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Review

Critical issues in chiral drug analysis in biological fluids by high-performance liquid chromatography

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Abstract

This review article focuses on the specificities of chiral liquid chromatography, with particular emphasis on stability, stereoconversion, enantiomeric separation, recovery and drug concentration determinations. In addition, the paper presents an overview of the different steps which have to be followed for a chiral method to be validated. Sensitivity, selectivity, linearity, precision and accuracy all have to be ensured for three chemical entities, the two enantiomers and the racemate. Only accurate and precise concentrations of the parent drug and its metabolites will lead to the reliable description of their in vitro stability and in vivo body disposition.

Keywords: Enantiomer separation; Drugs

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1. Introduction

Important recent advances in stereoselective assays make it possible to determine the enantiomeric composition of virtually any racemic mixture, whatever the biological fluid analyzed. However, chiral methods are often delicate and inter-laboratory transfer of analytical methods may prove difficult. Rigorous and thorough validation studies undoubtedly contribute to improve method ruggedness and ensure accurate and reproducible results for the parent drug and its metabolites in pharmacokinetics, metabolism, bioavailability and stability studies.

The validation of a chiral analytical method is similar to that of any achiral method [1] but has additional specificities. This review will not cover the details of method validation but will focus on the particularities of chirality. This paper will also present the different steps which have to be followed for a chiral method to be validated.

Ideally, every step of a classical method validation would have to be followed for three chemical entities, the two enantiomers and the racemate. This would ensure the lack of interferences between both enantiomers or document the extent of putative interferences. For clarity purposes, only molecules with one chiral center will be considered. However, the same principles apply to any chiral molecule, regardless of the number of chiral centers.

2. Specific issues in chiral analysis of biological samples

Stability studies will provide information concerning the optimum conditions for storage to minimize drug degradation or stereoconversion. They are conducted according to the same principles whether the drug is chiral or not.

2.1. Stability

2.1.1. Stock solutions

The stability of the separate and combined enantiomers has to be determined, since the presence of one antipode could possibly alter the stability of the other. Stability has to be investigated in appropriate solvents, in the same tubes as the ones used to collect and store the patient samples, at 4°C and eventually at −20 or −80°C, depending on the storage conditions.

2.1.2. Mobile phase

Stability of the enantiomers should be determined in the mobile phase in order to avoid interconversion during chromatography. In the case of sequential achiral–chiral chromatography, stability should also be studied in the fractions collected after achiral chromatography if they are stored before injection into the chiral system.

2.1.3. Extracted samples

Stability in extraction solvents has to be ensured for each enantiomer separately and for the racemic mixture. If extracted samples have to be evaporated, either following extraction or after a first-step chromatographic separation, the stability of each enantiomer has to be verified. If the samples have to be evaporated under heating, any adverse influence of heating has to be ruled out.

2.1.4. Biological samples

Enantiomeric stability has to be determined for the separate enantiomers and their racemic mixture in the same biological fluids as those in which drug levels will be measured. Since enantiomers are not expected to possess the same affinities towards biological proteins (circulating proteins, enzymes, etc.,), their presence can lead to specific drug–protein interactions and influence the degradation of each enantiomer in a different fashion.

If there is a delay between sample collection and analysis, one needs to evaluate:

1. The stability of the compound of interest in the same biological medium, under identical storage conditions.
2. Any adsorption on glass/plastic/filters, collection containers or container stoppers. Although enantiomers have similar physicochemical properties and are expected to have similar adsorption characteristics, if adsorption is concentration-dependent, any variation in the enantiomeric ratio will affect the extent of adsorption.
3. If the enantiomeric ratio is sensitive to repeated freezing–thawing cycles.

Therefore, sampling as well as storage conditions must be carefully described (sampling material, tubes, anticoagulant, centrifugation, etc.,). Control solutions, spiked at the same time as the biological fluids and conserved under identical conditions (according to the anticipated period of storage), will serve as controls to compare the percentages of degradation due to the matrix.

To evaluate short- or long-term stability during storage, stored and freshly prepared quality control samples can be compared. Dadgar et al. [2] recently suggested the comparison of two sets of quality control samples prepared on the same day. The reference set would be frozen in liquid nitrogen while the other set would be stored at the selected temperature.

To evaluate any concentration-dependent degradation (either in the mobile phase, in extraction solvents or in biological samples), the percentage of degradation has to be assessed at different concentrations within the range of the standard curves. If ignored, variable percentages would lead to errors in the concentration determinations.

2.2. Stereoconversion and racemization

The analytical method needs to serve as a tool to measure the extent of racemization/stereoconversion in vivo, and should not introduce additional variability or bias by causing in vitro conversion. In all cases, measured concentrations or enantiomeric ratios should reflect in vivo levels and not in vitro degradation or stereoconversion.

Chemically, the reversal of the chirality of a tetracoordinate atom is hard to achieve, requiring drastic conditions and/or a lengthy series of reactions [3]. Therefore, the in vivo inversion of a chiral centre is rare, with few documented examples [3,4]. Stereoconversion is substrate and species-dependent [4]. For a detailed discussion on the subject, particularly on the chiral inversion of non steroidal anti-inflammatory agents, the reader is referred to previously published reviews [5,6]. For example, unidirectional chiral inversion (from the *R*- to the *S*-enantiomer) is not a general occurrence for all 2-arylpropionic acid derivatives [7].

However, Reist et al [8] recently pointed out that configurational stability and lability are relative phenomena and that given the proper conditions (e.g., temperature, medium or pH) [4], no stereoisomer is configurationally stable. Some molecules will racemize at physiological or acidic pH (econazole, oxazepam) while others (zopiclone, hyoscynamine or scopolamine) will do so at increasing pH or temperature [9–13]. The racemization of ceftibuten is a good example of a medium-dependent isomerization. In human serum, it correlated highly with albumin concentrations, suggesting that drug binding to albumin might be the driving force for isomerization under in vitro conditions [14]. Similarly, thalidomide enantiomers bind albumin with different affinities resulting in different rates of racemization [15].

From a pharmacological standpoint, a long half-life of isomerization (e.g., several months) is essentially inconsequential in view of the usually shorter residence time of the drug in the body (e.g., several hours) [8]. In contrast, in analytical investigations or

in the pharmaceutical industry, a long isomerization half-life can lead to manufacturing difficulties or to drugs with limited shelf-lives [8]. Hence, the optical purity of reference chemicals must be periodically verified (see Section 5.1.4).

In addition, in vitro stereoconversion studies must be carried out, in the same medium as that of the biological samples. In vitro testing samples must be handled in the same way as patient samples. The biological material employed in in vitro studies must be of sufficient quality to reflect biological conditions (freshness, etc.,). For example, heat-inactivation of plasma samples against the AIDS/hepatitis viruses may damage plasmatic enzymes and modify enzyme-catalyzed stereoconversions. Similarly, anticoagulants may also affect enantiomeric ratios. Fernandez et al. showed that zopiclone stereoselective protein binding is reversed with heparine compared to citrate phosphate dextrose (unpublished data).

Thus, in order to prevent in vitro racemization, sample collection procedures may need to be validated. Enantioselective studies of ibuprofen and fenoprofen require that plasma or urine samples be acidified rapidly to pH 5 [16,17]. If in vitro stereoconversion/racemization occurs and is unavoidable, its extent has to be quantified and taken into account in further determinations.

Since racemization can also occur during sample preparation (derivatization, protein precipitation, extraction, etc.,), conditions should be optimized to avoid this situation. Some chiral drugs, for example molecules which are transformed into carbocation intermediates, are particularly unstable. The extent of stereoconversion in derivatization and extraction solvents should be verified by carrying out the procedures with the separate enantiomers.

Furthermore, racemization may occur during the chromatographic process. For instance, Fujima et al. [18] described a peak coalescence due to lorazepam racemization on an ovomucoid chiral column (Fig. 1). Because of this phenomenon, a baseline separation could not be obtained. The unresolved portion of chromatogram D is due to the coalescence of the two enantiomers, which do not necessarily represent a 50:50 mixture. This can bias peak integration and concentration determinations. As demonstrated by Fujima et al., this phenomenon may be avoided by adjusting the chromatographic conditions.

3. Chromatographic separation

3.1. Chiral stationary phases

3.1.1. Direct injection into a chiral system

Direct injection of an enantiomeric mixture into a chiral column can only be used when the spiked solution/biological material is sufficiently clean to allow clear chromatograms and when the chiral column is sufficiently stable towards impurities. However, this method is rarely feasible since it shortens the column life and leads to decreased efficiency and selectivity. Thus, when salbutamol enantiomers were directly separated on an α_1-glycoprotein column [19], only very small volumes (1–7 μl) could be injected into the chiral system, leading to an unacceptably high limit of detection (250 ng/ml).

3.1.2. Achiral-chiral chromatography

When the chiral drug has one or more metabolites (which could also be chiral) with similar chemical structures, achiral chromatography is used as a first-step separation, to obtain good resolution between the parent compound and its metabolites. This method is also recommended for dirty biological extracts, when direct injection of biological material is susceptible to damage the chiral column or modify its separation properties (e.g., plasma proteins vs. protein-bound columns).

This technique combines two chromatographic separations. The first step consists of the achiral determination of the total concentration of each analyte (the sum of both enantiomers of the parent drug and the sum of both enantiomers of each metabolite, if applicable). Analytes are separated from the biological matrix, and in a second step, injected into the chiral system to determine enantiomeric ratios.

Sequential achiral–chiral chromatography

Following achiral separation, the fractions corresponding to the drug and its metabolites are collected, evaporated to dryness, reconstituted into the appropriate solvent or the chiral mobile phase and separately injected into the chiral system. This technique has been used to separate the enantiomers of hydroxychloroquine [20,21] and halofantrine [22].

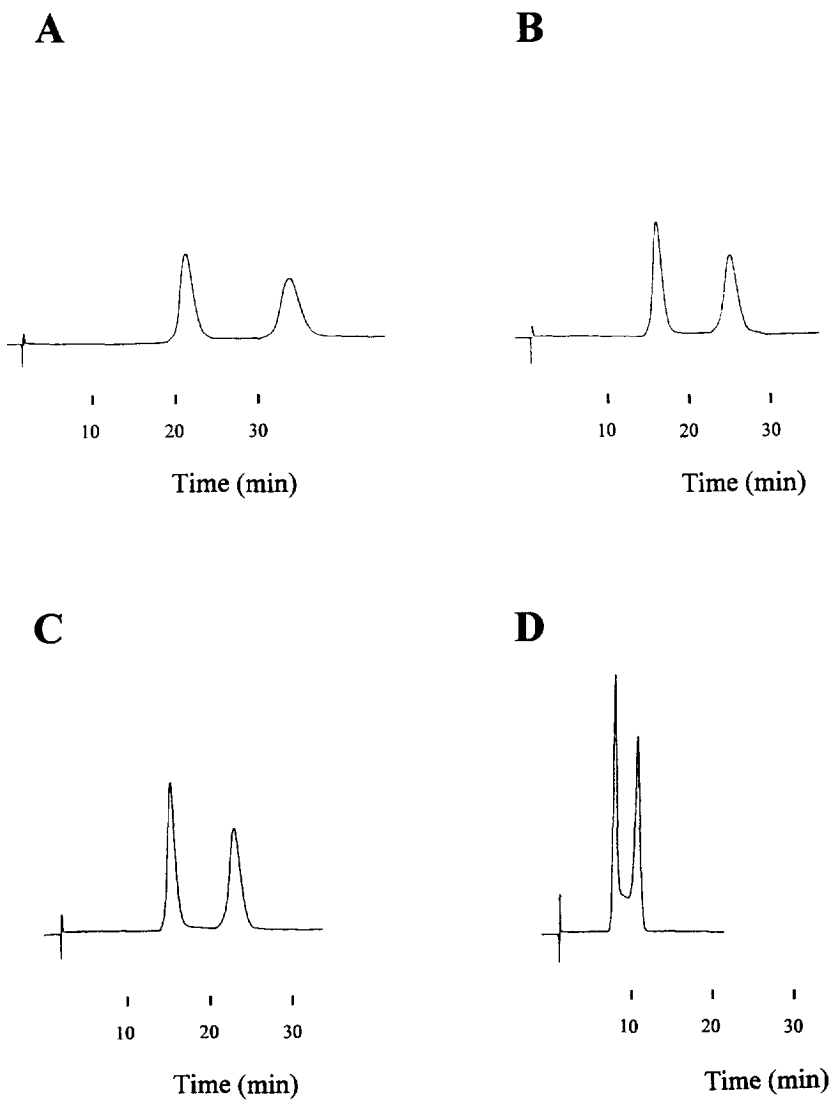

Fig. 1. Separation of lorazepam enantiomers on an ovomucoid chiral column. Mobile phase: 20 m*M* KH_2PO_4 (pH 4.6)–2 propanol (100:10, v/v). Flow-rate: 1.0 ml/min. Detection: 254 nm. Column temperature: 7°C (A), 10°C (B), 15°C (C) and 25°C (D). (From H. Fujima et al. [18], with permission.)

Coupled achiral–chiral chromatography

Following achiral separation, the effluent containing the drug and its metabolites is directly transferred to a chiral system via a switching valve (Fig. 2). Applications of this technique can be found for sotalol [23] or zopiclone [24]. However, column switching can be very delicate and difficult to carry out. Several factors must be optimized. The guard column must be chosen to retain the chiral compounds while eliminating the others. The trapping time must be a compromise between trapping efficiency and peak broadening. Moreover, mobile phases used in the achiral and chiral systems have to be compatible, that of the chiral system being preferentially more eluting.

The complexity of the validation procedures will depend on the selected chromatographic methods. For achiral–chiral chromatography, two analytical

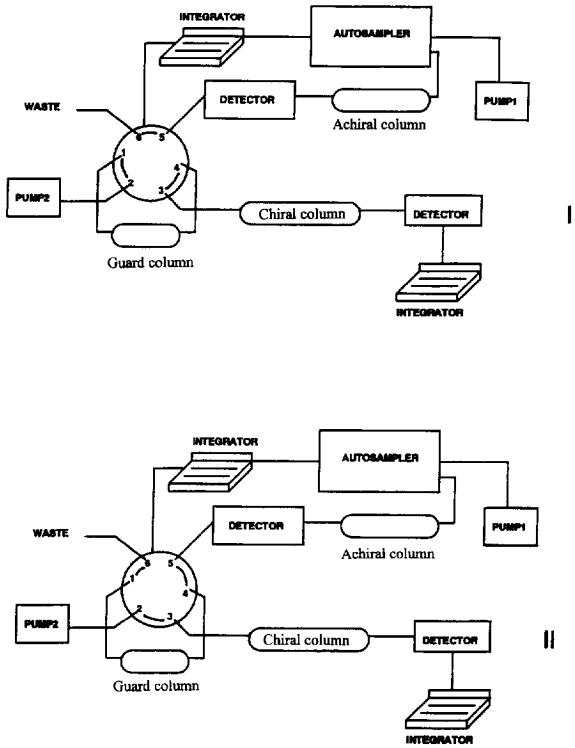

Fig. 2. Schematic description of coupled achiral–chiral chromatography (From Fernandez et al. [23]).

methods need to be validated. Obvious limitations of these techniques are the potential loss of analytes during fraction collection or system switching. In addition, when one enantiomer is non detectable, the concentrations of its antipode are likely to be overestimated. Hence, in such cases, one must consider the proportion of the non-detectable enantiomer to be 0% (underestimation) of the total and that of its antipode becomes 100% (overestimation).

3.2. Achiral stationary phases

When chiral separation cannot be directly achieved on a chiral stationary phase, diastereomeric derivatives can be prepared, before (derivatization agent) or during (mobile phase additive) the chromatographic separation. Ion pairs, metal complexes or covalent-bonded compounds are formed between one of the enantiomers of the drug on one hand, and one of the enantiomers of the derivatizing agent on the other. The resulting diastereomeric compounds present different physico-chemical properties and can therefore be separated using achiral stationary phases.

This technique avoids the additional step of a second chromatographic separation but nevertheless requires two validations, that of the chromatographic separation and that of the derivatization. The same requirements apply to chiral and achiral derivatizations [25]. Briefly, the derivatization reagent must be selective, non-toxic and, together with its degradation products, either non-detectable or well separated from the derivatized analytes [26]. Both the reagent and the reaction product should be stable under the reaction and chromatographic conditions [26]. The reaction should be as simple and as mild as possible, and yield a single product from one enantiomer or a pair of diastereomers from a racemic mixture [25]. The time needed for a reaction decreases on increasing the reagent/analyte concentration up to a ratio of approximately 10 [26]. Therefore, a larger excess of reagent would not improve the speed of the reaction and could lead to resolution problems.

The enantiomers of sotalol were also separated using this technique. The diastereomeric derivatives formed with *S*-(+)-1-(1-naphthyl)ethyl isocyanate or *S*-(−)-α-methylbenzylisocyanate were resolved in normal-phase [27] or in reversed-phase [28] chromatography.

In all cases, specific chromatographic conditions have to be clearly stated (i.e., column temperature, mobile phase recirculation, between runs injections, etc.,). For example, salbutamol enantiomers were separated on a chiral column maintained at 0°C [19]. For the chiral analysis of mexiletine enantiomers in urine samples, the Pirkle-1A column had to be washed with 2-propanol–hexane (10:90) for 10 min after every second injection to remove any endogenous material [29].

4. Recovery

In chiral chromatography, the compound of interest has to be extracted from biological fluids, and/or derivatized to separate the enantiomers, and/or transferred from a non-chiral to a chiral system. Loss of analyte can therefore occur during any of those steps.

4.1. Extraction

As for non chiral drugs, the extraction procedure isolates the compound of interest from biological impurities, proteins or metabolites. If separate enantiomers are available, extraction recovery has to be determined for each compound. If only the racemic mixture is available, peak heights or areas and *R/S* ratios have to compared before and after sample preparation, since extraction procedures may lead to modifications of enantiomeric ratios. Hence, McErlane et al. [29] showed an alteration in the natural *R/S* ratio of racemic mexiletine following protein precipitation with 2 *M* sodium hydroxide, the *R*-enantiomer being less efficiently extracted than its antipode. Protein precipitation with 10% trichloroacetic acid enhanced recovery and restored the natural racemic ratio.

Also, recovery experiments should compare peak heights or areas and *R/S* ratios in the biological matrix vs. pure solutions (e.g., water, organic solvents, etc.,), to evaluate any matrix effect.

4.2. Derivatization

When developing a derivatization procedure, the influences of reaction conditions (choice of solvent, concentrations of reactants and catalysts, temperatures, reaction times, etc.,) [26] must be thoroughly investigated to obtain optimal reaction conditions and information concerning reaction selectivity and the derivatization yield. Absolute peak heights can be compared between the various derivatization conditions tested. If the derivatization reaction is not carried out to completeness, an internal standard is needed to account for any variability.

Any enantioselectivity in the rate of the derivatization reaction can lead to quantitation errors, i.e., the peak height/area ratios of the diastereomers would not reflect the real enantiomeric ratio [25]. This can be avoided by careful optimization of the reaction conditions and by measuring the peak height/area of the derivatized racemate.

4.3. Column switching or fraction collection

Any loss of analyte during system switching or fraction collection needs to be detected by comparing peak areas/heights between the achiral and chiral systems, provided the internal standard does not interfere with the chiral separation. The latter can be ensured by spiking biological samples with the internal standard only and by sequential injection of the extracts through the achiral and chiral systems.

However, when working in achiral–chiral chromatography, the use of different mobile phases for each system may lead to alterations in peak shapes/heights, not necessarily associated with any loss of analyte. Therefore, comparison of absolute peak heights or areas alone could be misleading.

5. Drug assays

5.1. Reference chemicals and solutions

Chromatographic methods rely heavily on the reference standards to provide accurate data. Therefore, the quality and purity of the standards are crucial.

5.1.1. Pure standards

Whereas the enantiomeric purity of each enantiomer is a prerequisite for any investigation carried out with the separate enantiomers, racemate administration requires careful verification of the enantiomeric ratio of the racemic mixture. This enantiomeric ratio has to remain stable throughout the studies, implying continous verifications. Evidently, purity has to be ensured for every new batch of compound [30,31]. Optical purity has to be confirmed by stereospecific assays. If the analytical method needs to determine low quantities of optical impurities, standards should contain a significantly lower degree of impurity than what needs to be measured, e.g., if 0.1% of the *S*-enantiomer should be quantitated from the *R*-standard, the latter must not contain more than 0.005% of *S*, whereas the purity of the *S*-standard would be less critical [32].

When pure enantiomers are not available, enantiomeric fractions should be collected to determine the optimal rotation of each compound. This will ensure the adequate description of the elution order of each enantiomer. Enantiomers can also be described as (*R*) or (*S*) [33].

5.1.2. Stock solutions

When separate enantiomers are available, stock solutions of individual enantiomers should be prepared. If they are not 100% pure, the extent of contamination has to be taken into account for the preparation of the standard curve. The purity correction factor should be mentioned in reports and publications.

Also, stock solutions for the separate enantiomers and the racemate have to be prepared on the same day and kept under identical conditions, to ensure similar degradation, if there is any.

5.1.3. Diluted solutions

Since racemic mixtures and separate enantiomer solutions may present differing solubilities [34], solubility studies have to be carried out beforehand. The solubility of racemic ketorolac in water is approximately two times higher than that of the separate enantiomers [35]. This unexpected difference is thought to result from differences in the crystal lattice energies of the single isomer and racemate crystals [36].

All solutions have to be prepared in the same solvent, to standardize the analytical conditions. Any discrepancy between enantiomers could lead to biased determinations. Hence, for a cholecystokinin-A antagonist, optical rotation was found to show considerable solvent dependence [37].

5.1.4. Derivatization chemicals and reagents

The chemical, but also chiral, quality and purity of the derivatization reagent need to be controlled since they could themselves undergo a racemization/stereoconversion, leading to suboptimal and variable derivatization of the analytes [38,39]. This optical purity should not change during storage or within the course of the derivatization procedure [25]. The extent of racemization or the enantiomeric purity of the reagent can be estimated by checking the relative peak height/area of the diastereomeric impurity. In most cases, an impurity of less than 1% is considered acceptable [25].

The conservation conditions of the derivatizing agent, as well as the exact sample preparation conditions, should be carefully mentioned. For example, solutions of the derivatization reagent *R*-(−)-1-(1-naphthyl)ethyl isocyanate (neic) have to be prepared in organic solvents and kept over a drying agent [23]. Although *R*-(−)-1-(1-naphthyl)ethyl isocyanate has been thought to work more effectively in a dry environment [40], Hooper and Baker [23] found that adding 2 μl of carbonate buffer to dried plasma extracts improved sotalol derivatization.

5.2. Calibration curves

For achiral–chiral chromatography, standard curves can be derived according to two different methods:

Method 1

Concentrations can be derived from the ratio of the signal of the compound of interest over that of the internal standard on the achiral column. Concentrations are therefore calculated according to the achiral system standard curve and the chiral system is used to obtain enantiomeric proportions. Linearity has to be ensured on the achiral column while accuracy of the enantiomeric ratio has to be validated on the chiral column.

Method 2

Concentrations can also be derived from the ratio of the signal of the compound of interest on the chiral column over that of the internal standard on the achiral column. Although technically feasible, provided the efficiency of system switching or fraction collection is known, this method is not advisable since it adds considerable variability to the analyses.

5.2.2. Non-availability of the separate enantiomers

If sufficient quantities of separate enantiomers are not available, standard curves will be obtained with the racemic mixture. However, this will not allow testing and validation for unequal proportions of enantiomers, which could lead to biased peak or area integrations.

5.2.3. Availability of sufficient quantities of the separate enantiomers

If standard curves are prepared with the racemic mixture, additional controls have to be prepared with unequal proportions of enantiomers (e.g., very high concentrations of one enantiomer with low con-

Table 1
Method 1 – standard curves prepared independently from separated enantiomers

	Concentration (units)						
Isomer (+)	0	5	10	25	50	75	100
Isomer (−)	0	5	10	25	50	75	100

centrations of the antipode). However, the method validation is more sound if standard curves are prepared with separate enantiomers. If both enantiomers are stable and do not interfere with one another, standard curves can be obtained by spiking biological samples with both enantiomers and the racemate.

Three methods are possible:

Method 1

Standard curves can be prepared independently, for each enantiomer, in separate biological fluid samples. Numerous publications have used this technique [41]. According to the example presented in Table 1, each standard curve contains 7 data points, for a total of 14 samples to assay. In addition, controls containing unequal proportions of enantiomers have to be prepared. For the example in Table 1, one control sample could contain *R*/*S* proportions of 95%/5% and the other of 5%/95%, adding 2 samples to the 14 to be assayed. Extreme peak size ratios, as observed in investigations of optical purity, require high resolution [32]. If resolution is suboptimal and the smallest peak is eluted second, peak tailing will result in integration inaccuracies. However, standard curves constructed with the anticipated enantiomeric proportions should compensate for errors resulting from peak overlap [32].

It is noteworthy that the slopes of the calibration curves may differ for both enantiomers. This could result from different peak integration (peak shapes, tailing, etc.,) [42]. For example, the separation of pindolol enantiomers on a reversed-phase cellulose-based chiral column showed sharper and more symmetrical peaks for *R*-pindolol [43]. As a result, limits of detection and standard curve slopes were significantly different between both enantiomers.

Method 2

Standard curves can also be prepared with varying proportions of each enantiomer in the same biological fluid sample. Table 2 presents an example adapted from the work of Fieger et al. [21]. Each standard curve contains 8 data points, for a total of 8 samples to assay. However, this method is only possible with direct injections into the chiral column or, in the case of achiral–chiral chromatography, when the enantiomers are quantitated on the chiral column (since on the achiral column total concentrations would be equal).

Method 3

Standard curves could also be prepared with varying proportions of each enantiomer and the racemate in the same biological fluid sample. This complex technique would allow the determination of varying enantiomer concentrations with a minimum number of samples. Table 3 shows an example which illustrates this technique. Our suggestion would allow the simultaneous construction of 3 standard curves (one for each enantiomer and one for the racemic mixture) with only nine samples. This technique would be applicable to any chiral method.

6. Parameters for validation of chiral chromatographic methods

6.1. Sensitivity and selectivity

The limit of detection (LOD) and the limit of quantitation (LOQ) have to be determined for each

Table 2
Method 2 – standard curves prepared with varying proportions of each enantiomer (same final total concentration)

	Concentration (units)							
Isomer (+)	100	95	75	60	40	25	5	0
Isomer (−)	0	5	25	40	60	75	95	100
Ratio (+)/(−)	100/0	95/5	75/25	60/40	40/60	25/75	5/95	0/100

Table 3
Method 3 – standard curves prepared with varying proportions of each enantiomer

	Concentration (units)								
Racemic	0	5	10	25	50	75	100	100	100
Isomer (+)	0	2.5	2	15	20	60	50	95	5
Isomer (−)	0	2.5	8	10	30	15	50	5	95
Ratio (+)/(−)	–	50/50	20/80	60/40	40/60	80/20	50/50	95/5	5/95

enantiomer. For racemic mixtures, provided pure enantiomers are available, specific proportions of each enantiomer can be combined to determine the lowest percentage of one enantiomer that is detectable (detection limit) or quantifiable (quantitation limit) in the presence of its antipode.

As with any achiral method, blank samples should not contain interferences, and cross-reactivity of the method to structurally related or concomitantly administered compounds should be assessed. The analysis of plasma samples from treated patients would allow testing for possible interferences with drug metabolites. For achiral–chiral assays, the internal standard used in the first achiral separation should not interfere with the subsequent chiral separation.

6.2. Linearity

As for any achiral method, linearity will be checked from standard curves constructed from data points covering the expected range of concentrations. The measured signal can be either peak height or area. Since enantiomers possess identical physicochemical properties, their UV absorption or fluorescence will be identical. However, separate injections of the racemate and the individual enantiomers may have different retention times, leading to alterations in peak shapes and thus adversely affecting peak heights. Therefore, in chiral chromatography, areas tend to afford more reproducible results.

6.3. Precision and accuracy

Both accuracy and precision have to be determined on the individual enantiomers, at relevant concentrations. The uniformity of the enantiomeric ratio of the racemic mixture has to be assessed with replicate measurements, also over the range of the standard curve. As for achiral analysis, minimum criteria cannot be defined since both precision and accuracy are extremely concentration-dependent and acceptability limits depend on the purpose of the analyses [44]. While an assay with low but reproducible recovery may be acceptable when samples are analyzed with appropriate calibration curves, good accuracy and precision will never be obtained if recovery is not reproducible [45]. Analysts should always attempt to improve precision and accuracy and obtain the highest recovery possible.

7. Conclusion

Validation studies are crucial since analytical results are often key to the development of new drugs. Although specific guidelines on chiral method validation have never been published, the increasing awareness of the importance of chirality in drug pharmacokinetics and pharmacodynamics will undoubtedly lead to their establishment by governmental, industrial and academic experts. Since chiral drugs require chiral analysis for a meaningful interpretation of data [46], innovations and refinements in chiral bioanalytical technologies will facilitate and accelerate their determination in biological fluids.

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