An immunochemical approach for the determination of trace amounts of enantiomeric impurities†

Oliver Hofstetter,*[ab] Heike Hofstetter,[b] Meir Wilchek,[b] Volker Schurig[c] and Bernard S. Green*[d]

[a] *Department of Chemistry and Biochemistry, Northern Illinois University, DeKalb, Illinois 60115, USA. E-mail: ohofst@niu.edu*
[b] *Department of Biological Chemistry, The Weizmann Institute of Science, 76100 Rehovot, Israel*
[c] *Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, 72076 Tübingen, Germany*
[d] *Department of Pharmaceutical Chemistry, School of Pharmacy, The Hebrew University of Jerusalem, PO Box 12065, 91120 Jerusalem, Israel. E-mail: csgreen@md2.huji.ac.il*

Received (in Cambridge, UK) 8th June 2000, Accepted 11th July 2000
Published on the Web 2nd August 2000

The application of stereoselective antibodies in an enzyme immunoassay enables the quantitative determination of enantiomeric impurities beyond the outer limits of currently available methods; thus, using an antibody raised against a derivative of D-phenylalanine, the D-enantiomer of the free amino acid can be detected in a 100 000 fold excess of the L-enantiomer (ee 99.998%).

The determination of low ees is exceedingly important in wide-ranging chemical studies, such as, asymmetric synthesis,[1] dating of material of biological origin,[2] and the analysis of material of extraterrestrial origin.[3] In experiments where the ee may be very low, *e.g.*, those based upon non-conservation of parity,[4] ultra-sensitive assays are essential. The increasing understanding of the stereoselectivity of drug actions is causing regulatory agencies to define guidelines for the development of stereoisomeric drugs and to demand the specification of their stereochemical purity.[5] In some cases, even minor enantiomeric impurities may cause severe pharmacological and toxicological side effects.[6] Commonly used methods for the determination of enantiopurity include polarimetry, NMR spectroscopy and chromatography, which may allow detection of 0.1% enantiomeric impurity (99.8% ee).[7] More sensitive analytical tools are desirable, and may be vital for health safety, with regard to the production and investigation of therapeutic or nutritional chiral compounds. Improved techniques require the development of highly selective chiral hosts that specifically bind to one enantiomer present in a large excess of the opposite enantiomer. Stereoselective interaction between enantiomers and biological macromolecules such as antibodies, enzymes and receptor proteins is well known and has found various applications in stereochemistry.[8] Recently, we have shown that enantiomeric impurities can be determined by a chiral immunosensor utilizing the exquisite stereoselectivity of antibodies.[9] Here, we demonstrate the determination of enantiomeric impurities at an unprecedented level of detectability using an enzyme immunoassay.

Stereoselective antibodies sensitive to the chiral center of α-amino acids[10] were used to detect D-phenylalanine in non-racemic mixtures by a competitive enzyme-linked immunosorbent assay (ELISA, Fig. 1).[11] Rabbit antibodies, raised against a conjugate, prepared by diazotization of *p*-amino-D-phenylalanine and coupling to the protein keyhole limpet hemocyanin (KLH), stereoselectively bind to D-phenylalanine, but not to L-phenylalanine (Fig. 2). Typical sigmoidal inhibition curves were obtained with D-phenylalanine diluted in phosphate buffered saline (PBS) in the concentration range between 0.01 μM and 500 μM, while no inhibition was observed using L-phenylalanine even at much higher concentrations (up to 50 mM). Antibody binding to D-phenylalanine in the presence of a large excess of L-phenylalanine was shown using the D-enantiomer diluted in a solution of 10 mM L-enantiomer in PBS (Fig. 2). The inhibition curves obtained with D-phenylalanine in the presence and absence of L-phenylalanine are virtually identical, indicating that the stereoselective interaction between the antibody and D-phenylalanine is not affected by the presence of the L-enantiomer. As seen in Fig. 2, D-phenylalanine concentrations as low as 0.1 μM can be detected in the presence of 10 mM L-phenylalanine. This is equivalent to one part of D-phenylalanine in 100 000 parts of L-phenylalanine, corresponding to an ee of 99.998%. The limit of detection is dependent primarily on the affinity of the antibody and the sensitivity of the assay.

The suitability of this antibody for the quantitative determination of enantiomeric impurities was demonstrated in com-

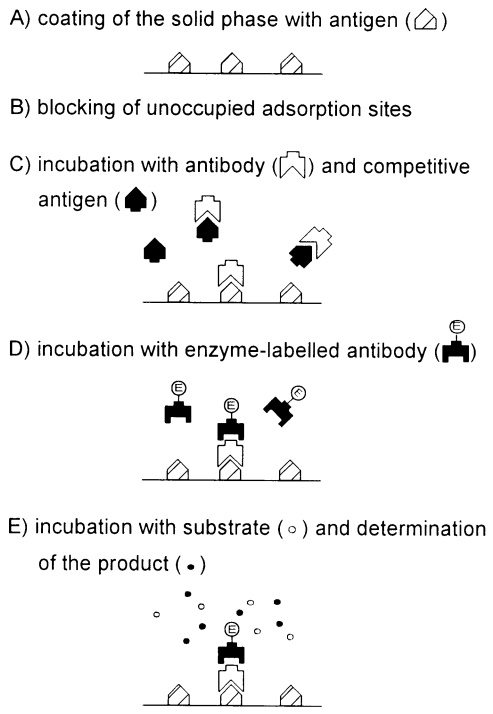

Fig. 1 Schematic depiction of the competitive ELISA procedure.[11] The antigen, in this case *p*-amino-D-phenylalanine, is bound to the wells of a polystyrene microtiter plate (A). After blocking of unoccupied adsorption sites on the plastic surface (B), antibody specific to D-amino acids is added to the wells along with the test sample (competitive antigen) (C). Enzyme-labelled antibody specific for the first antibody is added (D), and a chromogenic enzyme substrate is dispensed (E). The colored product which indicates the amount of the first antibody bound to the solid-phase immobilized antigen is determined photometrically. Between steps A–E, excess reagents are removed by washing.

† This paper is dedicated to Prof. Guenter Wulff on the occasion of his 65th birthday.

DOI: 10.1039/b004587j

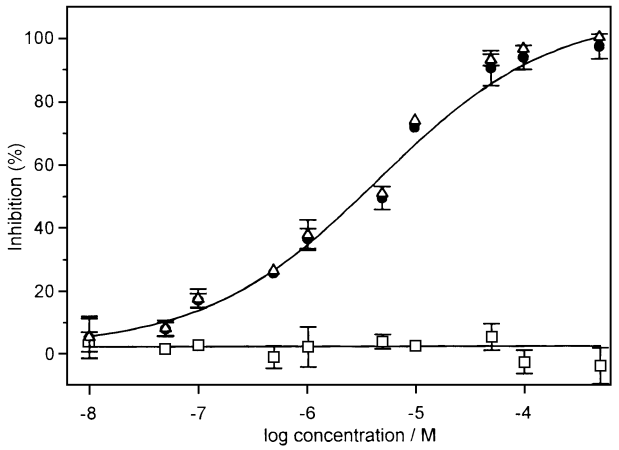

Fig. 2 Inhibition of antibody binding to solid-phase immobilized *p*-amino-D-phenylalanine by increasing concentrations of D-phenylalanine (■); by D-phenylalanine in the presence of 10 mM L-phenylalanine (△); by L-phenylalanine (□). Absorbance values were converted to % inhibition by: % inhibition = $(1 - (A/A_0)) \times 100$; A represents values in the presence of competitor, while A_0 is the absorbance without competitor. Here and in Fig. 3 error bars indicate standard deviations of triple determinations (missing error bars are obscured by the symbols).

petitive assays employing non-racemic mixtures of phenylalanine. Enantiomeric impurities of 0.1% and 0.01% were simulated by the addition of D-phenylalanine (2 μl of a 100 mM stock solution in PBS) to L-phenylalanine (1998 μl and 19 998 μl, respectively, of a 100 mM stock solution in PBS). Fig. 3 shows the inhibition curves obtained with these mixtures. The concentrations of total phenylalanine necessary to cause 50% inhibition of binding (I_{50}) were determined to be 3.12 ± 0.25 mM and 29.12 ± 2.26 mM, respectively. These results are in good accord with the I_{50} value of 3.1 ± 0.3 μM obtained from the calibration curve using enantiomerically pure D-phenylalanine in PBS (Fig. 2), and indicate that, within the accuracy of the method, the test mixtures indeed contain one part D-enantiomer in 1000 parts and 10 000 parts, respectively, of the L-enantiomer.

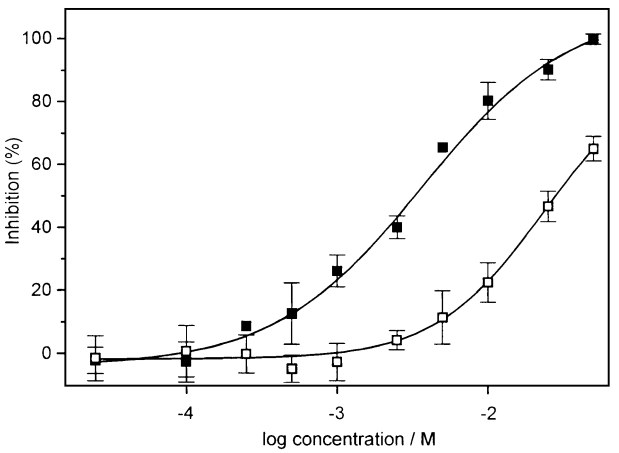

Fig. 3 Inhibition of antibody binding to solid-phase immobilized *p*-amino-D-phenylalanine by increasing concentrations of test mixtures containing 1 part of D-phenylalanine in 1000 parts of L-phenylalanine (■), and 1 part of D-phenylalanine in 10 000 parts of L-phenylalanine (□).

We have demonstrated the value of stereoselective antibodies for the detection of trace enantiomeric impurities and the determination of ee for the α-amino acid phenylalanine. Stereoselective antibodies have been raised for a variety of purposes, including the measurement of degree of racemization.[12] Enzymes have been used to determine enantiopurity[13] but their use is confined to natural substrates and suitable analogs, and is restricted by the number of appropriate enzymes. In contrast, antibodies can be raised against virtually any compound of interest[14] and can be selected and engineered according to one's needs.[15] Their use is routine and they are suitable for automated assays. Thus, the use of antibodies for the determination of enantiomeric impurities at extremely low levels may be extended to other compounds. This approach should be particularly valuable in chemical or pharmaceutical studies where the highest enantiopurity is essential.

We thank the G.I.F., the German-Israeli Foundation for Scientific Research and Development, for financial support, D. Then for technical assistance, and Prof. J. Gal for comments.

Notes and references

1 B. L. Feringa and R. A. van Delden, *Angew. Chem., Int. Ed.*, 1999, **38**, 3418.
2 J. L. Bada, in *Chemistry and Biochemistry of the Amino Acids*, ed. G. C. Barrett, Chapman and Hall, London, 1985, p. 399; A. Maroudas, M. T. Bayliss, N. Uchitel-Kaushansky, R. Schneiderman and E. Gilav, *Arch. Biochem. Biophys.*, 1998, **350**, 61.
3 J. R. Cronin and S. Pizzarello, *Science*, 1997, **275**, 951; M. H. Engel and S. A. Macko, *Nature*, 1997, **389**, 265.
4 S. F. Mason, in *Circular Dichroism. Principles and Applications*, ed. K. Nakanishi, N. Berova and R. W. Woody, VCH Publishers, New York, 1994, p. 39.
5 FDA, *Chirality*, 1992, **4**, 338.
6 M. Eichelbaum and A. S. Gross, *Adv. Drug Res.*, 1996, **28**, 2.
7 V. Schurig, *Methoden Org. Chem. (Houben-Weyl) 4th ed.*, 1995, **Vol. E21a**, p. 147.
8 E. L. Eliel and S. H. Wilen, *Stereochemistry of Organic Compounds*, Wiley, New York, 1996, pp. 201, 257, 273, 409.
9 O. Hofstetter, H. Hofstetter, M. Wilchek, V. Schurig and B. S. Green, *Nature Biotechnol.*, 1999, **17**, 371.
10 O. Hofstetter, H. Hofstetter, V. Schurig, M. Wilchek and B. S. Green, *J. Am. Chem. Soc.*, 1998, **120**, 3251.
11 O. Hofstetter, H. Hofstetter, D. Then, V. Schurig and B. S. Green, *J. Immunol. Methods*, 1997, **210**, 89.
12 C. E. Cook, in *Drug Stereochemistry: Analytical Methods and Pharmacology*, ed. I. W. Wainer and D. E. Drayer, Marcel Dekker, New York, 1988, p. 45; K. L. Rominger, A. Mentrup and M. Stiasni, *Arzneim. Forsch./Drug Res.*, 1990, **40**, 887; A. Huhtikangas, T. Lehtola, R. Virtanen and P. Peura, *Finn. Chem. Lett.*, 1982, 63.
13 J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, vol. 2, Wiley, New York, 1961, p. 1738; H. Scannone, D. Wellner and A. Novogrodsky, *Biochemistry*, 1964, **3**, 1742.
14 K. Landsteiner, *The Specificity of Serological Reactions*, Dover Publications, New York, 1962; D. Pressman and A. Grossberg, *The Structural Basis of Antibody Specificity*, Benjamin, New York, 1968, p. 30.
15 *Antibody Engineering, A Practical Approach*, ed. J. McCafferty, H. Hoogenboom and D. Chiswell, IRP Press, Oxford, 1996.