ELSEVIER

Journal of Chromatography B, 678 (1996) 73–92

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Review

Investigation of xenobiotic metabolism by CYP2D6 and CYP2C19: importance of enantioselective analytical methods

Antonio Marzo[a,*], Luc P. Balant[b]

[a] *I.P.A.S. S.A., Clinical Pharmacology Department, Via Giulia 43, 6855 Stabio, Switzerland*
[b] *Département de Psychiatrie, Unité de Recherche Clinique, 47 Rue du XXXI Décembre, Geneva, Switzerland*

Abstract

Investigations into the genetic polymorphism of drug metabolism have involved specific models to screen poor and extensive metabolisers of xenobiotics. Debrisoquine, sparteine, *S*-mephenytoin and dextromethorphan are particularly well known. They have been extensively described in the literature and are used to phenotype human subjects before performing investigations with new drugs which are believed to be under the control of a genetic polymorphism. Dextromethorphan, debrisoquine and sparteine are good substrates for CYP2D6, whereas the *S*-enantiomer of mephenytoin is a good substrate for CYP2C19, both being two isozymes of cytochrome P-450. In many drugs, the hepatic microsomal oxidative metabolism involving stereogenic centres congregates either with CYP2D6 or with CYP2C19 or, in certain cases, with both of them. The availability of both CYP2D6 from poor and extensive metabolisers and an enantioselective assay would allow genetic polymorphism in drug biotransformation to be investigated in vitro ex vivo at an early stage of drug development before the IND (investigational new drug). Single-dose investigations in vivo can also be performed when only minimal pre-clinical toxicological data are available and produce more reliable results than in vitro studies. This paper focuses on the problem of genetic polymorphism in drug development and specifically discusses some relevant knowledge gained in the last two decades on enantioselective bioassays specific examples; are given.

Keywords: Reviews; Enantiomer separation; Xenobiotics; Debrisoquine; Sparteine; *S*-Mephenytoin; Dextromethorphan; CYP2D6; CYP2D19; Enzymes

Contents

* Corresponding author.

0378-4347/96/$15.00
SSDI 0378-4347(95)00229-4

1. Introduction

Genetic polymorphism is a crucial problem in drug development, mainly because efforts to obtain an NDA (new drug application) for an innovative drug are fully justified only after multistate approval [1].

Several examples exist of drugs which display different biotransformation rates due to genetic polymorphism [2–4]. During investigations on an innovative molecule some probe drugs are available to phenotype a given population into extensive (EM) and poor (PM) metabolizers of debrisoquine/sparteine/dextromethorphan (good substrates of CYP2D6) or of *S*-mephenytoin (good substrate of CYP2C19) type [4] (Fig. 1).

As genetic polymorphism can seriously complicate the development of a new drug, and in certain cases can even lead to its discontinuation, much effort continues to be expended by pharmaceutical companies and investigators to gain useful information on this crucial problem as early as possible in drug development stages, possibly before the IND (investigational new drug) [4]. This is possible if investigations are performed in vitro on liver samples from PMs and EMs.

As genetic polymorphism very often involves stereogenic metabolism [5], enantioselective bioassays are mandatory for in vitro ex vivo and in vivo studies. Enantioselective assays are fully achievable with chromatographic techniques, namely with high-performance liquid chromatography (HPLC), gas chromatography (GC) either by chemical derivatisation leading to diastereoisomers or by chiral columns, and with capillary electrophoresis (CE).

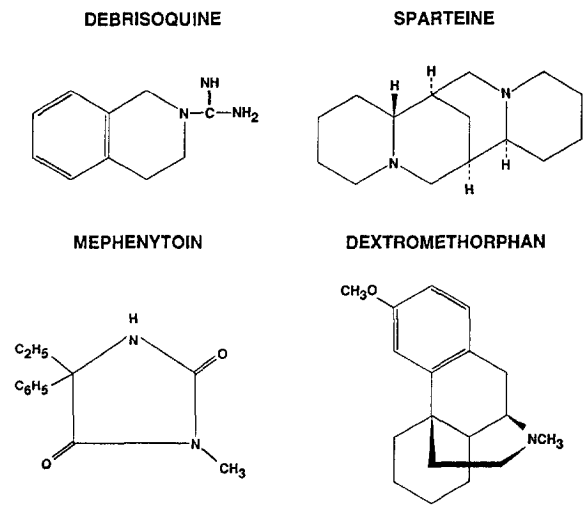

Fig. 1. Chemical structures of debrisoquine, sparteine and dextromethorphan, good substrates for CYP2D6, and of mephenytoin, a good substrate for CYP2C19.

In some more complex cases a two-step analytical approach is used, e.g. an achiral quantitative assay followed by evaluation of the enantiomeric ratio, or a first chiral separation of the enantiomers individually collected and quantitatively assayed by a second analytical approach.

The overall issue of genetic polymorphism in drug metabolism is extensively discussed in this paper, and includes several up-to-date examples and specific references to enantioselective assays involving products of oxidative metabolic steps catalysed by CYP2D6 and CYP2C19.

2. The role of genetic polymorphism in drug development

Among the factors which hinder the introduction of a drug on the market, a narrow therapeutic range combined with large interindividual variations in pharmacokinetics are of major concern. With a compound with these features, it can be presumed that part of the patient population receiving a "standard dose" of the drug will develop unwanted side effects related to "higher-than-normal" blood concentrations of the active principle, whereas other patients might show an "apparent non-response" due to unusually low concentrations. It is now well recognised that some drugs are eliminated by metabolic routes that are under genetic control. Some of these metabolic pathways are distributed in such a way that patients and/or healthy volunteers can be classified into distinct subpopulations. Among the best-studied routes showing genetic polymorphism are sulfonamide-type acetylation and debrisoquine-type and mephenytoin-type hydroxylation.

The clinical relevance of such genetic variability depends markedly on the therapeutic class of the compound and on the concentration–effect relationship of the desired and the unwanted effects. β-Blocking agents which display a large therapeutic margin can thus be used almost regardless of the patient's phenotype, as large interindividual variations in steady-state blood concentrations are probably of minor clinical significance and certainly do not warrant blood concentration monitoring. In contrast, it is now generally recognised that in tricyclic antidepressants, blood level monitoring is important to prevent unwanted side effects and therapeutic failures although this point of view is still not universally accepted. In the current viewpoint, the benefit-to-risk ratio for a new drug has to be very carefully assessed. This may lead the pharmaceutical industry to stop developing potentially important new chemical entities, for which early studies in man indicate that there may be a "higher-than-expected" incidence of side effects or difficulties in managing differences in dose requirements between different patients.

2.1. Typical examples of genetic polymorphism

2.1.1. N-Acetylation

2.1.1.1. Historical and mechanistic considerations. First discovered by Bonicke and Reif [6] and established on a sound basis by Evans et al. [7], the polymorphic acetylation of isoniazid is the oldest known polymorphism of drug metabolism. It was discovered as a result of studies on the fate of isoniazid in tuberculous patients and later was shown to be genetically controlled by observations in healthy families. Two phenotypes have been found to exist, namely rapid and slow acetylators. Numerous reviews [8–13] have discussed the various implications of acetylation polymorphism. In addition to isoniazid, other polymorphically acetylated drugs are aminoglutethimide, 7-aminonitrazepam, amrinone, caffeine, dapsone, hydralazine and hydrazine, procainamide, sulfamerazine, sulfamethazine and sulfapyridine. The molecular genetic and enzymatic basis of this human polymorphism has been determined and it is now possible to genotype healthy volunteers and patients.

Due to the type of chemical group transformed by N-acetyl transferase, no stereogenic centre is involved.

2.1.2. Debrisoquine/sparteine oxidation

2.1.2.1. Historical and mechanistic considerations. As is often the case, the discovery of so-called debrisoquine/sparteine oxidation polymorphism was not the result of any planned

Table 1
Selection of compounds whose metabolism congregates with that of sparteine/debrisoquine by means of enzyme CYP2D6

Antidepressants
Tricyclic compounds:
Amitriptyline
Nortriptyline
Imipramine
Desipramine
Clomipramine
N-Desmethylclomipramine
Mianserin
Trimipramine
Serotonine-specific compounds:
Fluoxetine
Norfluoxetine
Paroxetine
N-Desmethylcitalopram
Neuroleptics
Haloperidol
Reduced-haloperidol
Perphenazine
Zuclopenthixol
Thioridazine
β-Blockers
Alprenolol
Bufuralol
Metoprolol
Propranolol
Anti-arrhythmics
Encainide
Flecainide
Mexiletine
Propafenone
Miscellaneous
Amiflamine
Guanoxan
4-Hydroamphetamine
Indoramin
Methoxyphenamine
Perhexiline
Fenformin
N-Propylajmaline
Tomoxetine
Opiates
Codeine
Dextromethorphan
Ethylmorphine
Probe drugs
Debrisoquine
Dextromethorphan
Sparteine

strategy but rather an incidental observation. Early in the development of bufuralol, a β-blocking agent, it was suspected that its metabolism was under the control of a polymorphic enzyme [14]. During a Phase I pharmacokinetic study a volunteer suffered from marked orthostatic hypotension with simultaneous very high blood concentrations of the parent drug and very low concentrations of the hydroxylated metabolite. Further work by the same group confirmed this hypothesis [15,16]. At about the same time, in the UK, researchers investigating the behaviour of debrisoquine, an antihypertensive drug, found that a marked hypotensive response in one volunteer was due to impaired 4-hydroxylation of the drug [17]. At about the same time a group of physicians in Bonn also observed increased side effects associated with decreased oxidative metabolism of sparteine, an anti-arrhythmic and oxytocic drug [18]. Since these early observations, numerous further investigations have indicated that the proportion of "poor" metabolizers is about 7% in Caucasian populations [19–21]. The molecular basis for the defect in debrisoquine/sparteine oxidation is well known and has been extensively reviewed [13,22–24]. The defect in PMs is the result of the lack of synthesis of a cytochrome P-450 isozyme named P-450 2D6 or CYP2D6. Table 1 indicates some drugs whose metabolism is known to be under the control of CYP2D6.

2.1.2.2. Clinical relevance. Debrisoquine/sparteine oxidation polymorphism is a determinant of the pharmacokinetic variability of several useful drugs [25–28]. Despite the ever increasing number of drugs shown to be dependent on this polymorphic enzyme system for their elimination, this monogenetically determined variability becomes clinically significant only in those cases where pharmacokinetic variability is considered important relative to the efficacy and clinical safety of the drug. This means in essence that it is applicable to those drugs for which plasma concentration monitoring is considered useful. At present this "reduced list" includes tricyclic antidepressants, certain neuroleptics and possibly one or two other non-psychotropic drugs. As an example, excessively high concentrations of imi-

pramine and clomipramine and their demethylated metabolites have been measured in PMs [29]. Finally, it is interesting to note that people lacking the isozyme CYP2D6 do not apparently suffer from ill effects, except of course when affected individuals are exposed to certain drugs. This contrasts with genetic diseases such as thalassemias, phenylketonuria and others that have dire health consequences. It must, however, be mentioned that extensive research is in progress to seek possible associations of pharmacogenetic differences and different diseases such as cancer [30,31] or diabetes. The same is true for other known polymorphisms, as extensively discussed in the book edited by Kalow in 1992 [32]. It is, however, difficult to predict at the present time what impact this type of research will have on the development of new chemical entities.

2.1.3. Mephenytoin oxidation

2.1.3.1. Historical and mechanistic considerations. Early studies in the 1950s showed that in man N-demethylation is a major pathway of biotransformation of the anticonvulsive agent mephenytoin. Evidence was also obtained that the metabolic profiles of the clinically available racemic drug product and its desmethyl metabolite were stereoselective. Almost 25 years later, this enantiomeric aspect was reexamined by Küpfer et al. [33,34]. Further studies to examine the more clinically relevant chronic dosing revealed the unexpected finding that one of the normal subjects complained of unacceptable sedation, whereas other volunteers took the medication without suffering from this side effect. Subsequent analysis of urinary metabolic profiles showed a marked impairment in the metabolic formation of one of the metabolites in the "special" subject. It was then discovered that this metabolic deficiency was familial [35]. Finally, based on a panel of Swiss volunteers, it was concluded that a new polymorphism had been discovered [36]. The biochemical consequences of this polymorphism have recently been reviewed [37]. Its molecular basis has now been elucidated, the enzyme responsible being CYP2C19, which catalyses 4′-hydroxylation of *S*-mephenytoin in the human liver [38]. For some time it was believed that this polymorphism had little effect on drug use, but this attitude will probably need to be revised. In fact, it has now been shown that this polymorphism affects omeprazole [39], proguanil [40], certain barbiturates [41,42] and citalopram [43]. The oxidation of propranolol [44], certain tricyclic antidepressants such as amitriptyline [45], imipramine [46] and clomipramine [47] are under the control of both CYP2C19 and CYP2D6. Diazepam is probably also affected, albeit to a lesser extent [48]. As a result, large inter-phenotypic differences occur in the disposition of these drugs, which may affect their efficacy and toxicity.

2.1.3.2. Clinical relevance. The frequency of mephenytoin PMs is approximately 3% in Caucasians and this regulates the metabolism of drugs which display other major sources of variability and/or display a large therapeutic margin. It is thus not surprising that studies performed in Europe and North America have failed to reveal any significant impact on the clinical use of drugs already on the market. This might change for drugs under development and is probably of relevance if some drug products are transferred from Europe to Asia and vice versa, since studies on Chinese and Japanese subjects have shown that 15 to 25% of these individuals are PMs of the CYP2C19-type [49,50].

2.1.4. Other polymorphisms

Numerous other polymorphisms in the metabolism of endogenous or exogenous compounds have been described and reviewed [32], but their relevance for new drug development and registration is less apparent than the three polymorphisms described in the preceding paragraphs. Among the various examples reviewed, trimethylamine (TMA) should be considered.

For humans direct sources of TMA are fishes, mainly marine species, while indirect sources are lecithin, betaine, citicoline, carnitine and its derivatives, namely acetylcarnitine, propionylcarnitine, etc. Indirect sources are all trimethylalkylammonium derivatives which are incompletely absorbed in the small intestine. The unabsorbed moiety in the caecal intestine is

almost entirely metabolized by enteric bacteria to TMA, which is quickly absorbed and N-oxidised in the liver. TMA N-oxidation is controlled by genetic polymorphism for an allele determining impaired N-oxidation in homozygous [32].

2.2. Pharmacokinetic consequences

2.2.1. First-pass metabolism

Experience gained with drugs that are under the control of the debrisoquine/sparteine polymorphism shows that major differences and high inter-individual variability in hepatic first-pass metabolism may be observed [51]. As an example, in one study of bufuralol, systemic clearance was found to be about 37 l/h in EMs and only about 15 l/h in PMs [52]. It was also shown that polymorphic metabolism had a major influence on the extent of hepatic first-pass elimination as judged by the large variability in systemic availability. Since for safety reasons, the intravenous doses used in the study were smaller than the oral doses, the calculated apparent absolute availability even exceeded "one" in a healthy volunteer phenotyped as a "very poor" metabolizer, reflecting the fact that non-linear kinetics during first-pass through the liver probably occur at the higher dose levels used orally in the study.

2.2.2. Non-linear kinetics

Kinetic linearity is a consequence of drug concentrations being well below the K_m of the route of elimination, and it follows that steady-state concentrations are therefore proportional to the dose given. Drugs metabolized via capacity-limited pathways, i.e. pathways partly saturated at drug concentrations obtained in clinical practice, have a potential for concentration-dependent (i.e. non-linear) kinetics. If both linear and non-linear pathways are involved, the non-linear elimination system should contribute significantly to overall kinetics. Moreover, the therapeutic concentrations have to approach or exceed the K_m of the capacity-limited elimination pathway. At dose rates exceeding the V_{max} of the saturable pathways, overall kinetics will again appear linear when the drug eliminated through the saturated systems is just a small fraction of what is cleared per time unit. Studies have shown non-linear kinetics at therapeutic doses of the antidepressive drugs amitriptyline [53,54], nortriptyline [55,56], imipramine [57], desipramine [58] and paroxetine [59]. Their metabolism is partly dependent on CYP2D6, which constitutes the high-affinity–low-capacity pathway. Non-linearity seems to be present only in EMs, who have significant amounts of the enzyme, but not in PMs, who lack this functioning isozyme.

2.2.3. Stereospecific metabolism

This aspect needs special attention, especially during exploratory pharmacokinetic and pharmacodynamic studies during Phase I. This may be exemplified with metoprolol, a β-blocking agent administered as a racemic mixture of its enantiomers. In preliminary reports, stereoselectivity in its disposition was reported before it was known that its metabolism is under the control of the debrisoquine/sparteine polymorphism [60]. Later, the pharmacokinetics of its enantiomers were investigated in panels of EMs and PMs following oral racemic administration [61]. Significantly higher plasma concentrations of the active S-(−)-enantiomer as compared to the inactive R-(+)-enantiomer were observed in EMs, whereas in PMs a decrease in this stereoselectivity was observed. Maximum plasma concentrations of both enantiomers were higher in PMs and the elimination half-life was longer. The results of a study in which EMs were given an oral dose of racemic metoprolol indicate that preferential O-demethylation of the inactive R-(+)-enantiomer contributes to its more rapid clearance [relative to the S-(−)-enantiomer] in this phenotypic group. This difference in metabolic rates is probably absent in PMs. Thus, not only is the overall clearance of metoprolol lower in PMs but the ratios of its active and inactive enantiomers also differ as between the two groups of subjects. This is certainly a good example of pharmacokinetically important differences in drug metabolism. The main question, however, is related to the clinical relevance of this finding if one tries to deduce guidelines for the potential impact of such behaviour on new drug development. As discussed by Lennard [62], the "total plasma" metoprolol concentration–β-

blockade profile is shifted to the left in EMs as compared to PMs, a finding compatible with the higher plasma concentrations of the active isomer in the EM phenotype. Since a greater pharmacological effect would be expected in EMs than in PMs at a given total (R + S) plasma drug concentration, the magnitude of the difference of drug effect between phenotypes would tend to be less than that indicated by the pharmacokinetics of the sum of the enantiomers. Confusion arises from the fact that there is a large absolute difference between the two phenotypes in β-blocking action as well as in total drug concentration, and Lennard [62] thus concluded that these differences cannot be explained by stereoselectivity in the pharmacokinetics of metoprolol.

2.3. Regulatory considerations

In its Note for Guidance "Pharmacokinetic Studies in Man" [63], the Committee for Proprietary Medicinal Products of the European Union recommends that "Pharmacokinetic studies should indicate whether the rate of biotransformation may be substantially modified in cases of genetic enzymatic deficiency and whether within dosage levels normally used, saturation of metabolism may occur, thereby inducing non-linear kinetics." It is thus evident that this aspect cannot be neglected during the development of a new chemical entity, at least in Europe. This is emphasised in the same Note in the following sentence: "Certain types of adverse reactions are due to unusual genetic pharmacokinetic variations: though it will rarely be possible to study such aberrant behaviour in a prospective manner, every effort must be put into elucidating the pharmacokinetic mechanism(s) if there is any reason to suspect that the adverse reaction is caused by altered pharmacokinetics of the drug." These recommendations have been made more explicit and suggestions have been made as to some methodological approaches related to the search for sources of variability during clinical testing [64,65]. The concept that a full understanding of the pharmacokinetics and pharmacodynamics of a new drug in preclinical animal species and humans provides a scientific framework for efficient and rational development will certainly prevail in the future. There is presently a general consensus on the content of the pharmacokinetic documentation needed to support the choice of a dosage regimen with respect to efficacy and safety. The information required will, of course, largely depend upon the kinetic properties of the active principle(s), their therapeutic margin, the indication(s) of the new drug, and the potential subpopulations such as aged persons or patients with renal insufficiency. In any case, if stereogenic situations are suspected or if the drug is commercialised as a racemate, stereospecific analytical methods will be required to elucidate the ADME (absorption, distribution, metabolism, excretion) of such compounds.

2.4. In vitro methods during drug development

Early in the development of a new drug, it is now customary to perform preliminary metabolic studies in animals. The data from such investigations (together with theoretical considerations) may help in determining whether a given compound is likely to be metabolized by pathways known to be under polymorphic genetic control in man. It must, however, be kept in mind that a given enzyme may metabolize a substrate in different positions. It is also possible, as demonstrated in the rabbit model of the acetylation polymorphism, that two drugs may be handled by the same enzyme, one in a polymorphic and the other in a non-polymorphic fashion. Accordingly, caution must be applied when extrapolating from in vitro or animal data to man. At this stage it might be useful to extend further the predictability of such findings for man by using in vitro techniques with human hepatocytes, liver microsomes or transfected organisms such as yeasts or microbes. The trend towards the establishment of human liver banks, comprising well characterised samples stored under defined conditions [66–68], should prove invaluable in applying such an approach. In the present situation, one has to rely mainly on experiments with samples from subjects of defined phenotypes or in which the competitive inhibition of drug substrates is assessed. Thus, by

using livers from subjects identified as PMs or by performing inhibition studies with marker drugs, it should be possible to obtain valuable predictive information on the qualitative and quantitative importance of a polymorphic enzyme in the metabolism of a new compound. Many clinically important drug metabolic interactions could probably also be detected by using such techniques.

If in vitro experiments indicate that polymorphic differences are indeed to be expected in man, it would be prudent to perform pharmacokinetic experiments, at the earliest possible stage in healthy volunteers, phenotyped as EMs and PMs for the metabolic pathway under consideration. This is now quite feasible by using relatively simple methodology for the debrisoquine/sparteine type of polymorphism and also for the mephenytoin type. One may even use a combination of probes to phenotype volunteers for different polymorphisms in one session. In addition, studies with specific inhibitors of a polymorphic enzyme such as quinidine, might be performed in extensive metabolizers [69,70]. Such single-dose studies can be performed at a time when only minimal animal toxicological data are available (i.e. mutagenic potential, one- or two-week toxicity studies in two animal species and safety pharmacology). The aim of such an investigation would be to confirm the findings in animals and in vitro and to determine the contribution of the polymorphic metabolic pathway to the overall elimination of the drug. Indeed, if the metabolic route represents only a small fraction of the overall disposition of the new compound, it is very unlikely that genetic polymorphism will be of clinical relevance as far as efficacy is concerned since it would not lead to increased variability in the elimination of the drug. However, a drug that is only slightly metabolized by a polymorphically regulated enzyme will still interact with it and may therefore inhibit the metabolism of other compounds, as is the case with quinidine, and this may be of clinical importance. As described above, such drug–drug interactions should be detectable at an early stage of drug development. Finally, such a drug could be metabolized to toxic products from which poor metabolizers would be relatively protected. The relevance of this last situation cannot be assessed from data presently available.

On the other hand, if preliminary metabolic studies in man confirm the potential importance of genetically determined pharmacokinetic variability, it will be necessary to consider the magnitude of the therapeutic margin. Only if the therapeutic margin is small should a NO-GO decision be considered. Such information can usually be inferred from the animal data available at this stage of the pharmacology, toxicology and safety pharmacology programmes.

If a positive decision is taken to proceed with the development of the new chemical entity, the design of the investigations to be performed during Phase I, II and III studies should be tailored to the potential problem represented by genetic polymorphism in the metabolism of the drug. As an alternative, chemical analogues which retain the pharmacological properties of the drug, but which are metabolized by other routes should be considered. It should, however, be emphasised that if a large proportion of the metabolism of a compound is subject to genetic polymorphism, this is not in itself an indication to stop developing the drug. A final decision should be made taking into account drug alternatives and the potential therapeutic progress offered by the new drug. Here again, it must be emphasised that if racemic substances or compounds with stereogenic centres are developed, it is of primary importance to use stereospecific methods (if feasible) for all pivotal pharmacokinetic studies.

3. Enantioselective analytical methods

3.1. General considerations

Enantioselective pharmacokinetics can be studied by using chromatographic (GC, LC) or capillary electrophoretic (CE) methods. As has been recently shown, even if methods involving enzymes are strictly enantioselective, they assay only one enantiomer and entirely ignore the other one [71].

In a chromatographic assay enantioselectivity

can be achieved by two approaches as follows: the formation of a diastereomeric derivative through the reaction with another chemical substance possessing a stereogenic centre, at least where derivatization is possible; the use of a chiral column capable of separating enantiomers.

In certain cases as described in Sections 3.2.2 and 3.2.3, the enantioselective assay requires a two-step approach, as follows: a first achiral chromatographic process separates the analytes which are individually collected after the detector and reassayed for the enantiomeric ratio; a first chromatographic process separates enantiomers which are individually collected and quantified by a second achiral process.

The extensive use of HPLC in pharmaceutical analysis and in pharmacokinetics has led to a marked reduction of the practice of derivatization reactions, although these were extensively used twenty years ago to render volatile in GC analysis substances possessing carboxylic, alcoholic, phenolic, aminic, amidic groups and/or to render such substances sensitive to selective detectors [72]. Two handbooks [73,74] that are now very hard to find on the market, comprehensively describe derivatization reactions. In fact HPLC now solves most analytical problems in pharmaceutics and pharmacokinetics without requiring derivatizations [72].

More recently, however, the need for enantioselective assays in pharmaceutics and in pharmacokinetics required inter alia by the note for guidance on chiral medicinal products [75] and increasing interest in genetic polymorphism involving stereoselective biotransformations have rearoused interest in derivatizations aimed at producing diastereoisomers from the reaction of two substances that each possess a stereogenic centre. As recently reviewed by Marzo [76] it is difficult or very difficult to set up enantioselective assays in some cases, and wellnigh impossible in others. This depends mainly on the dose strength, distribution volume and clearance of the drug concerned. In several cases a low or very low limit of quantification (LOQ) is required to describe pharmacokinetic behaviour. Just as an example, β-adrenergic agonists should be considered, which are administered at very low doses and are present in plasma at a concentration range of 10–2000 pg/ml. It is already very difficult to set up a non-enantioselective method for these compounds, while it is impossible to validate an enantioselective assay [76].

Bioassay sensitivity is being continuously enhanced, due to improvements in analytical apparatus, chromatographic columns and detectors. The MS coupled with GC and HPLC and the tandem MS have significantly improved the sensitivity and the selectivity of bioassays. Capillary electrophoresis (CE) is a technique that is more useful in pharmaceutics than in pharmacokinetics. This is due to the fact that, compared to HPLC, CE is in itself on average ca. 100 times more sensitive but requires a volume ca. 1000 times lower to be injected, so that it is ultimately not more sensitive than HPLC and thus is of little use when sensitivity is a rate-limiting step, as usually occurs in pharmacokinetics. However, in pharmaceutics and in some toxicokinetic applications CE is extensively used as it is more selective than HPLC [77]. CE could also be useful in the metabolic screening of urinary metabolites of drugs, where their concentrations are high enough. Figs. 2 and 3 show the separation of diastereomeric derivatives of D- and L-carnitine achieved by HPLC and CE, respectively.

3.2. Some relevant chromatographic applications

3.2.1. Adrenergic β-blocking agents

This class includes a large number of substances that have been extensively investigated, most being marketed to treat hypertension, myocardial ischemia, *angina pectoris* and glaucoma. With only a few exceptions, e.g. labetalol, β-blockers possess one stereogenic centre which leads to *S*- and *R*-enantiomers, with activity residing in the *S*-form [78]. This class includes lipophilic drugs like propranolol, which are cleared mainly or solely by biotransformation processes, hydrophilic drugs like sotalol, which are mainly cleared by urinary excretion and drugs with intermediate characteristics. With a few exceptions, e.g. levomoprolol [79] and timolol maleate, these drugs are synthesized in racemic form.

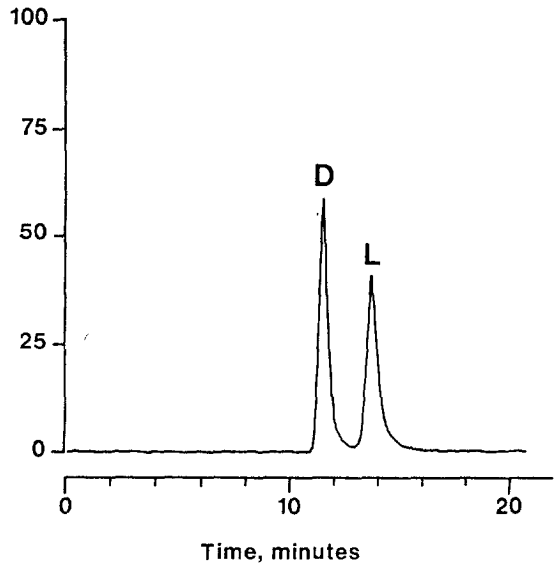

Fig. 2. HPLC separation of D- and L-carnitine after pre-column derivatization with (+)-1-(9-fluoronyl)ethyl chloroformate leading to two diastereometric derivatives. The analytical conditions were as follows: column: reversed-phase C_{18}, 150 × 3.9 (I.D.) mm, 4 μm; eluent: 25% acetonitrile–75% water solution of 5 m*M* tetrabutylammonium hydroxide and 50 m*M* KH_2PO_4 at pH 7.0; flow-rate: 0.75 ml/min; fluorimetric detection: 260 nm (excitation), 315 nm (emission). From De Witt et al. [77].

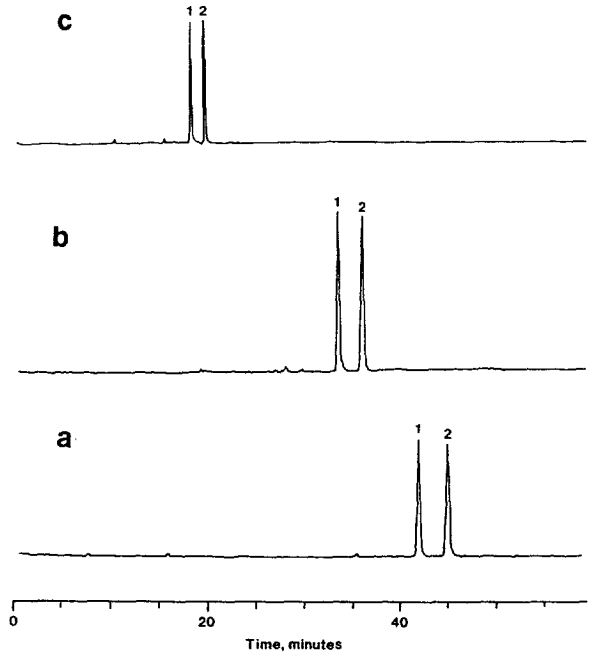

Fig. 3. Separation of L-carnitine (peak 1) and D-carnitine (peak 2) by capillary electrophoresis. Derivatives are the same as described in Fig. 2. Analytical conditions were as follows: capillary column: 50 cm (L_d) × 50 cm (L_t) × 50 μm (O.D.); temperature: 25°C; detector: UV = 214 nm; buffer: 0.05 *M* KH_2PO_4, pH 3.4 with H_3PO_4; voltages: (a) 10, (b) 15 and (c) 20 kV. From De Witt et al. [77].

In 1982 Hermansson and Von Bahr [60] published an HPLC enantioselective assay for *R*- and *S*-enantiomers of alprenolol and metoprolol (Fig. 4). In 1983, Lennard et al. [61] published a method, in part overlapping with that of Hermansson and Von Bahr, for the stereoselective assay of the *R*- and *S*-enantiomers of metoprolol. Both the above methods achieved enantioselectivity by derivatising the β-blocker moiety with symmetrical anhydrides of *tert.*-butoxycarbonyl L-leucine and by analysing the diastereoisomers by HPLC with a reversed-phase column and fluorometric detection. Table 2 summarizes analytical details of the above methods. This method was applied by the authors to the enantioselective assay of β-blockers in human plasma and urine. Lennard et al. [61] studied the enantioselective pharmacokinetics of metoprolol in six extensive and in six poor debrisoquine metabolizers to test the hypothesis that variability in metoprolol metabolism stereoselectivity was related to the debrisoquine oxidation phenotype. As noted in Section 2.2.3., in EMs these authors encountered plasma AUC values for the *S*-enantiomer 35% higher than those for the *R*-enantiomer, whereas in PMs AUC values of the *S*-enantiomer were lower than of the *R*-enantiomer. They concluded that the enzyme system responsible for polymorphic oxidation of the debrisoquine-type was stereoselective.

Enantiomers of propranolol after a diastereomeric derivatization were separated and assayed by Roux et al. [80] by HPLC with a reversed-phase column, as summarized in Table 2.

3.2.2. Stereoselective disposition of mianserin

Mianserin is a tetracyclic antidepressant possessing a stereogenic centre and is marketed as a

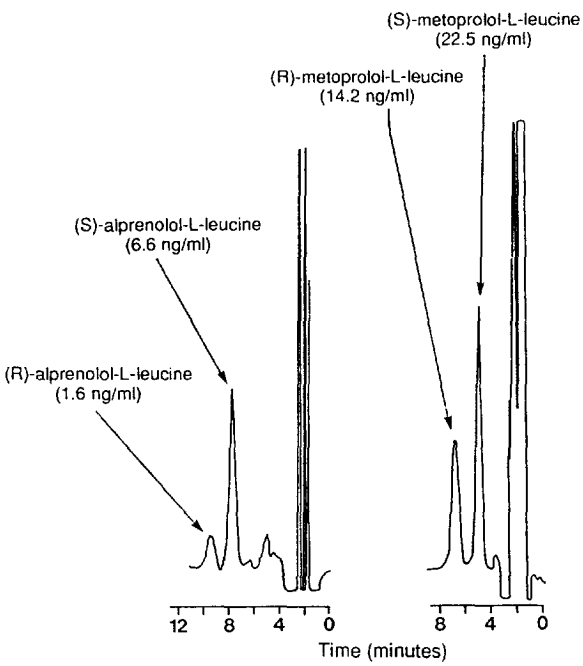

Fig. 4. HPLC separation of *R*- and *S*-enantiomers of alprenolol and metoprolol obtained after diastereomeric derivatization with *tert.*-butoxycarbonyl-L-leucine on human plasma. Analytical conditions are listed in Table 2. From Hermansson and Von Bahr [60].

racemic mixture of *R*- and *S*-enantiomers. *S*-Mianserin is more active than *R*-mianserin. Mianserin is metabolized by N-demethylation, 8-hydroxylation, N-oxidation and N-glucuronidation [81].

Stereoselective disposition of mianserin was studied in 1994 by two teams of investigators, namely Dahl et al. [82] and Eap et al. [83]. Table 3 summarizes analytical details of these two methods.

Dahl et al. [82] administered racemic mianserin (30 mg) to a total of 15 PMs and EMs of debrisoquine and *S*-mephenytoin, i.e., 5 PMs of debrisoquine and EMs of mephenytoin, 5 PMs of mephenytoin and EMs of debrisoquine and 5 EMs of both probe drugs. Mianserin's metabolites were assayed with a non-enantioselective method. The metabolic ratio of debrisoquine proved to be significantly correlated with AUC in racemic mianserin and its desmethyl metabolite. The enantioselective assay showed that debrisoquine's metabolic ratio was related to AUC in the *S*-form but not in the *R*-form of mianserin. The ratio of AUC between *S*- and *R*-mianserin proved to be higher in PMs than in EMs. No differences attributable to PMs and EMs of the *S*-mephenytoin-type were encountered. The authors concluded that the elimination of both mianserin and its desmethyl metabolite is dependent on CYP2D6.

Eap et al. [83] have validated an HPLC method for the simultaneous assay of the enantiomers of mianserin, desmethylmianserin and 8-hydroxymianserin in plasma and urine (Figs. 5 and 6). Using this method the authors have investigated the stereoselective metabolism of mianserin in ten patients treated for depression. A previous genotyping ascertained that none of the patients enrolled in the study exhibited a genetic deficiency of CYP2D6. The authors encountered *S*/*R* ratios of mianserin and desmethylmianserin from 1.0 to 4.06 and from 0.19 to 0.64, respectively. The authors concluded that the wide interindividual variability in the *S*/*R* ratios of mianserin and desmethylmianserin observed in EMs of debrisoquine-type could be even wider if PMs were included.

The fact that the mianserin enantiomers differ in pharmacological activity could partially explain the lack of correlation between therapeutic response and total (S + R) plasma levels of mianserin.

3.2.3. Amitriptyline: a highly complex pharmacogenetic metabolism

Amitriptyline (AT) is a non-chiral tricyclic antidepressant producing a complex metabolic cascade in the body. Part of its metabolism is under the control of CYP2D6 [54,84]. The main biotransformations of AT are hydroxylation in position 10, producing two geometric isomers (*Z*-10 and *E*-10 or *cis* and *trans* isomers) and N-demethylation which produces desmethylamitriptyline (NT = nortriptyline) possessing the same therapeutic target as AT [85]. Minor metabolites are AT-N-oxide and NT-N-glucuronide [86–89]. Fig. 7 depicts an AT metabolism scheme.

AT does not possess stereogenic carbon atoms,

Table 2
Analytical details related to the enantioselective assay of *R*- and *S*-enantiomers of alprenolol, metoprolol and propranolol achieved through diastereometric derivatives

	Hermansson and Von Bahr [60]	Lennard et al. [61]	Roux et al. [80]
Analytes	*R*- and *S*-alprenolol *R*- and *S*-metoprolol	*R*- and *S*-metoprolol	*R*- and *S*-propranolol + methyl-4-propranolol (I.S.)
Matrices	Plasma, 1 ml	Plasma and urine, 0.5–1.0 ml	Blood, 1 ml
Diastereomeric derivatives	The amide obtained by the reaction with the symmetrical anhydride of *tert.*-butoxycarbonyl-L-leucine		FLEC = (+)-1-(9-fluorenyl)ethyl chloroformate on secondary amino group
Column	RP C_{18}, 100 × 3.2 mm I.D., 10 μm	RP C_{18}, 100 × 5 mm I.D., 5 μm	RP C_{18}, 100 × 8 mm I.D.
Mobile phase	Acetonitrile–phosphate buffer at pH 3.0 (35:65) + N,N-dimethyloctylamine	Acetonitrile–water at pH 3.0 (30:70) with H_3PO_4 + triethylamine	Acetonitrile–water (75:25)
Flow-rate	0.5 ml/min	2.0 ml/min	2.0 ml/min
Detection	Fluorimetric, excitation 198 nm for alprenolol and 193 nm for metoprolol without cut-off emission filter	fluorimetric, excitation 193 nm without cut-off emission filter	fluorimetric, excitation 260 nm, emission 340 nm
Quantification	peak-height vs. concentration in the range 0.5–48.5 ng/ml with alprenolol and 0.5–52.0 ng/ml with metoprolol; external standardization		From analyte/I.S. peak-height ratios in the range 5–100 ng/ml

Table 3
Analytical details related to the enantioselective HPLC assay of *R*- and *S*-mianserin achieved through chiral columns

	Dahl et al. [82]	Eap et al. [83]
Analytes	*R*- and *S*-mianserin	*R*- and *S*-mianserin (MNS), desmethyl-MNS and 8-hydroxy-MNS and (+)-3-methoxymorphinan (I.S.)
Matrices	Serum, 2 ml	Plasma, 1 ml; urine, 0.5 ml
Chiral column	α_1-Acid glycoprotein, 100 × 4 mm I.D.	β-Cyclodextrin, 250 × 4.6 mm I.D.
Mobile phase	Acetonitrile–20 m*M* potassium phosphate buffer at pH 5.2 (5:95)	Acetonitrile–phosphate buffer at pH 3.0 + 1% TEA (6:94)
Flow-rate	0.9 ml/min	1 ml/min
Detection	UV 215 nm	Fluorimetric; excitation and emission 270/430 nm for analytes, and 275/360 nm for I.S; a photochemical reaction unit was inserted between the column and the detector
Quantification	Two-step method: total [(*R*) + (*S*)] concentration was achieved by an HPLC achiral assay; mianserin was manually collected after detection and reassayed for enantiomeric ratio with the above chiral column	Through the internal standard
Limit of quantification for both enantiomers	10 nmol/l (=2.6 ng/ml)	Plasma MNS 4.0 ng/ml; plasma desmethyl-MNS 2.5 ng/ml; urine mianserin and 8-OH-MNS 5.0 ng/ml; urine desmethyl-MNS 2.5 ng/ml

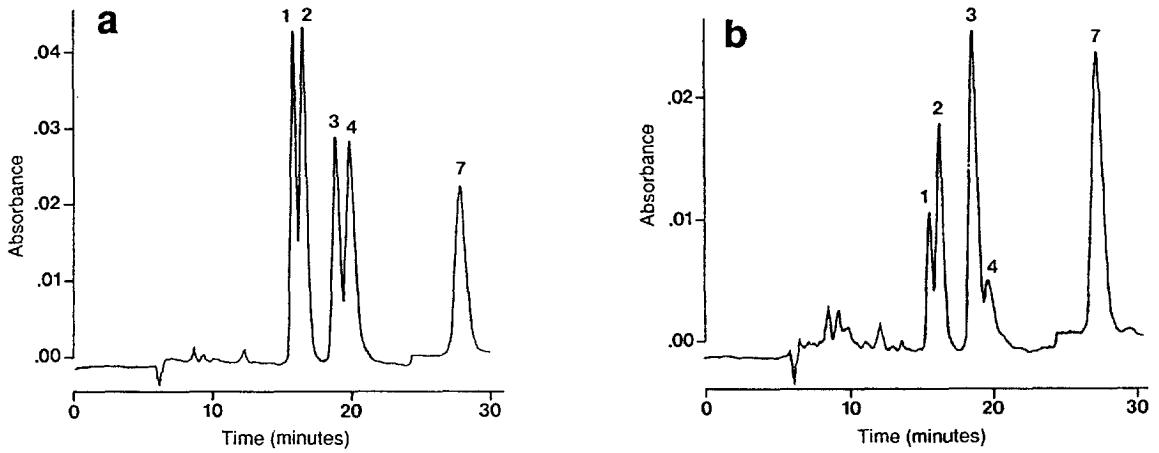

Fig. 5. HPLC separation of individual enantiomers of mianserin (MNS), desmethyl-MNS and 8-hydroxy-MNS in human plasma. (a) Standard plasma containing 100 ng/ml of each analyte (peaks 1–4) and 300 ng/ml of I.S. (peak 7), (b) a patient's plasma. 1 = *S*-desmethyl-MNS; 2 = *R*-desmethyl-MNS; 3 = *S*-MNS; 4 = *R*-MNS; 7 = internal standard (methoxymorphinan). Analytical conditions are listed in Table 3. From Eap et al. [83].

but it becomes chiral following hydroxylation in C-10. All the following isomers: *Z*-10-OH-AT, *E*-10-OH-AT, *Z*-10-OH-NT, *E*-10-OH-NT thus exist as coupled *R*/*S* enantiomers.

The non-enantioselective analysis of geometric isomers of AT and its metabolites has been attempted by several authors with various methods as follows. An initial approach by Borgå and Garle [90] was applied to hydroxylated NT but was not able to distinguish between the two geometrical hydroxylate forms as these metabolites were dehydrated to 10-11 dehydro-NT in the derivatization process with heptafluorobutyric anhydride to the related amide (Fig. 8), which was analyzed by GC.

In order to gain information on the relative amounts of geometric isomers of hydroxy-NT, Bertilsson and Alexanderson [91] in a steady-state study with NT (0.4 mg/kg, three times daily for two weeks in six healthy volunteers), first

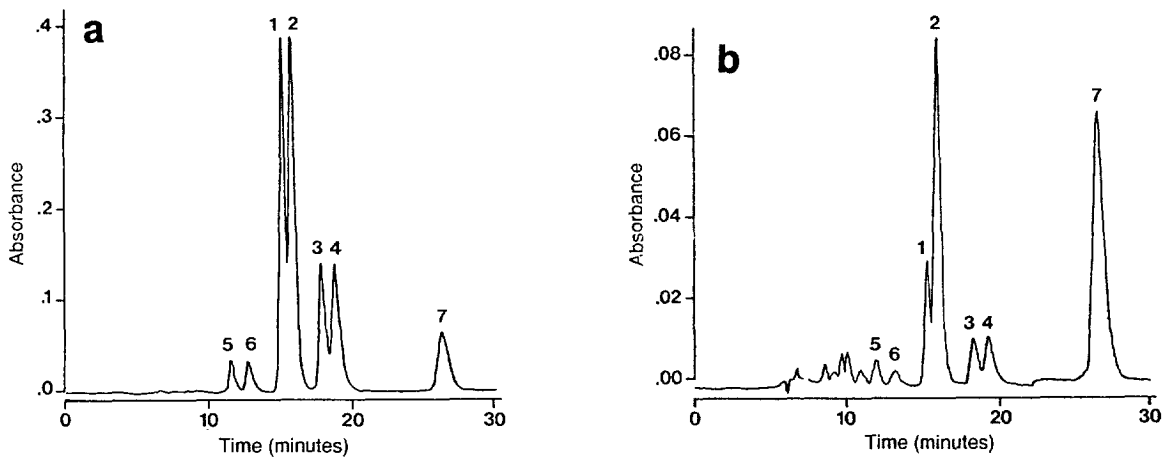

Fig. 6. HPLC separation of individual enantiomers of mianserin (MNS), desmethyl-MNS and 8-hydroxy-MNS in human urine. (a) Standard urine containing 1000 ng/ml of each analyte, (b) a patient's urine. 1 = *S*-desmethyl-MNS; 2 = *R*-desmethyl-MNS; 3 = *S*-MNS; 4 = *R*-MNS; 5 = *S*-8-hydroxy-MNS; 6 = *R*-8-hydroxy-MNS; 7 = internal standard (methoxymorphinan). Analytical conditions are listed in Table 3. From Eap et al. [83].

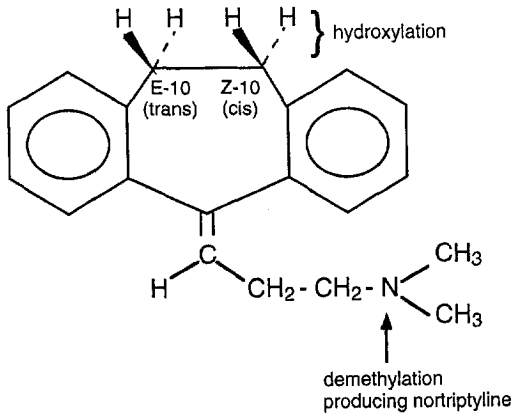

Fig. 7. Schematic presentation of the hydroxylation process of amitriptyline (AT) producing two geometric isomers, namely *E*-10-OH-AT (or *trans* isomer) and *Z*-10-OH-AT (or *cis* isomer), and demethylation producing desmethyl-AT (=nortriptyline) and bisdesmethyl-AT.

separated these isomers by TLC and then quantified them by the GC–MS method of Borgå and Garle [90]. They concluded that NT undergoes stereospecific hydroxylation in man and that there is no correlation between the steady-state plasma concentration of NT and the proportion of the two geometric isomers formed.

Using a GC–MS method Mellström et al. [92] determined NT and conjugated and unconjugated hydroxy-NT after an acid hydrolysis in plasma and urine, whereas *E*- and *Z*-isomers were determined only in urine by HPLC using the method of Mellström and Braithwaite [93]. Using the above procedures Mellström et al. [92] investigated the *E*- and *Z*-hydroxylation of NT in

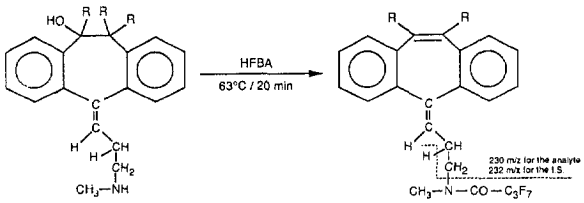

Fig. 8. Scheme of nortriptyline derivatization with heptafluorobutyric anhydride. The dehydrated derivative can be analysed by GC–MS monitoring the *m/z* 230 ion; the *m/z* 232 ion is monitored with deuterated nortriptyline, used as an internal standard. HFBA = heptafluorobutyric acid, R = H in the analyte, namely 10-OH-NT and R = deuterium in the I.S.

eight healthy subjects phenotyped with debrisoquine hydroxylation test to cover a wide range in the ratio of debrisoquine and its 4-hydroxymetabolite in the urine. A single dose of NT was given orally to the subjects, namely 28.5 mg to seven subjects and 57 mg to the remainder. Slow hydroxylators of NT excreted less 10-hydroxy-NT and had lower plasma clearance of NT than extensive hydroxylators. The correlation with debrisoquine hydroxylation was restricted to only the *E*-10-OH-NT, the *Z*-10-OH-NT being uncorrelated.

AT and the geometric isomers of its metabolites were assayed by Bock et al. [94] by means of HPLC with a reversed-phase column and UV detection at 205 nm reaching a LOQ of 10 ng/ml of plasma. In a steady-state study on 27 patients receiving routine treatment with AT to treat depression, Bock et al. [94] found *trans*-10-0H-NT to be the principal AT metabolite in plasma, with interindividual variations proving to be much greater than day-to-day variations.

The enantioselective assay of geometric isomers of hydroxylated AT and NT has recently been achieved by two different approaches. Dahl et al. [95] assayed enantiomers of *E*-10-OH-NT by combining a previous HPLC separation with a further GC–MS assay, and using trideuterium-labelled racemic *E*-10-OH-NT as an internal standard (Fig. 8). Table 4 summarizes analytical details of this approach. HPLC with an α_1-acid glycoprotein chiral column separated the enantiomers, which were collected separately and quantified by GC–MS. This method was used to investigate the enantioselectivity of NT hydroxylation to *E*-10-OH-NT in vitro in human liver microsomes and intestinal homogenate and in vivo in urine of patients under treatment with NT. The rate of formation of the (−)-enantiomer was higher than that of the (+)-enantiomer in both liver microsomes and intestine homogenate. Quinidine, a competitive inhibitor of CYP2D6, proved to inhibit the formation of the (−)-enantiomer according to a linear-concentration scale, whereas formation of the (+)-enantiomer was unaffected. In fact, another isozyme seems to catalyse the formation of the (+)-enantiomer in the liver. In the urine of six patients treated with

Table 4
Analytical details related to the enantioselective assay of hydroxylated metabolites of nortriptyline (NT) from Dahl et al. [95] achieved through a two-step method

Analytes	(−)-*E*-10-OH-NT and (+)-*E*-10-OH-NT; I.S. = labelled racemic *E*-10-OH-NT
Matrices	Human liver microsomes and intestinal homogenate in vitro studies and urine of patients treated with the drug
Chiral separation	Enantiomers separated by HPLC with an α_1-acid glycoprotein chiral column were separately collected and quantified according to the following procedure
Quantification steps	Derivatization on the secondary amino group with heptafluorobutyric anhydride producing the 10-11 unsaturated amido derivative (Fig. 8). GC separation on a 12.5 m cross-linked methyl silicone (0.2 mm I.D.) capillary column at 260°C using helium as carrier gas at a flow-rate of 1 ml/min after a 1:20 split system. Mass spectrometry with selected ion monitoring system at 20 eV quantified the analytes at *m/z* 230 and I.S. at *m/z* 232.

NT, the (−)-enantiomer proved to be predominant in both unconjugated (91%) and conjugated (78%) *E*-10-OH-NT, thus demonstrating that NT hydroxylation both in vivo and in vitro is a highly enantioselective process catalysed by CYP2D6.

Another approach to the enantioselective assay of enantiomers of both hydroxy-AT and hydroxy-NT has been devised by Nusser et al. [96,97]; analytical details are summarized in Table 5. Enantiomers of hydroxy-AT were derivatized with *S*-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (formerly labelled as the *R*-(+)-enantiomer by the manufactures) on the hydroxy group. Enantiomers of hydroxy-NT were derivatised with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate on the secondary amino-group. According to Becher et al. [98], in both cases, the diastereomeric compounds formed were separated and quantified by HPLC on a reversed-phase column and UV detection (Fig. 9). Using the above techniques Breyer-Pfaff et al. [99] have extensively investigated enantioselective amitriptyline metabolism in patients phenotyped for CYP2D6 and CYP2C19. Twenty-six in-patients suffering from depression were phenotyped with two tests, namely dextromethorphan (a substrate of CYP2D6) and mephenytoin (the *S*-form of which is a substrate for CYP2C19), before treatment with AT, which consisted of 75 mg once a day the first two days and 150 mg once a day thereafter. The metabolites were determined in urine collected over a 24-h period on day 8. The formation of (−)-*E*-10-OH-AT and (−)-*E*-10-OH-NT proved to depend on the activity of CYP2D6, because a negative correlation was encountered between the amounts of these two enantiomers and the log metabolic ratio of dextromethorphan, whereas this isozyme was not involved in the formation of (+)-*E*-10-OH-NT, (−)-*Z*-10-OH-NT or (+)-*Z*-10-OH-NT. Mephenytoin hydroxylase probably participated in side-chain demethylation.

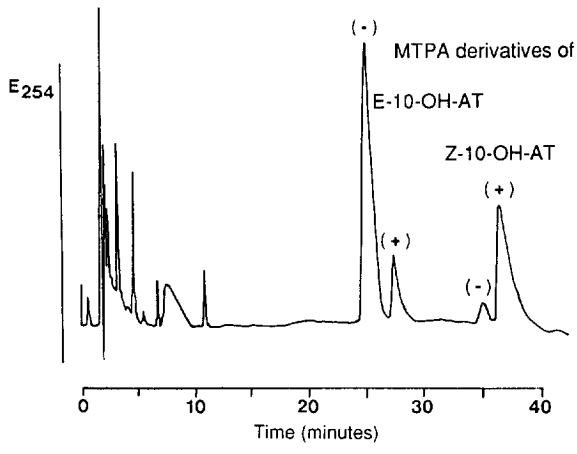

Fig. 9. HPLC separation of diastereomeric derivatives of *E*- and *Z*-10-OH-amitriptyline. Analytical conditions are detailed in Table 5. From Nusser et al. [97].

Enantiomers of trimipramine and its metabolites, a homologue of amitriptiline, were assayed by Eap et al. [100] through a two-step HPLC method; during the first step a CN-column separated the analytes which were manually collected after the detector and reassayed for the enantio-

Table 5
Analytical details related to the HPLC enantioselective assay of amitriptyline (AM) and nortriptyline (NT) metabolites achieved through diastereomeric derivatives

	Nusser et al. [96]	Nusser et al. [97]
Analytes	(+)- and (−)-*E*- and *Z*-OH-NT	(+)- and (−)-*E*- and *Z*-OH-AT
Matrix	Urine, 0.2–0.5 ml for conc. ≥ 1 μg/ml, 10–30 ml for conc. < 1 μg/ml	Urine, 150 ml, extracted through a cascade clean-up procedure
Diastereometric derivatives	TAGIT = 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate	MTPA = *R*-(+)-α-methoxy-α-trifluoromethylphenylacetyl ester
Column (two in series)	RP C_{18}, 150 × 3.9 mm I.D., 3 μm	RP C_{18}, 250 × 4.6 mm I.D., 5 μm
Mobile phase	Acetonitrile–50 m*M* NaH_2PO_4 at pH 2.5 containing 10 m*M* heptanesulfonic acid (38:62)	Acetonitrile–50 m*M* PCA at pH 2.5 (45:55)
Flow-rate	0.8 ml/min	1.5 ml/min
Detection	UV 254 nm	UV 254 nm
Quantification	From the calibrated variation of enantiomeric ratio resulting from the addition of the (−)-enantiomer to a constant racemate	

meric ratio with HPLC on an α_1-acid glycoprotein column and UV detection at 210 nm.

4. Genetic polymorphism in bioequivalence studies

In bioequivalence studies the formulations to be compared (reference from the innovator vs. experimental from the replicative company) are administered according to a Latin square design with a balanced crossover sequence. Possible differences in disposition due to genetic polymorphism are well managed by the crossover analysis of variance (ANOVA) which is able to analyse variations due to subjects, formulations and periods separately [101].

A problem however arises in extrapolating AUC to infinity ($AUC_{0-\infty}$).This requires the slope of the terminal phase of the plasma concentration–time curve to be carefully evaluated in order for the area added in the extrapolation to be $\leq$20% of the $AUC_{0-\infty}$. An empirical rule which usually allows this requirement in the EU guidance note to be met [101–103] is to sample blood for a period $\geq$3 times terminal half-life. In cases of drugs under genetic polymorphic metabolism a few of the volunteers selected can be PMs, possessing a longer half-life. In such volunteers the area added in the extrapolating procedure could be >20% of $AUC_{0-\infty}$ which would not comply with above guideline.

Propafenone is an example in point as it shows a debrisoquine-like polymorphic metabolism [104,105]. In EMs vs. PMs of debrisoquine, propafenone does in fact have a $t_{1/2}$ of 5.5 vs. 17.2 h and a clearance of 1115 vs. 268 ml/min. In addition, its active metabolite appears only in EMs [105].

5. New frontiers in chromatography

The above discussion on the approaches of various authors aimed at clarifying the role of genetic polymorphism and that of CYP2D6 and CYP2C19 in drug metabolism has mentioned the powerful analytical improvements that have been achieved in the last two decades. Very sophisticated techniques, namely GC–MS, HPLC–MS, MS–MS, capillary electrophoresis and the renewed interest in chemical derivatizations have permitted breakthroughs to be achieved which were unthinkable 30 years ago.

Some problems, however, remain, which have proved very hard, if not impossible to solve. These include, for instance, the enantioselective assay of drugs that are active in the systemic circulation at concentrations of tens or hundreds pg/ml of plasma [76]. If these problems are to be solved, further efforts and new approaches will be needed, posing a formidable challenge for scientists in the coming decade.

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