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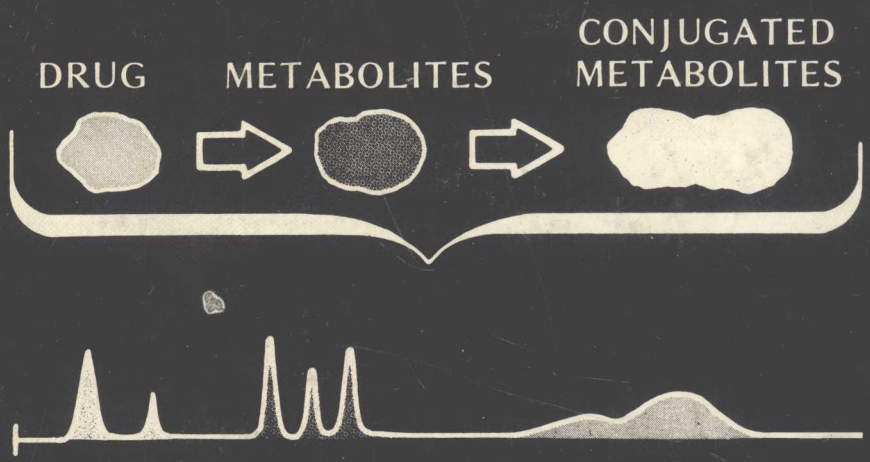
Drug Metabolite Isolation and Determination



Edited by
Eric Reid and J. P. Leppard

Drug Metabolite Isolation and Determination

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Senior Editor's Preface

In drug metabolism, as in kindred fields, sound conclusions hinge on good methodology with special skills which, however, have had less than their share of recognition and 'kudos'. This series of books is earning regard for its description and encouragement of good methods, not least because of emphasis on difficulties and on rationale rather than recipes such as prevail in the literature.

Each book is based on a Forum – in this case the 4th Bioanalytical Forum held at the University of Surrey in September 1981 – and mirrors some of the cut-and-thrust of the debates, but is not to be regarded as a symposium record. There has in fact been strong editing, partly for the sake of clarity and of relevance to analytical practice (thus, pharmacokinetic data have been excluded). The aim has been to produce a balanced desk book, well cross-referenced and complementing earlier 'Analysis' volumes, whose material on particular analytes is cumulatively re-indexed here (Index of Compound Types); the concluding article and the General Index aid retrieval of lore on problems and approaches.

The evolution of the *Methodological Surveys* twin series (outline facing title page) has undergone yet another discontinuity, in respect of publisher and format. For Vols. 10 and 11, conventional typesetting by the publisher was obligatory; it led to a generally good appearance, although there were errors that the page-proofing should have eliminated, but it contributed to the slowness of publication. Moreover, it entailed an intolerable editorial burden, now reduced by reversion to 'camera-ready' text produced at the Guildford end. The initiative of switching to Plenum Press, with marketing and other strengths and with pricing that appeals to American and other customers, has entailed sad parting from the U.K. publisher whose involvement could have started with Vol. 5 (illness precluded this) and did start with Vol. 6. If the now widening readership can get past volumes back-ordered, this will help not only bibliographic searching but also future Forum financing. Here too there is a discontinuity: from 1983 each Forum will be under the auspices of a new Trust (an 'educational charity'), although the normal venue will still be the nearby campus of the well-liked University of Surrey. Hopefully there will still be company support, which for the 1981 Forum came from Ciba-Geigy (U.K.), Glaxo and ICI Pharmaceuticals. It has been a boon that many contributors made little claim on Forum funds.

Scope of the book, and acknowledgements.- The articles are focused on 'real problems' in body-fluid analysis, typically with a final chromatographic separation of µg or often ng amounts if the aim is quantitation. The pitfalls may not be realized by a typical chemist (cf. remarks in #A-3), but he may excel in metabolite identification - which this book covers to a fair extent. Where identity is known, and the metabolite could interfere in therapeutic drug monitoring or in diagnosis or itself have clinical relevance, useful guidance will come from articles that follow. Authors have gone to much trouble, and are not to be blamed by any reader who would have liked an introduction to chromatography or to metabolic pathways (cf. list of conjugation reactions at end of concluding article). Appreciation is also expressed for permission to reproduce published material; the acknowledged sources include *J. Chromatog.* (Elsevier; e.g. in #A-1), *Anal. Chem.* (American Chemical Society; #A-2) and Wiley.

HPLC nomenclature.- With regard to the terms 'NP' and 'RP' (normal-, reverse-phase) the Editors have respected authors' preferences (cf. comment following Table 2 in #A-2) but share misgivings expressed by J.H. Knox and (in *LC in Practice)* by P.A. Bristow, who writes: "Reverse phase is a term which could well be retired gracefully". Thus, an 'uncapped' packing with a low content of bonded alkyl groups may behave adsorptively. Some authors exclude unbonded ('straight') silica from the 'NP' category that includes polar liquid stationary phases and polar bonded phases such as cyano. The designations 'hydrophobic' and 'hydrophilic' chromatography (Knox) have merit, and the concept of 'surface zone' rather than 'phase'. Some comment on so-called ion-pairing is made in #E (cf. p. 91).

Various abbreviations.- Nowadays the terms TLC (HPTLC = 'high performance' variant) and HPLC need no definition. In GC (gas-liquid in the present context), detector types include FID = flame ionization, AFID = alkali flame ionization ('nitrogen detector') which can detect phosphorus (hence NPD, N-PD), and ECD = electron capture but unfortunately connoting electrochemical detection in HPLC work. Mass spectrometry is denoted MS; EI = electron-impact, and CI = chemical ionization. In HPLC, t_R (preferred; otherwise R_t) = retention time.

Non-chromatographic abbreviations include i.s. = internal standard (usually 'processed'; see #A-6 in Vol. 7), RIA = radioimmunoassay, UV = ultraviolet [absorptiometry], IR = infrared, and NMR = nuclear magnetic resonance.

An example of metabolite jargon is a dialkylated amine drug, 'D', that has lost one alkyl group: it might variously be termed de(s)alkylD, monodesalkylD, desmonoalkylD, norD..... A conjugate is 'Phase II'.

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ERIC REID

1 April 1982

List of Authors

'Notes' (headed 'NC.....' in the text) are not distinguished from main contributions.

Contents

The NOTES & COMMENTS ('NC' items) at the end of each section comprise some comments made at the Forum on which the book is based, together with some supplementary points besides those in the concluding survey.

†*Sub-listed here: comments on particular articles in the section; likewise on pp. 135, 189 & 231.*

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* *Analytical case histories*

Section #A

TECHNIQUES APPLICABLE TO METABOLITE INVESTIGATION

#A-1

STRATEGIES CENTERED ON HPLC

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The choice of an HPLC system will depend on its application and on the nature of the metabolites to be measured. Normal-phase (NP) chromatography is limited to the measurement of drugs whose physico-chemical properties are similar to the parent drug, whereas reverse-phase (RP) chromatography in its various forms has made possible the simultaneous measurement of metabolites varying widely in pK_a, lipophilicity and polarity. Either alone or in combination with other chromatographic techniques, HPLC has aided the isolation of some polar metabolites which may be difficult to isolate from biological material. In contrast with other types of chromatography, the in vitro *formation of metabolites can be monitored directly by HPLC, with minimal sample preparation. Thus HPLC will aid the study of enzyme kinetics and reaction mechanisms.*

The biotransformation of a compound may result in a diversity of metabolites with widely differing physico-chemical properties. It is therefore desirable to know the properties of the parent compound and its possible metabolites in order that a suitable HPLC system may be chosen. Its choice also depends on whether it is being used primarily as an analytical method for the identification of a metabolite and the determination of its concentration, or preparatively, when substantial quantities of one or more of the metabolites are to be isolated for further structural characterization. HPLC has been used extensively for studying the metabolism of drugs, particularly drugs of high relative molecular mass and polar metabolites [1].

Recently the 'State of the Art' of HPLC has been reviewed [2,

3], and the theoretical and applied aspects discussed, in addition to newer developments. Consequently I will focus upon particular ways in which HPLC has been exploited in metabolic studies.

The subject of sample preparation and clean-up is discussed by other contributors (e.g. # A-2, #E). It suffices to say that RP materials are useful for sample clean-up and enrichment. Thus, a liquid-solid extraction with ODS material in cartridges removed the sparingly water-soluble mebendazole and its metabolites from plasma [4], yielding a chromatographically cleaner extract in a single operation without substantial loss of compound. The authors state that they could re-use the cartridges without loss of performance.

The first decision to be made is the type of chromatography to be adopted. NP (adsorption and bonded-phases) and RP systems are applicable to the study of Phase I metabolites and methylated and acetylated metabolites, whereas RP systems with either ion-pair or ion-suppression modes are suitable for separating the polar, water-soluble conjugates (glucuronides, sulphoconjugates and amino-acid conjugates). This article will concentrate more on the latter type.

SELECTION OF HPLC SYSTEM

Paracetamol is an example of a drug which is eliminated from the body predominantly as its highly polar and water-soluble glucuronide and sulphate conjugates. The cysteine and *N*-acetylcysteine conjugates are two other polar metabolites which are excreted in significant amounts when a substantial overdose of drug (20 g) is ingested. In this case filtered aliquots of urine can be directly

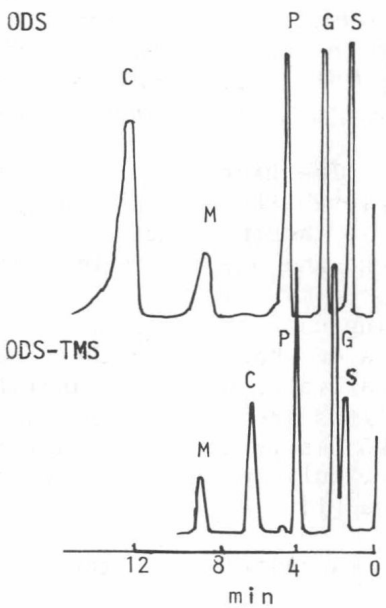

Fig. 1. Comparison of ODS silica *(upper)* with ODS-TMS silica packing *(lower;* TMS = trimethylsilyl*)* for separating paracetamol and its metabolites ([5], *by permission*).
Eluant: water - methanol - formic acid (86:14:0.1 v/v/v).
S = sulphate conjugate; **G** = glucuronide; **P** = paracetamol; **C** = cysteine conjugate; **M** = mercapturate.

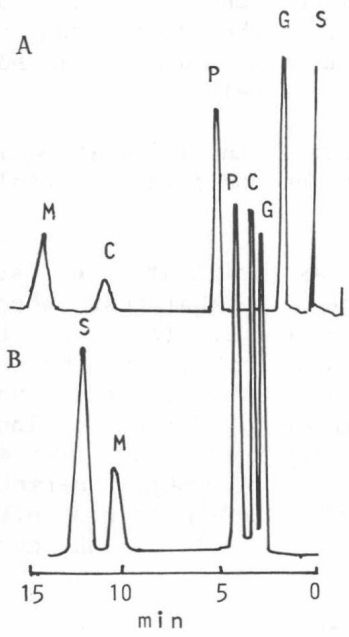

Fig. 2. Separation of paracetamol metabolites on ODS-TMS silica in the absence (**A**) and presence (**B**) of an ion-pairing agent ([6], *by permission*).
Eluant: **A** as for Fig. 1
B as for Fig. 1 but with dioctylamine (DOA; 0.7 mg /l) and KNO_3 (3 g/l).

injected onto the HPLC column, since there is a sufficient concentration of metabolites. With methanol-water-formic acid, Knox & Jurand [5] compared conventional ODS-silica with ODS-silica capped with TMS, and found the latter superior for the determination of paracetamol and its polar metabolites (Fig. 1). The effect of varying the concentration of methanol, formic acid and added salts on the k' values of the 5 compounds was thoroughly examined. The presence of acid in the eluting solvent markedly increased k' for these compounds, partly by suppressing the degree of ionization of the acidic metabolites. Acid in the eluant also decreased the possibility of hydrolysis of the conjugates. Slight pH changes of the eluant around the pK_a value of the metabolites can markedly alter k'. With straight methanol, water and formic acid mixtures as eluant, the elution order was sulphoconjugate, glucuronide, paracetamol, cysteine and *N*-acetylcysteine conjugates. However, upon the addition of salt (KH_2PO_4) to the eluant the capacity factor of the sulphoconjugate increased from zero to 0.8, and the elution order was glucuronide, sulphoconjugate, cysteine conjugate, paracetamol and mercapturate.

The addition of either dioctylamine (DOA) or tetrabutylammonium hydroxide (TBAOH) to the eluant to form hydrophobic ion-pairs with the highly ionized sulphate conjugate dramatically increased its k' value [6], the elution order now being glucuronide, cysteine conjugate, paracetamol, mercapturate and sulphoconjugate. To obtain reasonable values of k' for the mercapturate and sulphoconjugate it was necessary (Fig. 2) to add a salt (KNO_3) to the eluant when DOA

was used. However, there was not such a pronounced effect on k' for the sulphoconjugate when TBAOH was added to the eluant, thus making the addition of salt unnecessary. The column loadings for DOA and TBAOH were up to 7 mg/l and 200 mg/l respectively.

After optimization for the separation of the reference standards the composition of the eluant may have to be modified when biological material is being examined.

The ability to use aqueous eluants has facilitated the isolation and characterization of polar water-soluble metabolites. When ion-suppression is used, then the eluate corresponding to the metabolite peaks can be collected and either freeze-dried [7] or the solvent removed under reduced pressure. If necessary the conjugate can then be reconstituted and further purified by HPLC. The use of ion-pair reagents in the eluant does not necessarily negate the use of MS, although there may be a considerable excess of reagent relative to the metabolite. Thus, DOA because of its low eluant concentration was preferred to TBAOH when MS was used to characterize the metabolites of paracetamol [6].

ISOLATION AND IDENTIFICATION OF METABOLITES

Usually an enrichment step is required prior to the use of HPLC for purification and isolation purposes. For sample enrichment, body fluids can be injected directly [8] onto fully automated HPLC possessing pre-columns, which is useful for the routine determination of drugs and metabolites when their structure is known. However, for identification purposes it may be necessary to extract the acidic conjugate from the biological fluid prior to using HPLC. Probenecid acyl glucuronide was identified by ^{13}C-NMR after its extraction from acidified urine and purified with other metabolites of probenecid using ODS material [9]. Similarly Veenendaal & Meffin [10] used this approach for the purification and identification of the glucuronide of clofibric acid. The evidence that the purified compound was an *O*-glucuronide was that it was hydrolyzed in the presence of β-glucuronidase to clofibric acid, and that this reaction was inhibited by D-saccharo-1,4-lactone, a β-glucuronidase inhibitor. These reactions were monitored by HPLC. Further evidence of the identity of the glucuronide was that its absorbance ratio, measured at 227 nm and 277 nm, was similar to the ratio observed for the aglycone, making the reasonable assumption in this case that the glucuronyl moiety did not alter the absorption spectrum. Plasma concentrations as low as 1.5 µg/ml of glucuronide were directly measured by HPLC, by adding phenolphthalein glucuronide as an internal standard, in a trichloroacetic acid solution (Fig. 3). Interestingly, 4 isomers of the glucuronide of clofibric acid have been reported on the basis of GC-MS results, viz. the α and β anomers of the pyranose and furanose forms of the glucuronide [11].

The diastereoisomeric glucuronides of the 1,4-benzodiazepine, oxazepam, have been quantitatively measured by HPLC using ODS-silica and ion suppression [12], after their isolation and separation with XAD-2 and DEAE-cellulose-DE-23. The rate of enzymic hydrolysis of the purified S-(+) and R-(-) isomers was studied by HPLC with various β-glucuronidases from different sources. This study elegantly illustrated that this enzyme assay does not always provide unequivocal evidence of the identity of a suspected glucuronide if the production of aglycone is being measured. The rate of hydrolysis of the S-isomer was 400 times faster than for the R-isomer, for *E. Coli* β-glucuronidase, and moreover this enzyme preparation was far more stereoselective than β-glucuronidases obtained from bovine liver, marine molluscs and *Helix pomatia*.

Similar observations were made in another study [13] where HPLC was used to monitor the hydrolysis of 1-naphthol glucuronide and 1-naphthol sulphate by β-glucuronidases and aryl sulphatases from different sources.

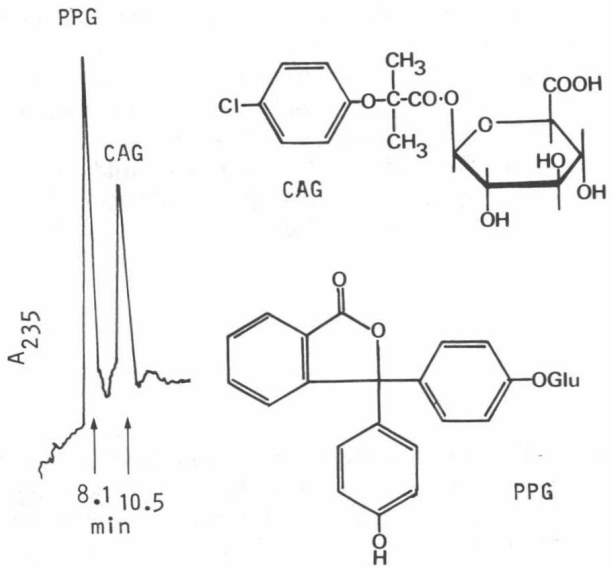

Fig. 3. Separation of the glucuronides of clofibric acid and phenolphthalein on ODS-silica [10].
Eluant: acetonitrile-glacial acetic acid-water (450:5:545).
CAG = clofibric acid glucuronide; **PPG** = phenolphthalein glucuronide.