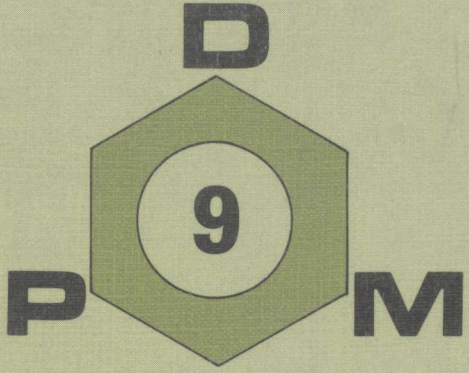
PROGRESS IN DRUG METABOLISM



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Progress in Drug Metabolism

Volume 9

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Preface

The process of percutaneous absorption is increasingly attracting the attention of cosmetic chemists, biopharmacists (especially those concerned with the skin as a site of drug delivery), toxicologists and occupational health specialists. A review of the principal factors which influence absorption through the skin, for example, species differences, is provided in this volume of *Progress in Drug Metabolism*.

In pharmacology and toxicology there is a growing appreciation of the role of extrahepatic biotransformation in the tissue, cell and cell component targetting of drugs and other xenobiotics. A review is included which discusses the ability of the lung to biotransform xenobiotics.

Prediction in drug metabolism depends on a good understanding of the relationship between chemical structure, biotransformation and pharmacokinetics. In furtherance of this important theme in the *Progress in Drug Metabolism* series, a comprehensive review of the biotransformation and pharmacokinetics of antipsychotic drugs is added.

All those involved with drug metabolism are keenly aware of the importance of mild reliable sample handling and analytical methods in many facets of their work. The problems in identifying and assaying conjugates of carboxylic acids are critically discussed together with improved approaches.

Finally, although the application of drug-metabolism and pharmacokinetic data is widely recognized in clinical pharmacology and occupational health, its usefulness in forensic medicine is far less well known. To remedy this, an article on this expanding area of application is included.

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CHAPTER 1

Methodology for the isolation and characterization of conjugates of xenobiotic carboxylic acids

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1. Introduction

Compounds containing the carboxylic acid group (−COOH), or which may be readily converted to carboxylic acids by metabolism (e.g., simple esters), are of considerable economic importance due to a variety of interesting biological activities. They find use as therapeutic agents (non-steroidal anti-inflammatories, hypolipidaemics, analgesics, diuretics, etc.), as pesticides and as herbicides. The potential for the exposure of human populations to such agents, either deliberately or accidentally, is thus considerable, and it is important that their safety is thoroughly evaluated.

In recent years, there has occurred a resurgence of interest in the acute and chronic toxicity of a number of carboxylic acids and their simple derivatives. Adverse reactions to a number of non-steroidal anti-inflammatory agents, for example, benoxaprofen, zomepirac and indoprofen, have attracted considerable attention and led to restrictions upon the use of these drugs, and aspects of the toxicity of aspirin have also been reassessed. Clofibrate and related hypolipidaemics have also been a cause of concern. The potential hazards of the herbicides 2,4-dichlorophenoxyacetic acid (2, 4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) have received notice, and the phthalate plasticizers, apparently ubiquitous in the environment, have been associated with chronic changes in various animal species.

It is now well understood that a knowledge of the metabolic disposition of xenobiotics may be of great value in understanding their biological effects, both desired and undesired. The literature contains much information about carboxylic acids, the metabolic pathways of which are diverse and may involve both reactions at the carboxylic acid grouping and/or elsewhere in the molecule (for reviews, see Caldwell, 1978a, 1982a; Hutt and Caldwell, 1983, 1984; Caldwell *et al.*, 1980, 1983). The principal metabolic reactions of the carboxyl group, first discovered in the nineteenth century, are conjugations with glucuronic acid and various amino acids. Many studies have since shown the versatility of these pathways in terms of species distribution and substrate requirements, and have discerned that their relative extents are a function of both the structure of the acid and the animal species in question. More recently, a number of novel metabolic transformations of the carboxyl group have been discovered, involving interactions with lipid biosynthetic processes and the formation of sterol esters. In general, these pathways are quantitatively minor.

The importance of the conjugation reactions of foreign-compound metabolism is sometimes underemphasized (Caldwell, 1978b, 1979, 1980). One reason contributing to this are the great difficulties which the products of these reactions pose in their analysis and characterization (Caldwell *et al.*, 1983). Conjugates are most frequently highly polar, water soluble and involatile, all features which hamper their study. Although the conjugation reactions of carboxylic acids are reasonably well understood, a perusal of the literature shows that even now the majority of studies on their disposition fail to characterize fully all of their metabolites.

The increased interest now shown in the toxicity of carboxylic acids has resulted in a need for comprehensive accounts of their disposition in animals and man in anticipation that the results may illuminate various aspects of their biological actions. The author's laboratory has been engaged in studies of the conjugation of carboxylic acids for many years, and in recent years we have evolved a logical approach to the characterization of the major conjugates of such agents. It is the purpose of this contribution to review in full this approach, illustrating it from our own experience and the published literature. Certain of the material from our own studies has been published, but some of the data have been obtained for the purpose of inclusion in this review: this is particularly the case for certain spectra and chromatographic data, which have been obtained under standard conditions to permit better comparisons to be drawn.

It will be appreciated that a given acid may undergo a variety of conjugation reactions, so that biological fluids may contain three or more different types of conjugate, as well as the free acid and other metabolites arising from transformations not involving the carboxyl group. Therefore the approaches which are described here are general methods which allow the characterization of different types of conjugate of carboxylic acids present in mixtures.

2. *Initial studies*

A complication frequently encountered in the characterization of conjugates of carboxylic acids is the presence of a mixture of both peptide- and ester-type conjugates together in a single biological sample. Before the individual members of the major classes of conjugates may be characterized, it is essential to assess the relative proportions of peptide and ester conjugates present. This may conveniently be achieved by simple chromatographic means, by high-performance liquid chromatography (h.p.l.c.) and/or thin-layer chromatography (t.l.c.) in systems described in a later section, before and after treatment with mild alkali. The sample of interest is examined before and after treatment with 1 M NaOH at 37°C for 30 min, which serves to cleave ester conjugates quantitatively but leaves peptide conjugates unaltered. The disappearance of a chromatographic band and its replacement by another, perhaps new, band upon alkali treatment is strongly suggestive of the presence of an ester-type conjugate.

This simple procedure requires little sophistication in the t.l.c. and h.p.l.c. systems. The systems should be chosen such that the acid has a high R_F (or long retention time in reversed-phase h.p.l.c.) and the more polar conjugates will have lower R_F values (or shorter retention times, provided they are not eluted in the void volume).

Thus it is possible at the earliest stage to determine the relative proportions of ester-type, mild alkali-labile conjugates and alkali-stable metabolites present. These two classes of metabolites may then be characterized separately.

3. Ester-type conjugates

In almost every case, ester-type conjugates of carboxylic acids are ester glycosides, involving various sugars or sugar acids. The most common of these is glucuronic acid, but it is known that glucose, xylose and ribose conjugates occur in certain circumstances (see Caldwell, 1982a,b).

A common error is the assumption that all alkali-labile conjugates of carboxylic acids are ester glucuronides. However, this is not justified, and the investigator should endeavour to identify not only the aglycone, or xenobiotic moiety in the conjugates, but also the conjugating agent. It is often thought that such studies may be time-consuming and technically demanding, especially when identifying the conjugating agent. However, although classical methods are laborious, simple tests now give reliable answers to the problems encountered.

By far the most commonly used procedure to identify xenobiotic glucuronides is their lability to the enzyme β-glucuronidase, which is commercially available at reasonable cost prepared from mammalian, molluscan and bacterial sources. However, problems arise in this approach: (i) from the failure to employ appropriate controls; (ii) from the chemical properties of ester glucuronides, which may lead to their resistance to enzymic hydrolysis or to spontaneous hydrolysis if samples are collected and/or stored inappropriately; and (iii) from the failure to consider the possibility that a glycoside may involve a sugar other than glucuronic acid.

The liberation of an aglycone upon incubation of a conjugate with β-glucuronidase may only be taken as presumptive evidence that the conjugate is a β-D-glucopyranosiduronate when adequate controls have been employed. Four control incubations should be performed in addition to the test:

(1) Demonstration of the complete inhibition of hydrolysis by the specific β-glucuronidase inhibitor, saccharo-1, 4-lactone (Levvy and Conchie, 1966). A suitable concentration of inhibitor is 2 mg per 5000 units of enzyme, or 1×10^{-4} M.
(2) Demonstration of the inactivity of a boiled enzyme preparation to show the absence of spontaneous hydrolysis under the incubation conditions.
(3) Confirmation of β-glucuronidase activity under the incubation conditions by including together with the biological sample an authentic glucuronide, for example *p*-nitrophenyl glucuronide or phenolphthalein glucuronide, both of which are easily and cheaply available, and assaying the incubate for the aglycone of the reference glucuronide. In the cases of the glucuronides mentioned, this assay is easily achieved by adjustment to alkaline pH followed by colorimetry (Sinclair and Caldwell, 1982a). This control incubation is important, as the occurrence of endogenous inhibitors of β-glucuronidase has been reported (Jayle and Pasqualini, 1966; Levvy and Conchie, 1966), which could cause an apparent failure of enzyme activity and thus a misidentification of a conjugate.

Table 1. Compounds whose ester glucuronides are known or are suspected to undergo pH-dependent intramolecular rearrangement to β-glucuronidase-resistant forms.[a]

Known	Suspected
Bilirubin	Benzoic acid
Clofibric acid	Buniodyl
Diflunisal	Diphenylacetic acid
Fenclofenac[b]	Fenoprofen
Isoxepac[c]	Hydratropic acid
Probenecid	Indol-3-ylacetic acid
Valproic acid[d]	Indomethacin
Wy-18,251 (3-(*p*-chlorophenyl) thiazolo-[3,2-a]benzimidazole acetic acid)[e]	1-Naphthylacetic acid
	2-Naphthylacetic acid
Zomepirac[f]	Tyropanoic acid

[a] For references, see Sinclair and Caldwell (1982a) and Faed (1984).
[b] Marsh *et al.* (1983).
[c] Marsh (1982).
[d] Dickinson *et al.* (1984).
[e] Janssen *et al.* (1982).
[f] Hasegawa *et al.* (1982).

(4) Confirmation of the role of β-glucuronidase in the cleavage of the reference glucuronide (see above) by complete inhibition of activity by saccharo-1, 4-lactone (see above).

In the case of ethereal glucuronides, the above controls will readily demonstrate that the metabolite is indeed a glucuronide, and since β-glucuronidase is specific for 1-*O*-substituted β-D-glucopyranosiduronates (Levvy and Conchie, 1966), little or no further confirmatory work is required. However, the situation is different when dealing with putative ester glucuronides, as it is commonly found that the extent of cleavage obtained with β-glucuronidase is less than that given by mild alkali treatment (see Table 1). In these cases it is important to use alternative methods to determine the nature of the conjugating agent.

Identification of the sugar-conjugating agent

The literature of carbohydrate chemistry contains a plethora of methods of potential usefulness for this purpose, but many of them require the availability of the conjugate in a pure form, and should a putative conjugate turn out to be a mixture of glycosides of the same aglycone with different sugars, such methods would give confusing results.

Heirwegh and his colleagues have described the extremely simple method of ammonolysis of ester glycosides on t.l.c. plates (Heirwegh and Compernolle, 1979), and we have found this to be the quickest and most convenient approach to the identification of the sugar involved. Ammonolysis may be performed directly upon the biological sample or on a partially purified sample of the conjugate,

obtained by preparative t.l.c. In either case, the sample of interest is spotted on t.l.c. plates along with reference samples of sugar-conjugating agents (glucuronic acid, galacturonic acid, glucose, ribose, xylose, etc.) and the aglycone. The plates are then placed overnight in a t.l.c. tank containing a small beaker of 0·88 sp. gr. ammonia. Upon removal of the plates from the tank, they are placed in an air current in a fume hood to remove ammonia vapour before development in solvents appropriate to the separation of sugars and the recognition of the aglycone. For sugar separations, we have found n-propanol–water (17:3, by vol.) useful with Merck silica gel F_{254} plates. After development and drying, the plates are sprayed with naphthoresorcinol (5 parts 0·2% w/v naphthoresorcinol in ethanol and 1 part 85% orthophosphoric acid, and the plates then heated to 100°C for five minutes). The sugars are revealed as blue spots, and comparison of R_Fs with the standards permits identification of the unknown. The method is sensitive to as little as 0·5 μmol of the sugars. T.l.c. for the aglycone in appropriate systems will reveal whether the conjugate involves the parent acid or its Phase I metabolite. If the acid has undergone metabolism before conjugation with the sugar, then the aglycone may be isolated and identified with suitable physicochemical methods.

β-Glucuronidase-resistant glucuronides

The at-least partial resistance of many ester glucuronides to hydrolysis by β-glucuronidase has been mentioned above. Two possible explanations may be put forward for this phenomenon: (i) that the conjugate is not a glucuronide at all, but a glycoside, involving another sugar, and thus having many of the physical properties and chemical reactions of a glucuronide, or (ii) that some chemical peculiarity of ester glucuronides leads to their resistance to the enzyme. An extensive search of the literature reveals that ester glycosides involving sugars other than glucuronic acid are extremely rare, the only examples being the ester glucoside and riboside of bilirubin, and the ester glucoside of the anti-inflammatory agent, oxinepac (see Caldwell, 1982a,b). This latter example, however, is not supported by full analytical evidence, at least in the English literature (Hakusui *et al.*, 1978).

We have found that several of the 'ester glucuronides' reported to be partially resistant to β-glucuronidase are indeed glucuronides by the ammonolysis method described earlier (see references in Table 1). What then is the chemical peculiarity of these ester glucuronides which leads to their resistance?

Carbohydrate chemists have long known that acyl groups attached to a sugar may migrate from hydroxyl to adjacent hydroxyl upon the sugar ring (Fischer, 1920; Haines, 1976). However, drug-metabolism workers have largely ignored this phenomenon, although in the 1930s there was controversy over the structure of benzoyl glucuronide: Magnus-Levy (1907), who first isolated the compound, proposed it to be the 1-*O*-acyl glucuronide, which was confirmed by Pryde and Williams (1933) by supposedly unequivocal techniques. Quick (1934) claimed