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LETTERS: THE USE OF LABELLED COMPOUNDS IN PHARMACOLOGY

Methodology problems associated with the use of radioisotopically labelled drugs in drug metabolism studies

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It is generally recognized that in the field of drug metabolism as in many other areas of biological research, radioisotopic tracers are very powerful and sometimes uniquely useful tools. This usefulness in experimental pharmacology in general is well documented in the recent book edited by Roth [1]. However, as with all laboratory tools, there are problems and disadvantages sometimes associated with the use of radioisotopic tracers. The purpose of this article is to review some of these problems which may lead to errors in interpretation of drug metabolism data. Unless otherwise indicated the discussion applies to ^{14}C and/or ^{3}H labelled drugs. While most of the problems discussed are not peculiar to the use of radioisotopes in the field of drug metabolism, it is hoped that presentation of these general problems in the specific context of drug metabolism will be especially useful to investigators in this field.

RADIOCHEMICAL PURITY

The well known problem of the presence of radiochemical impurities is an important one. In drug metabolism work, serious problems of this type are most frequently encountered in tritium labelled drugs prepared by the Wilzbach or similar radiation-induced exchange procedures [2]. These procedures frequently produce very small weight amounts of impurities which contain a large fraction of the total incorporated ^{3}H. The question of radiochemical purity and many other tritium-associated problems are discussed in the forthcoming book by Evans [3]. Any effort made *before* the metabolic work starts, to demonstrate that the presence of radioactive impurities is unlikely, is well worthwhile. The usual criteria for chemical purity (melting point, ultraviolet analysis, elemental analysis, etc.) are helpful but not sufficient. Similarly, with complex biological materials the demonstration of reasonable specific biological activity of the

labelled material is necessary; but it is not a good criterion for determining radiochemical purity since the methods used to determine biological activity are usually less sensitive on a weight basis than even the usual physical-chemical methods. Chromatographic separations used in conjunction with sensitive radioactivity detection techniques are generally the most useful method for detecting (and sometimes identifying) radiochemical impurities. Even here several different chromatography systems should be used. In our synthesis and characterization work we rely heavily on thin layer and paper chromatography followed by analyses of sections of the plate or paper strip with liquid scintillation techniques. For ^{14}C and ^{3}H labelled compounds with specific activities in the range of a few μc's per mg, this method is readily capable of detecting and estimating the amounts of radiochemical impurities at the level of a few tenths of one percent. Radiochemical impurities at the level of 1 or 2 percent are frequently of little consequence in drug metabolism studies. (This is fortunate since upon close examination impurities at this level are frequently found.) However, it is important to know of their presence so that the degree of uncertainty with respect to excretion, tissue localization, etc. of small amounts of radioactivity may be recognized. The problem of radiochemical purity of a labelled drug sometimes comes to the fore in the question of trace metabolites. Parke [4] has recently given an example in which the presence of a presumed urinary metabolite was actually the result of the presence of a labelled impurity in the administered drug. It should be kept in mind too that the same chromatography systems used to detect metabolites should also have been used to characterize the administered drug.

One aspect of the overall radiochemical purity problem is that of radiation-induced chemical decomposition of an originally pure radioactive drug. It is difficult to generalize about the likelihood of this problem occurring or about how to minimize this likelihood. This problem has been reviewed recently [5, 6]. Again some type of chromatographic separation combined with sensitive radioactivity detection techniques is usually the best way to detect radiation-induced impurities. From the overall point of view, the most important point is to know the radiochemical purity of the drug at the time the experiment is started and, depending upon the objectives of the experiment, to undertake further purification as necessary.

Analytical methods

The question of what methods to use for analysis of biological samples obtained from a labelled-drug metabolism experiment is an important one. Occasionally measurement of total radioactivity content is all that is necessary, e.g. total ^{14}C content in urine fractions will suffice to measure the rate of urinary excretion of a drug and its ^{14}C-containing metabolites. Much more frequently however, it is desirable to measure something in addition to total radioactivity content. Failure to do so may lead to gross misrepresentation of the true meta-

bolic picture. In general, radioactivity measurements are most useful in drug metabolism experiments when they are used in conjunction with other more chemically-specific analytical methods, e.g. colorimetry, fluorimetry, gas chromatography, solvent partition. The choice of methods should be governed by the objectives of the experiment. For example, it is almost always true that a better accounting for the total dose of a drug can be made using labelled drug and measuring total radioactivity. On the other hand, attempts at correlation of drug concentration with degree or duration of pharmacologic effect are more likely to be fruitful if methods more specific for the unchanged drug are used. If a reasonably complete picture of the metabolism of a drug in both the chemical and physiologic sense is desired, several analytical techniques are usually needed.

A discussion of non-radiometric methods of analysis is outside the scope of this discussion. However a few comments on radiometric methods are appropriate. In general, liquid scintillation counting has become the method of choice in drug metabolism work. However, a variety of techniques are used to get the sample into the counting vial [7]. Again the choice of method depends upon the particular experiment. However, our experience suggests that in gross physiologic disposition experiments in whole animals, as compared, for example, with more detailed studies in *in vitro* systems, the oxygen flask combustion method as basically described by Kelly *et al.* [8] and Kalberer and Rutschman [9] is the method of choice for measuring total ^{14}C and ^{3}H content. Several modifications of this method have been proposed [10] and we have incorporated some of these modifications into the method we currently use [11]. Some examples of potential analytical problems minimized by this method are: [1] it can be demonstrated that good analytical results can be obtained for a given compound (drug) in urine simply by adding urine containing the drug directly to an appropriate, water-miscible scintillation mixture; however it is much more difficult to be sure that the frequently unidentified urinary metabolites of this drug, which are often more water soluble and hence probably less soluble in scintillation solution, are going to behave in the same way; [2] similarly, good analytical recovery checks for many drugs in tissues can be obtained by adding a known amount of drug to a tissue homogenate, extracting with a water immiscible solvent and counting the extract; however, if one is interested in measuring the total amount of drug and its metabolites in this tissue, it is difficult to be sure that the metabolites will behave similarly because of solubility and/or tissue binding differences. Indeed the unchanged drug itself added to a tissue homogenate *in vitro* may not become bound in the same way and thus not be extracted in the same way as the unchanged drug in the same tissue *in vivo*; [3] the combustion technique is less subject to surface adsorption effects at low concentration of labelled compound than procedures involving solvent extraction or direct addition of, e.g., plasma or urine to scintillator solution. While there are other techniques available for getting « whole tissues » into the counting vial [7], the oxygen flask combustion method appears to us to be the best for drug metabolism work where the method should be capable of handling a wide variety of chemical compounds (drugs

and their metabolites) in a wide variety of sample types (tissues and body fluids). One subtle and frequently unrecognized problem with the combustion procedure is the unstable quenching of ^{3}H counts apparently resulting from oxygen dissolved in the scintillator solution. This problem was recently documented by Conway and Grace [12] and may account for the low recoveries of ^{3}H encountered by some investigators. We have looked for, but not detected, oxygen quenching in the procedure we use where the alcohol adsorbant is in the flask during combustion and the scintillator solution is added to the alcohol-tritiated water mixture in the counting vial.

Label stability

Implicit in the use of radioactive atoms as tracers in biological systems, is the assumption that the radioactive atoms remain attached to the molecules being traced. In drug metabolism work the cases where this assumption is not true fall into two general categories: those in which loss of the radioactive atom results in a *net chemical change* in the molecule and those in which *no net chemical change* occurs. The former type can be exemplified by oxidation of a ^{14}C-labelled N-methyl group to $^{14}CO_2$ or by replacement of an aromatic tritium atom by a hydroxyl group. This type of label instability can generally be anticipated and recognized, especially with ^{14}C. In fact, it can be advantageous or not, depending upon the purpose of the experiment. However, the loss of radioactive atoms accompanied by no net chemical change, i.e., an isotope exchange reaction, is more difficult to recognize and can lead to serious interpretive errors. In drug metabolism work this kind of problem is most likely to arise with tritium labelled compounds. *In vivo*, the instability may involve a general, gross exchange of readily labile tritium with the hydrogen of, e.g., body water. This type of problem can usually be avoided by repeated exposure of the labelled drug to a hydroxylic solvent before introduction into the biological system. On the other hand, the *in vivo* exchange may be a much more subtle and specific process such as described by Evans, *et al.* [13] for the enzymatically catalysed exchange of ^{3}H for ^{1}H at the alpha carbon of an L-amino acid. While isotope exchange problems are most frequently encountered with tritium, examples are known of similar problems with other isotopes. For example, in studying the metabolism of benzothiazole-2-sulfonamide, Colucci and Buyske [14] have demonstrated the metabolic replacement of a group containing ^{35}S by other similar groups containing non-radioactive sulfur.

It is apparent that when isotope loss of either type occurs, measurement of radioactivity may no longer be a valid indication of either the absence of the original compound, since all of the label may have been removed, or the presence of the original compound, since, e.g., any radioactivity detected may be due simply to the presence of tritiated body water or to compounds which have become labelled by biosynthetic incorporation of tritium from tritiated body water. Okita and Spratt [15] have described methods for determining if *in vivo*

tritium exchange has occurred. The extent of recovery of tritium in urine and feces in non-volatile form can also be used as a gross index to the extent of exchange with body water [16].

Once it has been established that label instability is a problem, its significance to the overall experiment must be evaluated. If the purpose of the experiment is to qualitatively detect major metabolites, fairly extensive instability may be of no consequence. If, on the other hand, the problem is to demonstrate the presence or absence of very small amounts of a drug in a particular tissue, limited exchange is probably important.

Isotope effects

Another basic assumption in the use of radioisotopically labelled compounds as tracers is that the molecules which contain the radioactive atoms have the same chemical (and physical) properties as those molecules which do not, i.e. that there are no isotope effects. There are many clear cut examples in biological systems to illustrate that, under certain circumstances, this assumption is not valid [17–21]. Thus, isotope effects with labelled drugs are a potential problem which should always be kept in mind. However, this problem is not one of the most frequently encountered or decisively important ones in studies with labelled drugs. The likelihood is generally small that a metabolic reaction will take place at or near the site of labeling and that the reaction will take place by a mechanism in which the breaking of the bond involving the isotopic atom is kinetically important. The magnitude of an isotope effect is likely to be small relative to other variables inherent in biological systems, especially in whole animals. This is particularly true with ^{14}C and to a lesser extent with tritium. For example, an isotope effect on metabolic routes and hence on the relative amounts of two metabolites found in urine is likely to be small in relation to the variation in relative amounts of the metabolite excreted by two individual animals.

Although isotope effects in gross drug metabolism studies *in vivo* are not likely to be important, these effects are more likely to become important as the experiments become more precise and sophisticated. For example, isotope effects are more likely to be important when a labelled drug is used *in vitro* to measure the activity of a given drug metabolising enzyme in the kidney than if it is used to detect urinary metabolites. Thus, as with other isotope-related problems, the seriousness of isotope effects depends upon the experimental objectives. The most important point is to be aware of the possibility of such an effect and its significance in the experiments at hand.

Tissue residues

Several of the general problems discussed above have particular application in situations where radioisotopically labelled drugs are used to determine the

amount of « drug residues » in tissues. The importance of radiochemical purity of the administered drug can be illustrated by pointing out that a one percent radiochemical impurity in a drug which is administered at a dose of 10 mg/kg body weight can give rise to a residue of radioactivity equivalent to 0.1 μg of drug/gm of wet tissue if the impurity is selectively retained and evenly distributed throughout the body. A second potential problem can be viewed as a kind of label stability problem. It is possible that traces of radioactivity found in tissues following administration of a labelled drug may be the result of incorporation of radioactive fragments from the labelled drug (e.g. $^{3}H^{+}$, $H^{14}CHO$, etc.) into normal body constituents. It is important to know if this fragment incorporation has occurred, since if it has, traces of radioactivity remaining in the tissues do not necessarily indicate the presence of a foreign and therefore potentially toxic substance. This problem has been discussed recently by Rosenblum [22] and, in a slightly different context, by McMahon [23]. The data of Berenbom [24] give an example of the ^{14}C from the N-methyl group of a drug being metabolically incorporated into a normal body constituent. It should be pointed out, however, that the traces of radioactivity in the tissues may in fact indicate the presence of small amounts of drug-derived, foreign, and therefore potentially toxic compounds. Direct demonstration that radioactive normal body constituents are present or that drug fragment likely to be incorporated into normal body constituents are formed should precede proposals that residues of radioactivity are the result of metabolic incorporation of drug fragments into normally occurring body constituents. While the use of high specific activity radioisotopically labelled drugs with their inherent analytical sensitivity is bound to increase in tissue residue studies, it might be well to consider that at least as much effort be devoted to making a technically sound biological (toxicological) interpretation of the almost inevitable residues as is currently being devoted to increasing analytical sensitivity.

Additional problems sometimes associated with the use of labelled drugs for tissue residue studies are the potentially high cost, possible health and contamination hazards and waste disposal problems resulting from the large amounts of radioactivity required for studies in many large animals. Although tritium has some disadvantages (label instability, etc.) as mentioned above, it has the advantage of relatively low cost when compared with ^{14}C and ^{35}S.

Clinical studies

A general problem which sometimes occurs in drug metabolism studies with radioisotopically labelled drugs is that of performing these studies in man. Drug metabolism data in man can usually be obtained readily with non-labelled drugs, frequently as a by-product of therapeutic or clinical pharmacologic studies. The drug administration and sample collection aspects of metabolism studies in man with labelled drugs require more effort. It is not appropriate to go into the many and varied reasons for this difficulty here; suffice it to say that

as more experience is gained in both the problems and potential usefulness of these studies, they will probably be carried out with increasingly less difficulty.

In closing it should perhaps be pointed out that after the analytical problems involved in the identification and quantification of a drug and its metabolites have been solved (something which sometimes cannot be done without labelled drugs), a major task frequently remains in making a meaningful and decisive interpretation of the biologica significance of these analytical results.

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L'Étude métabolique des médicaments marqués: description d'un plan expérimental

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INTRODUCTION

Le métabolisme des médicaments est actuellement une discipline qui s'intègre dans la science pharmacologique. Plusieurs articles ont déjà exposé l'importance, l'ampleur et les informations que ces études peuvent apporter dans l'étude des nouveaux produits pharmaceutiques [1, 2]. Cette extension est d'ailleurs prouvée par la littérature scientifique qui s'accroît de jour en jour.