example, the definition recognizes that test sensitivity can vary with the point on the standard curve. If one of the points used is "zero," then the sensitivity estimate can be either the level of smallest quantitation or the level of detectability of the method. The intended use of the method will dictate the statistical requirements for the determination of the level of detectability or quantitation. Also, the definition allows a semi-quantitative reference level to be set for the test. This reference level could be the regulatory tolerance for a drug or marker residue.

Two sets of data should be developed to determine sensitivity. The first would be the determination of the analytical sensitivity of the method. This phase of the work is usually done in the laboratory using calibration standards and tissue or feed matrices that have been fortified or "spiked" with the test analyte.

The second phase is the determination of the number or percentage of true positive results achieved with the test in a population that has been dosed with the drug article. This is an essential phase in the development of residue methods and the rigorous assessment of the true positive rate requires confirmation by a separate assay method(s). In addition, part of this study may need to be performed under field conditions, particularly if the test is intended to be used in a non-laboratory environment.

8. The Variability Associated With Each Standard Point On The Analytical Curve. (Raw Data With Any Mathematical Algorithm Or Transforms Used)

9. Data To Show That Components From Drug Free Matrix Do Not Interfere With The Determination Of The Test Analyte Or Its Metabolite(s). If There Is Interference, The Methods Of Control Should Be Shown.

- A. Baseline variability in drug free samples. This data should be presented for both dosed and fortified tissues.

10. Specificity

- A. Demonstrate "parallelism" between parent analyte and metabolite(s) in immunoassay curves. This is an important piece of evidence for total residue claims.
- B. Show the complete array of chemicals that were tested for cross-reactivity. Show how cross-reactivity was calculated.
- C. Data should be presented showing the number or percentage of true negative results obtained by testing samples from animals that have not been exposed to the drug or chemical. As in the determination of test sensitivity, this evaluation may need to be performed in a field environment. However, unlike sensitivity, the determination of the negative rate in the estimation of specificity does not require a separate confirmatory analysis.

The data on the true positive and true negative rates in conjunction with the drug use in the target population can be used to calculate the predictive value of the test. The predictive value is the percentage of true positives in a positive test population.

11. Interferences

- A. What is the environment in which the test will be used?

Interference and specificity are closely associated but distinct concepts. Specificity is the extent to which the biological agent, i.e., antibody, exclusively reacts with the test analyte. Specificity is very difficult to assess in absolute terms since specificity can only be measured by testing suspect cross reactant molecules in the analytical system. When the number of compounds that are potential cross reactants are finite and known, a good estimate of specificity can be determined. In order to make the number of cross reactants finite and known to a reasonable degree, the conditions and applications of the method must be defined. Thus, specificity has a parochial meaning and does not have general meaning. The determination of specificity is even more difficult to determine with certainty in the forensic or residue area. In this case, the compound universe is not finite. Nonetheless, by

carefully choosing the panel of compounds to be used in testing the specificity of a biochemical reagent, a useful estimate of its reactivity can be achieved.

Interferences, on the other hand, identify specific compounds or conditions that adversely affect the optimum performance of the method. Sources of interferences can be quite broad and diffuse. It is essential that the milieu in which the method is to be used be well understood so as to select potential interfering substances or conditions with some practicality and meaning. For example, if a rapid test is to be used on a farm environment, then the chemicals that typically are found in this environment should be tested for interference in the test.

The sample extraction and cleanup methodology can also be a source of interference, especially from solvent residuals or adverse effects of the processing on the analyte stability.

In the case of analytes that are contained in complex matrices, e.g., feeds or tissues, a sufficient number of matrix blanks must be run to determine what the population blank really is and whether there are any interferences to be expected from this source.

12. Validation Studies

- A. Optimization of performance
- B. Identification of critical steps
- C. Recognition and control of interferences
- D. Assessment of method performance using authentic samples under authentic conditions
- E. Confirmatory analysis

There are several approaches that can be used for confirmation.

- a. Use a definitive reference method to confirm the proposed method, e.g., confirm with M.S. Mass spectrometry is an ideal confirmatory procedure since it gives specific information about the structure of the test analyte.
- b. Confirm with several alternate tests. All test results should be consistent with each other and with the proposed method results.

Where a definitive confirmation is not available or feasible, other methods can be used. In this case, more than one confirmatory test may be required to provide the certainty of identification. The confirmatory tests selected should be based on different principles from the primary test. For example, if an immunoassay is to be confirmed, multiple chromatographies under different conditions may be suitable.

- c. Define an existing method as a standard or reference and compare the proposed test with it.

A New Approach to Tissue Residue Analyses

Steven A. Barker, PhD; Austin R. Long, PhD and
Charles R. Short, DVM, PhD

*Analytical Systems Laboratory and the Department of Physiology, Pharmacology and Toxicology,
School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803.*

Introduction

The previous presentations illustrate a consensus that a new approach to the analysis of tissues for drug residues is needed. The approach of analysis for individual compounds by individual methods cannot keep pace with the ever-increasing need to analyze more samples for more drugs and their metabolites in the future.

In part, the problem is parallel in nature to that which occurs in the screening of urine samples for the presence of drugs of abuse, such as occurs in laboratories which conduct equine medication surveillance of racing thoroughbreds as occurs in our laboratory. In these analyses, generic counter-current extraction methods are used to isolate compounds according to class (such as acid, base, neutral or amphoteric). Once isolated, generic methods for the screening of the extracts for the presence of drugs are utilized. The simplest and least expensive is thin layer chromatography (tlc) and can be highly effective in detecting a wide range of drugs or drug metabolites. However, the sensitivity of tlc is inadequate for the detection of trace levels of drugs that may be used to effect the performance of the equine. In these cases, radioimmunoassay (RIA) or variants of RIA type assays are used to detect the presence of drugs at levels of less than 2 ng/ml of sample. Compounds for which no such assays are available may be detected by gas chromatography (GC) analysis using nitrogen/phosphorous (NPD) or electron capture (EC) detection with and/or without derivatization of the sample. Thermally labile samples may be assayed by high pressure liquid chromatography (HPLC) using fluorescence, UV and/or electrochemical detection, individually or in series.

Can such an approach be applied to drug residue analysis in food animals? Most likely. Screening of marketed animals by such methods could be adapted, examining urine, blood or biopsied tissue samples prior to slaughter or post slaughter. This will require generic extraction methods and characterization and validation of the more generic screening methods for these purposes. Immunoassays for such applications may be class specific, such as for sulfonamides or benzimidazoles, in general. Subsequent quantitation and confirmatory methods can then be conducted on those samples which are of a suspect nature. As new drugs come to the market they could first be examined by such generic metho-

dology by their sponsors. If found to be detectable by such methodology, at levels required, then further development would not be required. Drugs that can not be analyzed by such approaches would require special consideration.

However, analyses of tissues by such an approach requires more sample preparation and manipulation than a urine or blood matrix. Further, the ability to conduct such general screening methods as tlc, IA, GC or HPLC on tissues requires an extract of high relative purity. For tlc, interfering substances must be absent and lipid must not be present at such levels as to cause streaking of a sample. For IA, isolation of the compounds of interest from the tissue matrix is required and removal of endogenous substances may reduce false positive results. For GC and LC, interfering peaks must be eliminated to assure the ability to detect what may be, in many cases, trace levels of drugs and their metabolites. Thus, new extraction methodologies are required that conduct multiresidue extractions for classes of compounds or that are generic in nature, such as isolation of compounds on the basis of their acid, base, neutral or amphoteric properties. These methods must be efficient, less laborious than classical methods and, ideally, amenable to automation.

Classical methods for tissue extraction involve homogenization of the tissues in an aqueous medium, removal of debris, precipitation of proteins by acids, bases or salts, adjustment of pH and counter-current extraction of the isolated supernatant. This invariably leads to emulsion formation and the need to conduct further manipulation of the sample. This must then be conducted again, after adjustment of pH, to isolate other classes of compounds.

A partial solution to this problem is offered by solid phase extraction (SPE) technology, in which a supernatant may be added to a SPE column, followed by elution of the sample with solvent, to isolate a specific compound or a class of compounds. This process eliminates emulsion formation and can often reduce the volumes of solvent required to isolate a given compound when compared to classical methods. This technology has grown explosively in the past decade and there are many different phases and column sizes now available.

SPE columns are packed with various materials, usually polymer-bound beads of various sizes. These polymer phases

range from reverse-phase (C₃,8,18) to normal phase (polar; SO₃, NH₂, CN, etc.) and the most appropriate material can be chosen for a given application.

For tissues, the homogenization and removal of cell debris, and often, proteins and lipids, is required prior to application to the column. This is performed in order to prevent plugging of the column or overloading of the polymer phase. However, of concern in this approach is the required pelleting of the sample prior to extraction. In such procedures, entrained or electrostatically bound drug may be lost to the pelleting process. To address this problem we have examined the more direct application of the sample to an SPE column.

In this process one conducts homogenization of the sample in an aqueous medium as before. However, rather than pelleting the sample or precipitating proteins, one blends the homogenate with a solid support. We have observed that one can homogenize liver tissues in aqueous ammonium hydroxide and absorb the homogenate onto pelicular diatomaceous earth (DE), thus yielding a semidry tissue-coated matrix which can be packed into a column and eluted with solvents to clean the sample and elute drugs from the tissues. The construction of such a column is shown in Figure 1. We have applied this approach to the extraction and analysis of benzimidazole anthelmintics from bovine liver tissues and have observed that this methodology can provide a simplified approach to multiresidue analyses of these drug residues in tissue samples. This approach eliminates the need to centrifuge, precipitate proteins from or adjust the pH of a sample prior to its addition to an SPE column. In this manner, the entire tissue is exposed to extraction and losses due to pelleting of the sample are reduced.

We found this method to be directly applicable as a multi-residue approach for the extraction and analysis of benzimidazoles. However, the DE was too polar for compounds such as the β -lactam antibiotics, which are highly retained. This may be an advantage in certain cases but fails to make the use of DE as a generic SPE matrix feasible. However, we have since developed a new approach that is generic and eliminates the need to homogenize, centrifuge or manipulate the sample as in classical methods as just described.

This approach is based on the classical idea of dissolving cell membranes so as to access membrane bound proteins or other components and to completely disrupt the cell structure, allowing one access to internal cellular components. This process has normally involved the addition of detergents or surfactants, such as sodium dodecyl sulfate, to tissue homogenates. While quite efficient, one must then isolate compounds in the presence of such a detergent and remove the detergent post-extraction. However, this principle may be applied and these difficulties can be overcome by use of a solid support-bound lipid-solubilizing material, so that the tissue's cell membranes would be dissolved onto a solid support. Such a material is available in the nature of SPE and HPLC column packing materials composed of oc-

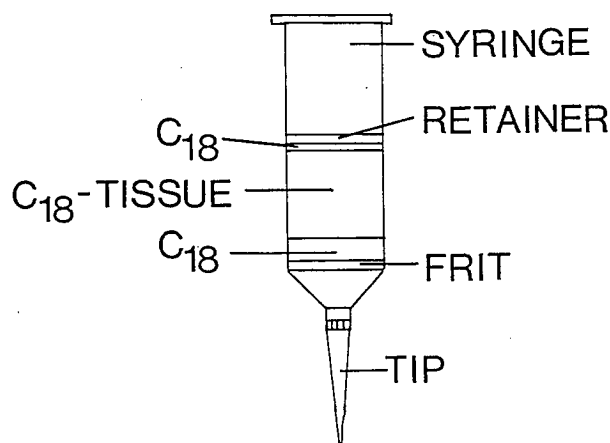


Figure 1. Construction of SPE column used to clean sample and elute drugs from the tissues.

tadecylsilane polymer bound to silica beads (C₁₈).

Thus, by adding whole tissues (0.5g) to C₁₈ packing (2.0g) in a porcelain mortar and gently grinding the material for 30 seconds, one obtains a near-homogenous mix of tissue cell membranes "dissolved" into the solid phase packing material. This provides a semi-dry material which can be packed into a column, as shown in Figure 1, from which compounds may be eluted based on their solubilities in the polymer/tissue matrix. We have observed that an elution profile is capable of isolating several classes of drugs from a single sample, with different classes being isolated in each of the different eluting solvents. For example, elution with hexane and benzene isolated the organophosphates; elution with methylene chloride, the sulfonamides; elution with ethyl acetate, the benzimidazoles; elution with methanol, the β -lactam antibiotics; and elution with water, cellular proteins and, possibly, DNA and RNA.

Each tissue type provides a different matrix, having unique elution properties, and is being characterized to determine the best ratios of C₁₈ to tissue. However, all preliminary data indicate that such a technique can be applied as a generic method for the isolation of drugs or natural compounds from tissues. In many cases no further clean-up of the sample has been required prior to analysis of the samples. However, the need to conduct such further steps is greatly simplified by the removal of the tissue matrix by this approach. Further, the entire sample is exposed to the extraction and the processes of classical homogenization, centrifugation, precipitation, and overall sample manipulation are eliminated.

In order to conduct generic screening, techniques such as tlc, IA or others on tissues, the compounds of interest must be removed from the tissue matrix. We report here a new approach to the isolation of compounds from tissues that is generic in scope and that can provide the ability to conduct such screening methods in a timely fashion. This capability may enhance the use of such an approach to drug screening of tissues in the future.