CHIRALITY IN A COMBINATORIAL AGE

Enantiomeric excess analysis is getting a high-throughput makeover.

BY DAVID FILMORE

IN A MARCH MEETING of the American Academy of Allergy, Asthma, and Immunology, the results of a survey were reported comparing patient satisfaction with two asthma medications. For one of the drugs, 75% of pediatric asthma patient caregivers (parents or others) reported symptom relief within 30 min. Only 49% of caregivers for children taking the other drug reported a comparable response. The less-favored medicine in the study was the well-known and widely used inhalant albuterol. The more-favored drug was, well, also albuterol. These findings were not the result of confused respondents or a medical mind game played by physicians. There was, however, some molecular sleight of "handedness" involved.

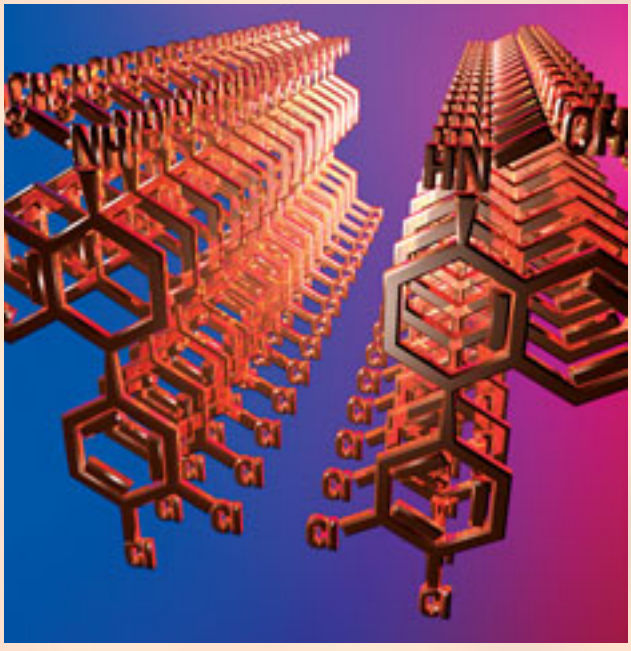
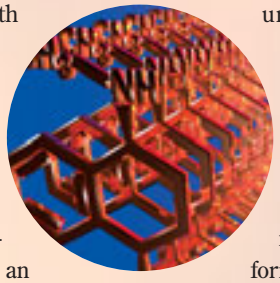


ILLUSTRATION: TONY FERNANDEZ

The preferred drug, which has the brand name Xopenex and was approved by the FDA for children ages 6–12 in February 2002 (and for adults in March 1999), was actually levalbuterol, the pure (*R*)-enantiomer of the molecule in question. Its less-effective partner in the study was the racemic mixture (50/50 mixture of the *R* and *S* isomers) of albuterol, which has been used by asthma sufferers since the 1980s. It turns out that all of the bronchodilatory activity resides in the (*R*)-enantiomer, which makes the pure isomer much more efficient. Furthermore, a large percentage of the Xopenex caregivers in the survey reported that they were highly satisfied with the drug because it had fewer side effects than racemic albuterol, pointing to potential deleterious activity coming from the (*S*)-enantiomer, a finding documented in previous studies as well.

This type of compelling differential effect from enantiomer-specific pharmacological interactions has led to a rising prevalence of enantioselective drug design. Seventy-six percent of the new drugs introduced in 2000 were single enantiomers, compared with 21% in 1991 (*1*).

And in 2001, single-enantiomer drugs represented 36% of the total drug market (*2*). High-profile drugs in this category include those produced in chiral switches—developing a single-enantiomer drug from an already marketed racemate, as with Xopenex or the antiulcerant esomeprazole (Nexium), the next generation of the racemate of omeprazole (Prilosec). Other drugs are produced from the start as pure enantiomers, such as the antidepressant sertraline (Zoloft) and the cholesterol-lowering drug atorvastatin (Lipitor). Suffice it to say, the expectation for enantiomeric purity in the development of new chiral drugs is escalating, causing an increased reliance on the challenging analytical task of quantifying enantiomeric content.



Analysis, in excess

For any chiral compound, the percent excess of one enantiomer over the opposite enantiomer in a chiral sample is referred to as the enantiomeric excess, or ee. For example, a 1% ee for levalbuterol indicates that there is a 1% surplus of the (*R*)-enantiomer in a mixture of the *R* and *S* compounds. A 100% ee for levalbuterol specifies the presence of the pure isomer, and a value of 0% identifies a racemic mixture.

The ee values directly correlate with the defining characteristic of enantiomeric compounds, that is, their optical activity. The only physical property that distinguishes an enantiomer from its mirror image is the direction in which it causes polarized light to rotate—clockwise or counterclockwise. In a racemic mixture, the opposing effects are canceled out, which results in optical inactivity. Therefore, the only direct means of determining ee is by polarimetry, which measures the degree of light rotation. However, techniques used for this purpose have traditionally shown low sensitivity and have not been very tolerant of the presence of chiral impurities.

More successful have been the tools that currently predominate in industrial ee analysis—specialized chiral stationary phases (CSPs) or other chiral additives used in HPLC and GC columns (and, to some extent, in CE). This approach takes a lesson from the biological implications of chirality rather than the purely physical. The natural world largely works in a "single-handed" manner—pure enantiomers are the rule rather than the exception for the biomolecules that make us, and the rest of the world, tick (which, of course, is the reason for the differential actions of drug enantiomers). CSPs—examples include macrocyclic antibiotics, crown ethers, and proteins—mimic biology by presenting single enantiomers that form a more stable interaction with one analyte hand than the other. This creates an equilibrium between mobile phase and stationary phase that is distinct for each enantiomer of the analyte and leads to differences in retention times.

In this manner, HPLC enantiomer impurity analysis, the most widely used pharmaceutical stereochemical analysis technique, can routinely measure ee down to the 0.01% level (*3*). This performance has made pharmacokinetics and pharmacodynamics analysis of each enantiomer of racemic drugs, which was encouraged by the FDA beginning in the 1990s, a common measurement in racemic drug development. Overall, chiral HPLC method development remains an important and vibrant component of pharmaceutical analytical chemistry. There is, however, a significant thrust of research investigating new methods of ee analysis.

Early-stage drug discovery has become predominantly a combinatorial endeavor. And with the growing significance of pure enantiomer drugs and, thus, pure enantiomer lead compounds, there is a greater need for highly parallel ee analysis. Furthermore, the major enabling technologies of the single-enantiomer market are the asymmetric transition metal catalysts and enzymes that make the synthesis possible. To keep this fuel burning, so to speak, a growing activity in pharmaceutical companies and their suppliers is combinatorial catalyst discovery, which also further emphasizes the need for high-throughput

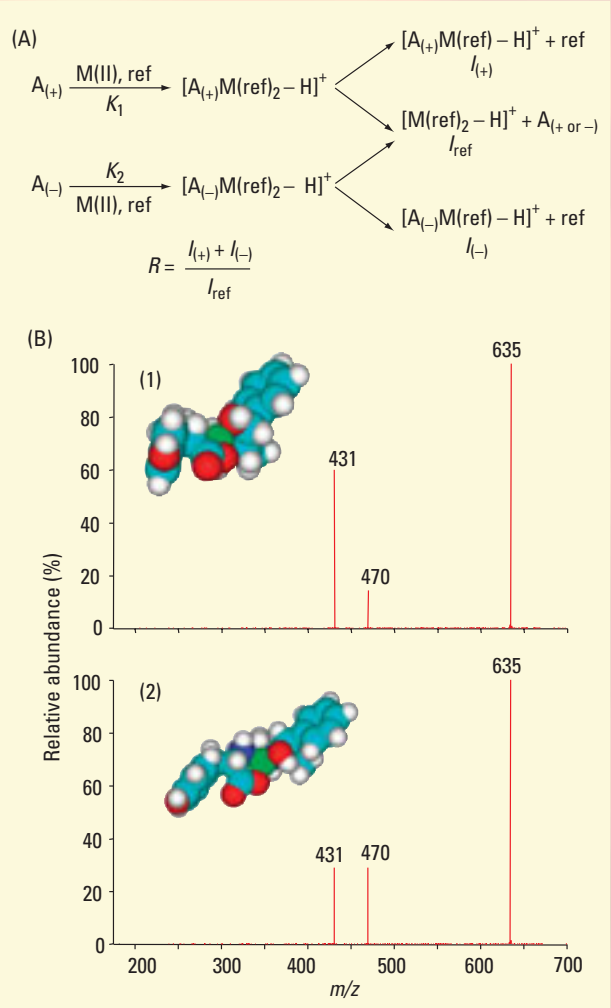


Figure 1. ee en mass. (A) Competitive dissociation of metal-ion-bound complexes ($A_{(+)}/A_{(-)}$, analyte enantiomers; ref, chiral reference; M, metal) and the ratio formula for *R*, which is proportional to ee. (B) MS spectra of dissociation products, $[Cu^{II}(L\text{-Trp})(ephedrine)\text{-}H]^+$ (m/z = 431) and $[Cu^{II}(L\text{-Trp})_2\text{-}H]^+$ (m/z = 470), of a copper complex of the analyte ephedrine and reference L-Trp, $[Cu^{II}(L\text{-Trp})_2(ephedrine)\text{-}H]^+$ (m/z = 635): (1) pure (+)-ephedrine enantiomer and (2) pure (–)-ephedrine enantiomer. Where there is a mixture of enantiomers, the peak ratios fall between these two extremes and can be related to ee through a linear calibration plot. (Adapted with permission from *Anal. Chem.* **2003,** *75,* 25 A–31 A.)

ee. Chiral HPLC, with recent modifications, can allow a throughput of more than several dozen measurements a day. The developing requirements of high-throughput pharmaceutical screening extend well beyond this limit, and thus, alternatives are sought.

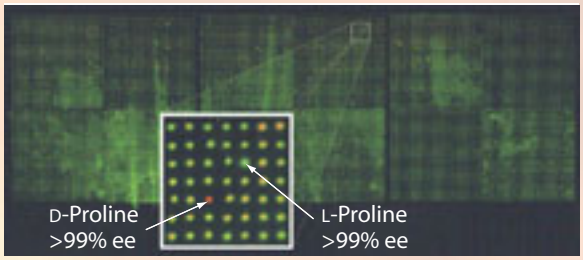


Figure 2. ee squared. Identification of two >99% ee samples of proline in a collection of 15,552 samples. (Adapted with permission from *J. Am. Chem. Soc.* **2001,** *123,* 361–362.)

Mass in the mirror

One of the more commonly investigated tools for this purpose is mass spectrometry. Of course, simply running a chiral compound through MS will in no way distinguish between its enantiomers, which have the same mass and have ion fragments with identical mass-to-charge ratios. However, modern MS techniques provide a staging ground, absent of any interference by solvents, for measuring subtle energy differences that arise from varying chiral interactions.

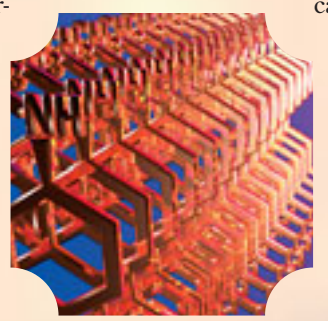
Using MS in this manner is illustrated by the successful electrospray ionization 2-D MS strategy recently developed by R. Graham Cooks and colleagues at Purdue University (West Lafayette, IN). Their technique (*4*; Figure 1) measures the competitive dissociation of trimeric metal cluster ions that contain a chiral drug analyte (A) and a chiral reference molecule (ref) as ligands (general formula: $[M(A)(ref)_2]^+$). The complex allows for multipoint interactions between the chiral molecules (of the type created with CSPs, for example) that differentiate the geometries of the two enantiomers of the analyte. So the reaction energetics of the dissociation of the complex, which the researchers induce in the mass spectrometer via electric current, differ on the basis of the enantiomer. And the product ratios, as measured by ion abundances, therefore directly depend on the ee.

This sensitivity, according to Cooks, is a result of what is called the "kinetic method", which was introduced in his lab in the 1970s. The sensitivity describes a logarithmic relationship between the ion abundances of cluster ion dissociation products and the critical energies involved in the fragmentation reactions. Translation: Even subtle differences in the dissociation caused by small chiral-based steric changes lead to relatively large adjustments in the MS peak intensities. These ratios can be directly related to ee values through a simple two-point-generated calibration plot.

This method has been used to measure ee for a range of chiral drugs, including beta-blockers, decongestants, antibiotics, and antivirals. "I don't know that this method is the ideal method for the very final drug or predrug determination of ee as required by FDA," says Cooks. This would require ee measurements of down to 0.1%, and the method has not accurately measured ee lower than 1% with their current instruments. However, the method shows definite promise for high-throughput screening of combinatorial synthesis products. "There is no prior chemistry," says Cooks. "It is just a matter of mixing. It is typically a 30-s experiment."

Also on the path to high-throughput ee MS analysis is work being done at The Scripps Research Institute (La Jolla, CA) by M. G. Finn and colleagues that is focused on the discovery of new asymmetric catalysts. They react catalyst-generated products with mass-tagged chiral derivatizing agents—an equimolar mixture of "pseudoenantiomers" that differ in a substituent remote from the chiral center (*5*). Assuming that each configuration of the derivatizing agent preferentially reacts with the matching analyte configuration, the mass of the final molecule will be related to its absolute configuration. The relative MS peak intensities are used to determine ee within a ±10% error.

High throughput is brought into the picture with Scripps' chip-based MS sampling method, in which MS-active spots are photopatterned to make silicon arrays onto which analytes can be deposited. More than 100 spots per chip can be created, separately addressed, and analyzed using MALDI (matrix-assisted laser desorption-ionization) instrumentation, and data acquisition rates of 5–10 s/sample are now routine.



Current efforts will further exploit the very real therapeutic advantages that single enantiomers have to offer

ee array!

As mentioned, chiral interactions of the type harnessed by CSPs and in the MS techniques reflect a biological reality. Thus, it follows that other techniques already designed for well-known biological interactions (e.g., DNA base pairing) are finding application to ee measurements.

For example, Matthew Shair and colleagues at Harvard University (Cambridge, MA) developed what they call reaction microarrays (*6*). They used DNA microarray fabrication techniques to spot chiral amino acids on functionalized glass slides—as many as 100,000 could be deposited on a single slide. Shair's team probed the slides with a pair of differently fluorescing chiral probes. The probes were "pseudoenantiomers" like Finn's MS derivatizing agents, with fluorescent tags instead of mass tags at remote points from the chiral center. Thus, at each spot, the resulting emission wavelength—between the two extremes of the probe wavelengths—was proportional to the ee at that spot, within ±10% ee (Figure 2). Notably, a single set of fluorescent probes was used to determine the ee of six structurally diverse α-amino acids, which, according to the Harvard researchers' paper describing the technology, provides a basis for reaction microarrays as a general method for high-throughput ee analysis, presumably for small molecules other than amino acids as well.

Another common genomic technique is capillary array electrophoresis, which is used for automated and highly parallel DNA separations and sequencing. With chirally modified electrolytes and a slightly modified version of a commercial DNA analysis device, Manfred Reetz and colleagues from the Max Planck Institute of Coal Research (Mülheim an der Ruhr, Germany) analyzed the ee of chiral amine compounds on 96-well systems (*7*). This, Reetz writes, makes possible 7000 ee determinations per day, and, by optimizing experimental parameters, he believes that up to 30,000 per day are possible.

Small, fast, and all-purpose

Numerous other strategies are under investigation as well, ranging from chiral molecularly imprinted polymers to infrared thermography, which has detected differential heat output from oppositely handed reactions, to a color assay based on the optical properties of cholesteric liquid crystals doped with a chiral analyte (*8*).

Even polarimetry-based measurements are overcoming their former difficulties and moving toward more practical functionality, particularly as highly sensitive detectors for achiral HPLC. A major limitation in increasing the throughput of chiral HPLC beyond current levels has been the inadequate detection sensitivity needed for use with smaller columns. More recent finely tuned polarimetry measurements, such as a microinterferometry backscatter method developed by Darryl Bornhop's lab at Texas Tech University (Lubbock) (*9*), can be used for chiral analysis with achiral capillary-scale columns. In a recent review in the journal *Chirality* (*5*), Finn says that "[Bornhop's] method holds great promise for bringing the direct measurement of optical purity to the necessary levels of scale, speed, and generality so as to be useful in high-throughput screening."

The need for scale, speed, and generality (to avoid extensive method development for each new compound class) is the driving force behind current efforts in ramping up the capacity of modern ee determination—efforts that will further exploit the very real therapeutic advantages that single enantiomers have to offer in this frequently one-sided world.

References

(1) Agranat, I.; Caner, H.; Caldwell, J. *Nat. Rev. Drug Discov.* **2002,** *1,* 753–768.
(2) Rouhi, A. M. *Chem. Eng. News,* June 10, 2002, pp 43–50.
(3) Armstrong, D. W; Zhang, B. *Anal. Chem.* **2001,** *73,* 557 A—561 A.
(4) Cooks, R. G.; Tao, W. A.; *Anal. Chem.* **2003,** *75,* 25 A—31 A.
(5) Finn, M. G. *Chirality* **2002,** *14,* 534–540.
(6) Korbel, G. A.; Lalic, G.; Shair, M. D. *J. Am. Chem. Soc.* **2001,** *123,* 361–362.
(7) Reetz, M. T.; et al. *Angew. Chem., Int. Ed.* **2000,** *39,* 3891–3893.
(8) Reetz, M. T. *Angew. Chem., Int. Ed.* **2002,** *41,* 1335–1338.
(9) Bornhop, D. J.; Hankins, J. *Anal. Chem.* **1996,** *68,* 1677–1684

David Filmore is an associate editor of *Modern Drug Discovery*. Send your comments or questions about this article to mdd@acs.org or to the Editorial Office address on page 3. ■

KEY TERMS: combinatorial chemistry, high throughput, screening, technique