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Journal of Chromatography A, 719 (1996) 3–13

JOURNAL OF CHROMATOGRAPHY A

Review

Importance of stereospecific bioanalytical monitoring in drug development

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Abstract

The role of the bioanalyst in the support of drug discovery and development is described with particular emphasis upon stereospecific assays for the individual optical isomers of chiral drugs. The significance of the stereochemical aspects of pharmacokinetics and drug metabolism in both preclinical and clinical development is summarized and illustrated with reference to the pharmacogenetic polymorphisms of drug oxidation existing in the human population. The significance of stereochemical considerations in drug metabolism and pharmacokinetics has recently become an issue for both the pharmaceutical industry and the regulatory authorities, driven to a great extent by recent developments in methodology for both the analytical and preparative resolution of racemic drug mixtures. This has led to the so-called 'racemate-versus-enantiomer' debate in recent years. The development of regulatory attitudes in the major jurisdictions of the world to the development of new drugs containing one or more chiral centres is outlined.

Contents

1. Pharmacokinetics, bioanalysis and drug development

Successful drug therapy requires reliable relationships between dose and response and a knowledge of factors affecting these relationships. Drug action is the result of interactions with target sites, for both desired (pharmaco-

0021-9673/96/$32.00
SSDI 0021-9673(95)00465-3

logical) and undesired (toxic) actions, modulated by the transfer processes, the pharmacokinetic variables of absorption, distribution, metabolism and elimination, by which the drug enters and leaves the body.

1.1. The purpose of disposition and metabolism studies

In general, there exist better relationships between effect and internal exposure, i.e., concentration at the targets, most frequently related to plasma concentration, than with the external dose offered [1]. The accurate and reliable determination of drug concentration in biological fluids is thus of paramount importance. This bioanalytical monitoring must determine the molecular species responsible for the activity of interest, be it the parent compound, a metabolite, the active stereoisomer, etc., within the relevant concentration range (which can be very wide) in the relevant matrices (urine, bile, plasma etc.) and be robust, economical in time and cost and with an acceptable specificity and reproducibility. The drug metabolism and pharmacokinetic studies which the bioanalytical monitoring programme underpins have essential roles to play in all stages of the pharmaceutical research and development process, ideally being involved from the pre-nomination phase in drug discovery right through to post-marketing surveillance. The pharmacokinetic sphere has immense significance in drug development because the ADME (absorption, distribution, metabolism and elimination) studies which it involves generate information on:

1. the chemical natures of molecules produced within the target organism to which specific target sites will be exposed, and
2. the quantities and concentration–time courses of these various compounds (parent and metabolites) within the target organism and at specific target sites within it.

The vast majority of drugs and other foreign chemicals which enter the animal body undergo enzymic metabolism before elimination, principally in the urine or the faeces, subsequent to biliary excretion [2]. The products thus may retain, or even have enhanced pharmacological activity and may be involved in toxic responses as a result of their enhanced reactivity, resulting in covalent binding to critical macromolecular targets in the cell [2]. There occurs far more inter- and intra-species variation, both in animals and humans, in the factors influencing the nature and extent of internal exposure, than in the sensitivity of drug targets and this pharmacokinetic variability is the cause of major problems in drug development and use [2–4].

1.2. Disposition studies in drug development

The objectives of the various pharmacokinetic studies which are performed during the course of drug development may be summarized briefly as follows, namely the determination of:

1. Drug levels and kinetics in blood, body fluids and tissues.
2. The rate and extent of absorption at different dose levels.
3. The distribution of the drug and its metabolites in tissues, organs and body fluids at different dose levels and after single and multiple doses.
4. The pattern and rate of metabolism and excretion at different dose levels.
5. Plasma protein and tissue binding of the drug and its metabolites at different dose levels.
6. Accumulation and/or retention of the drug and its metabolites upon chronic dosing.
7. Enzyme induction and inhibition potential of the compound.
8. Characterization, identification and pharmacokinetics of metabolites.

From the foregoing, it will be apparent that the work of the bioanalyst underpins all of the drug metabolism programmes, which have the longest contact with a drug molecule during its research and development. Drug metabolism and disposition studies have critical roles to play in the process of nominating new compounds into development. Through the development phase, the work of metabolism and kinetic

scientists is absolutely critical to the successful development programme and extends to post-marketing support. In each case, the accurate and reliable determination of the concentration of the drug and, where relevant, its metabolites, in biological fluids is thus a sine qua non for successful drug development.

2. Stereochemical aspects of drug action and disposition

Recent years have seen a re-discovery of the significance of stereochemistry in drug action [5]. There may be large differences between the receptor affinities of stereoisomers of chiral drugs, at least when the chiral centre(s) of the drug are important in its interaction with its target. Living organisms are made up of macromolecules abundant in chiral centres of defined configuration e.g., D-sugars, L-amino acids. It was a matter of early experience in biochemistry and pharmacology that the receptors and enzymes which are the targets of drug action are able to discriminate between stereoisomers, e.g., adrenaline and related sympathomimetics, cholinesterase inhibitors. Recent insights into macromolecular structure given by X-ray crystallography and molecular biology account for this discrimination at the molecular level: particular successes have been obtained with the β-adrenergic [6] and muscarinic cholinergic [7] receptors and cyclooxygenase (prostaglandin H_2 synthetase) [8].

In contrast, the realization of the importance of chiral discrimination for the pharmacokinetic phase of drug action is more recent [5]. The availability of novel analytical modalities has led to substantial growth in our knowledge of the magnitude and consequences of such discrimination. The pharmacokinetic importance of drug stereochemistry depends upon the mechanism of the process under consideration: passive processes such as diffusion across membranes do not involve macromolecular interactions and stereochemistry has little influence, but when the drug interacts with an enzyme or a transporter system, then discrimination may be seen [9]. There is now a range of examples showing differences between stereoisomeric forms of numerous drugs in terms of their absorption, distribution, metabolism and excretion [9–12]. Although often of biological importance, the magnitude of these differences is generally much less than those exhibited by receptor and enzyme targets.

Much currently available metabolic and pharmacokinetic data on racemic mixtures is derived from the non-selective assay of the total drug present in biological media, i.e., the sum of the individual enantiomers. Such data have at best limited value and can be highly misleading, particularly when attempting to relate plasma concentrations to pharmacological effect or therapeutic benefit [13,14].

2.1. Pharmacological and toxicological significance of enantioselective drug disposition

The examination of the pharmacokinetics of individual isomers enables the determination of the 'true' pharmacokinetic parameters of the active agent and provides a basis for the determination of enantiomeric potency ratios and, if required, rational therapeutic drug monitoring. Enantiospecific pharmacokinetic studies in patients and/or human volunteers have explained apparent anomalies in drug concentration-effect relationships with route of administration. Thus, the cardiotonic agent verapamil (Fig. 1) appears to be more effective when given intravenously than orally, while the reverse is true for the β-blocker propranolol (Fig. 2). Both these findings are due to stereoselective first-pass metabolism which results in the enantiomers having different bioavailability after administration of the racemates. In the case of verapamil, this metabolism is selective for the more active enantiomer [15,16] whilst the reverse is true for propranolol [17,18].

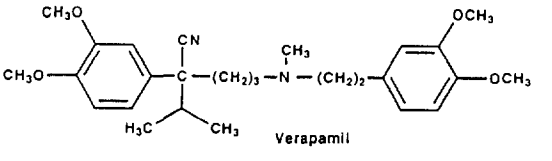

Fig. 1. Structure of verapamil.

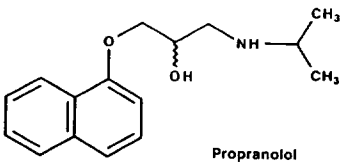

Fig. 2. Structure of propranolol.

Toxicity testing of drugs and other xenobiotics is carried out using animal models and there are a number of examples where the disposition of the enantiomers of racemic drugs differ markedly between species (vide infra). Examination of the pharmacokinetics of the enantiomers of racemic drugs in various species is thus necessary for the effective extrapolation of preclinical safety data to the human situation.

Data obtained from in vivo studies in animals and humans must be treated with some care if the enantiomeric composition of a drug or metabolites determined in excreta is to be used as an indication of stereoselectivity in metabolism. In such cases the observed enantiomeric excess may reflect a number of stereoselective processes, e.g., absorption, protein binding, selective tissue uptake, renal and/or biliary excretion in addition to metabolism. Hence the enantiomeric composition of metabolites may not be a reflection of enzyme activity (see, for example, Winter and Caldwell [19]).

2.2. The clinical significance of enantioselective drug disposition: the case of propranolol

Propranolol metabolism and excretion provides a useful example of the above. This compound undergoes three major metabolic pathways (Fig. 3), namely direct glucuronidation at the free hydroxyl group, oxidative metabolism of the aminopropanol side chain to yield 3-naphthoxylactic acid, and aromatic oxidation to yield 4-hydroxypropranolol which then may also undergo conjugation with both sulfate and glucuronic acid [18,20,21]. Each of these pathways exhibits stereoselectivity. An examination of the enantiomeric composition of the material excreted in urine indicates a slight preferential metabolism of *R*-propranolol to yield *R*-4-hydroxypropranolol, while glucuronic acid conjugation and side chain oxidation are apparently selective for the *S*-enantiomer. However, consideration of the partial metabolic clearances of the enantiomers along each pathway indicate that the primary metabolism of propranolol is domi-

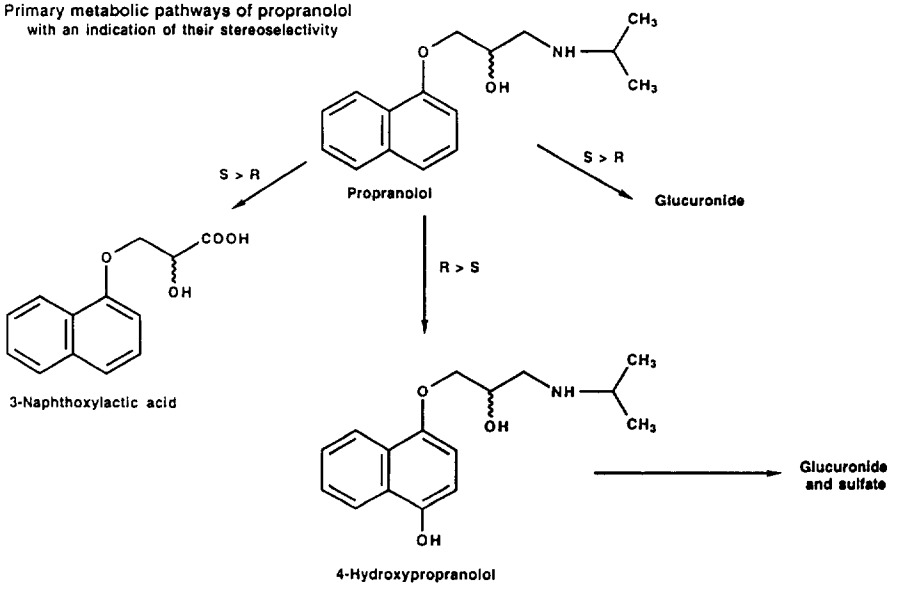

Fig. 3. Major metabolic pathways of propranolol.

nated by the first pass (presystemic) 4-hydroxylation which is highly selective for *R*-propranolol. This preferential first pass metabolism of the inactive *R*-enantiomer enriches the plasma concentration with respect to its active *S*-antipode, accounting for the observations mentioned above of the apparently 'greater potency' of the racemate when given orally [17]. The partial metabolic clearances via the other two pathways (which are systemic) show only slight stereoselectivity, which in neither case reaches statistical significance. The enantiomeric composition of the urinary excretory products thus arises from the increased concentration of *S*-propranolol available to undergo glucuronidation and side chain oxidation [18,20,21].

3. Enantiomer–enantiomer interactions

That different stereoisomers of the same compound can have different activities is well established, e.g., the (−)-opiates are analgesic while their (+)-antipodes are antitussive, but interactions between enantiomers, in which one stereoisomer can interfere with the actions of another, are less well understood. One major origin of these is in their pharmacokinetics, where enantiomers are mutually inhibitory of each other's disposition. Most studies on enantiomer–enantiomer interactions have examined the effect of one enantiomer on the pharmacokinetics rather than the metabolism of its antipode.

3.1. Use of pseudoracemates: indobufen as a case example

Pseudoracemates, i.e., mixtures of equal amounts of one enantiomer which is labelled with a stable or radioisotope and its differently labelled or unlabelled antipode, introduced by McMahon and Sullivan [22], have provided a potent tool for the investigation of enantiomer–enantiomer interactions. It has the advantage over other techniques of allowing the direct quantitation of one or both parent enantiomers and all their metabolites, and may involve stable or radioisotopes. The relative abundances of the various isotopic forms thus provides a direct indication of the ratio of the stereoisomers without the necessity for their physical separation by chromatographic or others means.

There occur metabolic and kinetic interactions between the stereoisomers of many drugs, so that the effects of the racemates are different from those of their constituent isomers. The possibility of such interactions can be explored by the use of pseudoracemic mixtures of labelled and unlabelled enantiomers and will be illustrated here with reference to indobufen, where a ^{14}C-labelled psuedoracemate has been used.

Indobufen is an inhibitor of platelet aggregation, acting by inhibition of thromboxane A_2 formation. Like its close relatives the 'profen' non-steroidal antiinflammatory drugs, its inhibitory actions on arachidonic acid metabolism are mediated through the *S*-enantiomer. Unlike the profens, this 2-arylbutyric acid does not undergo metabolic chiral inversion in laboratory animals or man [23]. There are marked interspecies differences in the metabolism of indobufen along the pathways shown in Fig. 4. In humans and mice, the major route is by glucuronic acid conjugation at the carboxyl group, but in rats, the molecule is hydroxylated at the 5-position of the isoindolinyl ring and the phenol so produced is excreted as a sulfate conjugate [24]. In the rat, *S*-indobufen shows a marked dose dependency of metabolism and excretion, with 48% of a dose of 10 mg/kg being excreted in the urine and faeces in 48 h as free and sulfated 5-hydroxyindobufen (5-OHIB) but only 18% in this form after 20 mg/kg [25]. In comparison, with racemic indobufen the excretion of 5-OHIB was 27% of the dose at 10 mg/kg and 11% at 20 mg/kg [24,26]. These data suggest that (i) the hydroxylation of indobufen is saturable in the rat, (ii) *S*-indobufen is preferentially hydroxylated, and/or (iii) the *R*-enantiomer present in the racemate inhibits the hydroxylation of its *S*-antipode. To resolve the situation, it was decided to apply the pseudoracemate approach to the in vivo metabolic enantiomer–enantiomer interaction of indobufen in the rat using high-performance liquid chromatographic (HPLC) separation of the en-

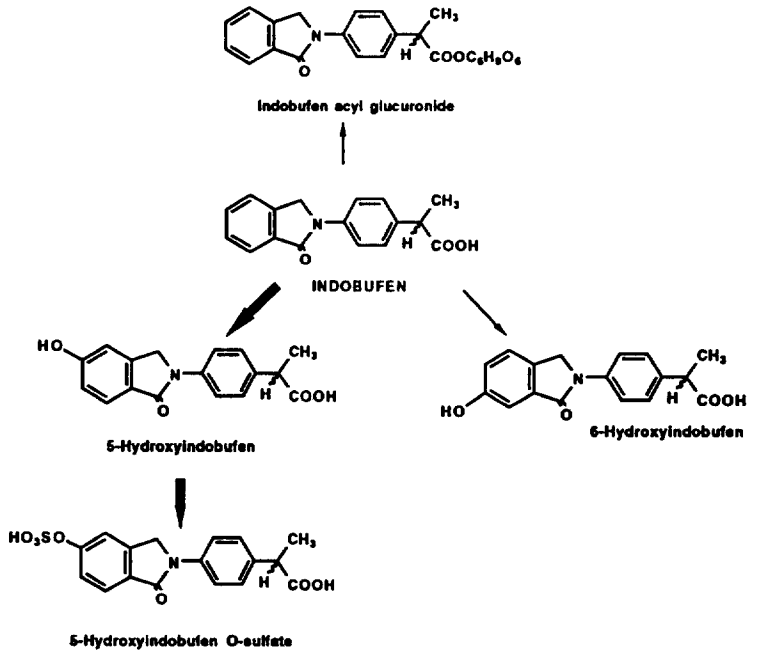

Fig. 4. Major metabolic pathways of indobufen.

antiomers of indobufen and 5-OHIB as their L-leucinamides, as illustrated in Fig. 5 [24].

Rats were given 10 or 20 mg/kg of a pseudo-racemate of [^{14}C]-*S*-indobufen mixed with unlabelled *R*-indobufen, their urine and faeces were collected for 48 h and analyzed for in-

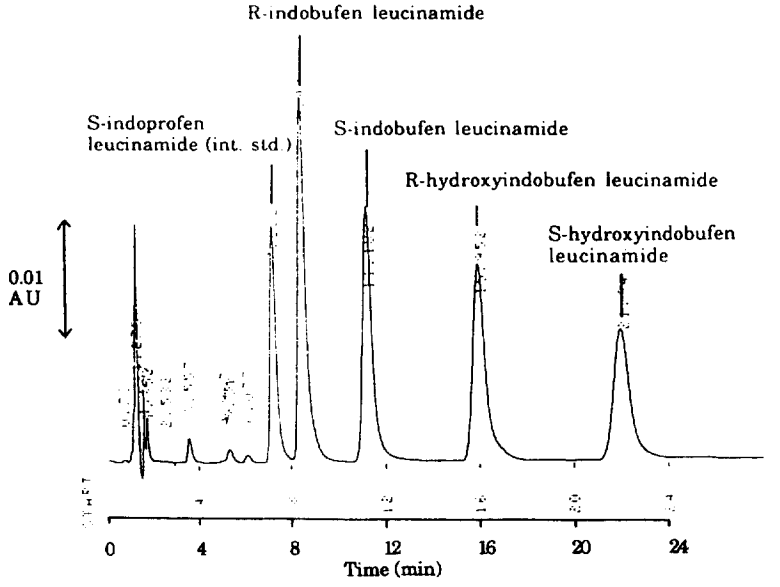

Fig. 5. HPLC separation of the enantiomers of indobufen and 5-hydroxyindobufen as L-leucinamides. Column: Merck LiChrosorb C_{18} cartridge, 250 × 4 mm I.D. Mobile phase: acetonitrile–10 m*M* phosphate buffer, pH 6.5 (35:65, v/v), flow-rate 2 ml/min. The L-leucinamides were formed via a carbodiimide reaction as described by Grubb et al. [25].

dobufen and its metabolites by radio-HPLC. After the 10 mg/kg dose, effectively the same as 5 mg/kg of [^{14}C]-*S*-indobufen, some 47% was excreted as free and sulfated 5-OHIB, but after the 20 mg/kg dose, equivalent to 10 mg/kg of [^{14}C]-*S*-indobufen, the excretion of 5-OHIB was unaltered at 45% of dose.

The recovery of 5-OHIB after 10 mg/kg pseudoracemic indobufen was markedly lower than after the *S*-enantiomer at the same dose level. However, at the 20 mg/kg dose level, the opposite was true, i.e., the recovery of 5-OHIB was less after the pseudoracemate. The saturation of the metabolism of *S*-indobufen at the higher dose complicates the interpretation of these data, but it is clear that at these doses, 10 mg/kg pseudoracemate being equivalent to 5 mg/kg *S*-indobufen and 20 mg/kg to 10 mg/kg *S*-indobufen, the fate of *S*-indobufen is essentially identical. The higher recovery of *S*-5-OHIB (detection of the ^{14}C label after HPLC measures only the *S*-enantiomer) after 20 mg/kg pseudoracemic than after racemic [^{14}C]-indobufen (where both enantiomers of 5-OHIB are assayed), 46% versus 33%, further illustrates the enantioselective hydroxylation of *S*-indobufen in the rat in vivo.

The use of the pseudoracemate approach to this problem, with the ease of quantitation of only the radiolabelled enantiomer, has enabled us to distinguish the separate effects of dose size, enantiomeric interaction and substrate enantioselectivity on the hydroxylation and supports the interpretation that the *R*-enantiomer has an inhibitory effect upon the 5-hydroxylation of *S*-indobufen. This is one of the few experimental approaches to this issue which can be applied reliably in vivo, and provides an important adjunct to the various methods applicable in vitro.

These studies show the utility of isotopically labelled pseudoracemic mixtures as tools giving considerable insight into the metabolism of chiral drugs and allow the easy discernment of enantiomer–enantiomer interactions in metabolism. There are relatively few adequately documented examples of enantiomer–enantiomer interactions and these have been underemphasized, largely due to the lack of, until recently, suitable analytical techniques. Interaction between enantiomers may be more common than the literature indicates, but is not easily detected unless specifically sought: the data reported here and to be found elsewhere in the literature show that the technical difficulties posed by such questions may be readily overcome by the pseudoracemate approach.

4. Pharmacogenetics and the stereochemistry of metabolism

'Pfeiffer's rule' [27], which generalizes that the more potent a drug is, the more likely it is to show stereoselectivity of action as a consequence of the greater steric demand for tight receptor binding, may have parallels in drug metabolism. However, the drug metabolizing enzymes have always been regarded as showing little substrate selectivity, making such generalizations difficult. In the past ten years, it has become clear that the relative lack of substrate specificity of the major drug metabolizing enzymes is illusory and that it is the net result of the activities of families of isozymes which often have marked, but overlapping, catalytic specificities. It may thus be the case that an enzyme with marked specificity may well show stereoselectivity and this is the case with two cytochrome P450 isozymes, CYP2D6, which catalyzes the hydroxylation of debrisoquine and sparteine (Fig. 6) and CYP2C19 which performs the aromatic hydroxylation of mephenytoin (Fig. 7).

4.1. Stereoselectivity and the 'debrisoquine polymorphism'

The best known of the human genetic polymorphisms of drug oxidation is that affecting the metabolism of debrisoquine and which arises from the virtual absence of CYP2D6 from the poor metabolizer phenotype. Drugs such as debrisoquine which are subject to this polymorphism are substrates almost exclusively metabolized by this isozyme. Debrisoquine itself is achiral, but a number of other compounds whose

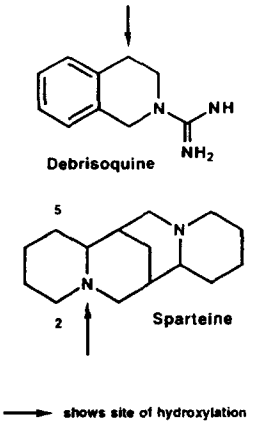

Fig. 6. Structures of debrisoquine and sparteine, archetypal substrates for CYP2D6.

metabolism is influenced by the polymorphism contain chiral centres and, in a number of these cases, CYP2D6, a high affinity, low capacity isozyme, is able to discriminate between the enantiomers [28,29].

A very significant example of the stereoselectivity of the CYP2D isozymes is given by their differential inhibition by the Cinchona alkaloids, quinine and quinidine, which differ in their configuration at C-8 and C-9 (quinine is 8*S*, 9*R* and quinidine is 8*R*, 9*S*; Fig. 8). Quinidine inhibits the human isozyme CYP2D6, converting the predominant extensive metabolizer (EM) phenotype for debrisoquine and sparteine into 'phenocopies' of the poor metabolizer (PM) phenotype, which are markedly deficient in CYP2D6 activity [30]. In contrast, quinine has no effect upon the metabolism of typical CYP2D6 substrates in humans [30]. The rat orthologue of CYP2D6 is CYP2D1, which shows comparable substrate selectivity. The female

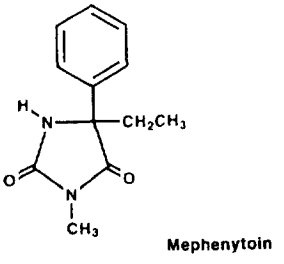

Fig. 7. Structure of mephenytoin.

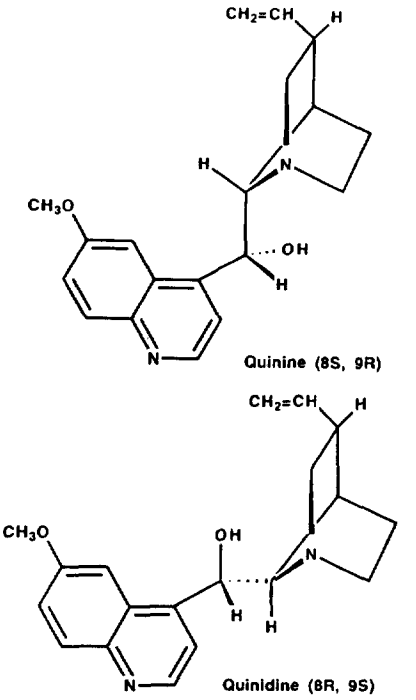

Fig. 8. Structures of quinine and quinidine, stereospecific inhibitors of CYP2D1 and 2D6, respectively.

Dark Agouti (DA) rat seemingly lacks CYP2D1 and provides a limited animal model of the human debrisoquine PM phenotype [31]. However, in the rat, the stereoselectivity of CYP2D inhibition by the Cinchona alkaloids is reversed, with quinine inhibiting CYP2D1 while quinidine has no effect [32].

5. 'Chiral pharmacology' and the regulation of new drugs

The significance of stereochemical considerations in drug metabolism and pharmacokinetics has recently become an issue for both the pharmaceutical industry and the regulatory authorities [33]. Recent developments in methodology for both the analytical and preparative resolution of racemic drug mixtures [34,35] have provided a major stimulus for the present considerable interest in stereochemical considerations in drug disposition.

More and more new drugs are designed to

interact with targets which can be described in atomic detail, and chiral discrimination by these targets has to be taken into account ab initio in the design process. Interest in this area has led to the re-examination of chiral drugs already under development or in use as racemic mixtures to see if they might be improved if used in stereochemically pure form [5,13,14]. The use of stereochemically pure drugs would be expected to be advantageous by:

(i) reducing the total dose given,
(ii) simplifying dose–response relationships,
(iii) removing a source of intersubject variability, and
(iv) minimizing toxicity due to the inactive isomer.

The steeper the dose–response curve, the greater the benefit to be expected.

The decision as to the relative value of racemate or pure enantiomer is multifactorial and is driven by the magnitude and significance of the pharmacodynamic and pharmacokinetic differences between the enantiomers and their clinical significance as well as marketing advantage. The cost penalty associated with the use of a stereochemically pure drug must be balanced against the extra development costs of a racemate, since emerging regulations (vide infra) now demand information about its component isomer as well as the racemate itself.

It was not until the mid 1980s that a scientific consensus emerged that the significance of stereochemistry of drug action and disposition was a topic worth examining on a general basis: while there are a few examples of drugs developed before 1990 as single isomers, the great majority of synthetic drugs reaching the marketplace were racemic mixtures of optical isomers. Since 1983–1984, there has been an ongoing 'racemate-versus-enantiomer' debate, examining the advantages and disadvantages of each. This has been driven in part by technical advances in analytical and preparative chemistry, making pure stereoisomers readily available in quantity and providing means for their separate assay in animal and clinical studies, and in part by developments in structural biology and computational chemistry, which have led to basic concepts in molecular recognition and allowed the visualization of molecular targets for drugs in three dimensions

While the first years of this debate were largely academic in character [13,14], since 1987 the main jurisdictions have explored these issues to consider whether additional guidelines were required [36–39]. The Food and Drug Administration (FDA) of the USA and the European Union Committee on Proprietary Medicinal Products (CPMP) have both issued formal guidelines, in 1992 and 1993 respectively, while the Japanese will not issue formal guidelines but deal with the matter on a case-by-case basis through the consultation procedure used for many other comparable points [39]. Amongst other authorities, those of Switzerland, Australia and the Nordic Countries have promulgated formal guidance, while a consultation on draft guidance is currently ongoing in Canada.

However, although different authorities have dealt with this matter in different ways, a broad philosophical commonality may be discerned. In all cases, there is a first requirement that the chirality of the drug substance be recognized and that the stereoisomer(s) responsible for the activity of interest be identified. Stereochemical considerations must be added to the specification of the drug substance, including assignment of absolute configuration, an enantioselective analytical method and a full description of the synthesis, notably the formation of the chiral centre.

The most important addition to the normal requirements for drug approval is the need to justify on chemical, preclinical and clinical grounds the choice of the stereoisomeric form(s) chosen for marketing. If this is a single isomer, then the only additional requirements are those for the discussion of reasons for choice of a single isomer, the documentation of the synthesis, especially the formation of the chiral centre, expansion of the relevant sections of the specification of the drug substance (purity, batch-to-batch variation, etc.) and the confirmation of optical stability in formulation, on storage and in vivo.

If a racemate is chosen, the requirements in

general are those for any new chemical entity, with the addition of the justification of the choice of a racemate over a single isomer, the relevant expansion of the specification of the drug substance (purity, batch-to-batch variation, etc.), the pharmacokinetic evaluation of individual isomers and the evaluation of any metabolic chiral inversion occurring in vivo. Even though there is a growing trend towards the development of single isomers, there remain a number of justifications for the development of a racemate. There may be little difference between enantiomers in activity, pharmacokinetics or toxicity, the enantiomers may be optically unstable in vitro or in vivo, while it may be impossible to obtain the desired enantiomer in sufficient quantity at an acceptable and reproducible optical purity. Thus far, no authority has indicated any intention to refuse approval to racemates or to conduct a retrospective evaluation of marketed racemates. However, the choice of a racemate must be justified and the criteria for this will become more demanding as time goes by. In particular, the justification becomes harder with steeper dose–response curves and narrower therapeutic indices.

The past ten years have seen great emphasis on the importance of stereochemistry in drug development, largely in the form of the 'racemate-versus-enantiomer' debate. It is now arguable that this debate is over, with the great majority of innovative drug companies worldwide focusing their activities on single stereoisomeric forms. While there are opportunities in the generic marketplace for 'racemic switches' from a previously marketed racemate to an active enantiomer, these are limited. There are probably only 20 or so drugs where such a switch would give genuine therapeutic benefit and be economically feasible.

The renewed attention paid to chiral issues in drug development in recent years makes a contribution to the goal of safer, more effective and more easily used drugs. The consideration of the importance of chirality in drug development is moving on from a first generation of issues, the 'racemate-versus-enantiomer' debate, to new perspectives, notably arising from fundamental insights into molecular recognition and the exploitation of these new levels of understanding in drug design. From the viewpoint of the bioanalyst, the present emphasis upon stereospecific assays of drugs and their metabolites will remain, driven both by the need to assess the relative merits of racemate and enantiomer and to assure the stereochemical stability of optically pure drugs in biological matrices.

Acknowledgement

I wish to thank Mrs. Sheila Rose for her help in the preparation of this manuscript.

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