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ADVANCES IN CHIRAL SEPARATIONS: A REVIEW

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ABSTRACT

Chiral separation in forensic science is chemical separations of optically active isomers of drugs and metabolites. Specific functions of D-amino acids in humans are bound to lead to the revelation of D-amino acid abnormalities in human disorders. Therefore, high-throughput analysis techniques are warranted to determine D-amino acids in biological fluids. Two chromatographic techniques, a nonchiral derivatization with chiral (chirasil-L-val column) separation in a GC-MS system and a chiral derivatization with Marfey's reagent and LC- MS analysis were developed. The techniques for D-serine, L-serine, and glycine determination in cerebrospinal fluid (CSF) were validated. Toluene monooxygenases (TMOs) have been shown previously to catalyze region selective hydroxylation of substituted benzenes and phenols. TMOs are also capable of performing enantio selective oxidation reactions of aromatic sulfides.

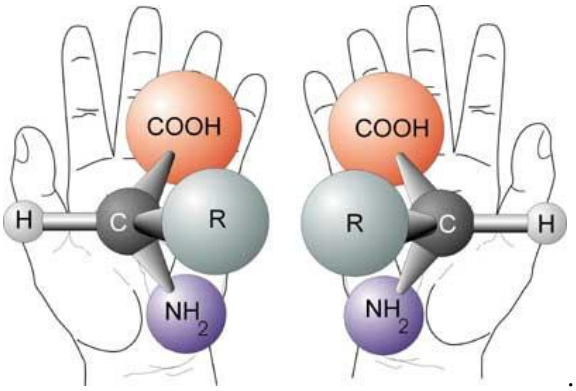
Key words: Enantiomers, GC-MS system, LC-MS System and Toluene Monooxygenases

INTRODUCTION

A chiral molecule is a type of molecule that lacks an internal plane of symmetry and thus has non-super imposable mirror images. The feature that is most common often the cause of chirality in molecules is the presence of asymmetric carbon atom.

Chiral in general is used to describe an object that is non-super imposable on its mirror image. New chiral selectors and innovative approaches for CE and CEC are introduced. Recent progress in column technology for CEC is highlighted and the development of new chiral stationary phases is discussed.

Chirality usually refers to molecules. Two mirror images of a chiral molecule are called enantimers or optical isomers. Chiral separation in forensic science is chemical separations of optically active isomers of drugs and metabolites. The ability to separate these isomers is becoming more important in drug analysis.



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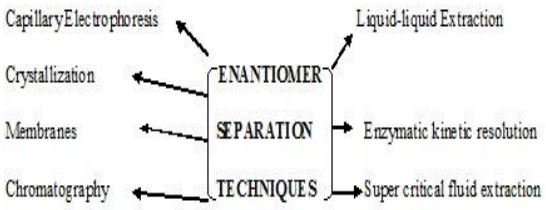

Two Mass-Spectrometric Techniques for Quantifying Serine Enantiomers and Glycine in Cerebrospinal Fluid: Amino acids are essential molecules for all living beings. All amino acids except glycine occur in an L-and D-form, depending on the tetrahedral configuration around the chiral center on the α-carbon atom. Incorporation of a particular amino acid enantiomer in proteins or (poly) peptides determines the spatial architecture of biological polymers and plays a major role in enzymatic specificity and structural interactions. [1] With the advance of chromatographic analysis techniques, improved determination of the different amino acid enantiomers was achieved; revealing the unexpected but undeniable presence of small quantities of D-amino acids in lower and higher animals, plants, and foods .Research has largely focused on the D-amino acid D-serine, which has been identified in surprisingly high concentrations in the mammalian central nervous system. [2] Subsequent studies demonstrated endogenous D-serine metabolism and synthesis from L-serine. [3] Like glycine, D-serine functions as neuromodulator through binding to the *N*-methyl D-aspartate (NMDA) excitatory amino acid receptor[4]. NMDA receptors are involved in central nervous system development, brain plasticity, memory, and learning. NMDA-receptor dysfunction has been implicated in various pathological conditions, including schizophrenia, epilepsy, stroke, and neurodegenerative conditions[5]. Recently, we reported that D-serine might be essential for human central nervous system development and provided the first example of human D-serine deficiency in patients with 3-phosphoglycerate dehydrogenase deficiency. [6] Other studieshave implicated altered D-serine concentrations in schizophrenia [7,8] amyotrophic lateral sclerosis, [9] and nociception. [10] Together, these studies imply that D-serine is important in human physiology and pathology.

Materials: Acetylchloride,2-propanol, D-serine, N_{α}-2,4-dinitro-5-fluorophenyl- L- alaninamide (Marfey's reagent), and ammonium formiate, chloroform, 5-sulfosalicylic acid, L-serine, glycine, disodiumtetraborate.$10H_2O$, formic acid, and hydrochloric acid , pentafluoropropionic anhydride; acetonitrile,acetone ,and labelled stable isotopes 3-^{13}C(99%) DL-serine and 1,2-C_2(99%)glycine .

CSF samples: D-Serine concentrations were determined in human CSF samples .These samples were kept at 4 °C for 1 week before being stored at –80 °C. Excluded samples with more than 100 erythrocytes/mL, meningitis, HIV, intracranial pathology, perinatal asphyxia, and serine biosynthesis disorders, and epilepsy, schizophrenia, and neurodegenerative disorders.

Quality control samples: Sample volumes of 100, 200, and 300 µL (QC1a–c) were derivatized following the normal GC-MS sample preparation procedure, which includes drying of the solution after addition of internal standard and derivatization of the complete residue. For recovery analysis, CSF from the metabolic and endocrine diseases was pooled (QC2) and spiked with aqueous amino acid solutions, theoretically increasing L-serine concentrations with 33.33 µmol/L and D-serine and glycine concentrations with 6.66 µmol/L, thus doubling the expected concentrations (QC3). Similarly, prepare QC4 samples by spiking pooled CSF with aqueous solutions of 15 µmol/LD-serine, L-serine, and glycine.

GC-MS analysis

Sample preparation procedure: CSF samples were thawed at room temperature and derivatized. [11] After stirring, transfer 200 µL CSF to 1.5 mL microtubes and add 50 µL internal standard solution (aqueous solution of 600 µmol/L 3-^{13}C-DL-serine and 60 µmol/L 1,2-$^{13}C_2$-glycine). Deproteinize the samples by adding 200 µL aqueous 5-sulfosalicylic acid, thoroughly mix, and centrifuge in a Heraeus Biofuge Pico centrifuge 4 min at room temperature. Apply the supernatant to Durapole microfilters (0.22 µm; Millipore) to eliminate remaining (oligo) peptides and transfer the filtrate to Pyrex culture tubes with screw caps and PTFE-faced rubber lining.Remove the solvents in a nitrogen stream at 40 °C. Add 250 µL of 2.5 mol/L HCl in 2-propanol (acetyl chloride in 2-propanol 1:4 v/v) to the dry residue. After heating for 45 min at 70 °C in heating blocks, remove the reagents in a nitrogen stream at ambient temperature. Added 400 µL chloroform and 100 µL pentafluoropropionic anhydride and heat the mixture at 100 °C for 20 min and remove the reagents in a nitrogen stream at ambient temperature. Dissolve the residues in 50 µL chloroform and subject 2 µL to GC-MS. For each analytical run, prepare a 7-point calibration curve with aqueous solutions of L-serine (0–112.5 µmol/L), D-serine, and glycine (0–26.25 µmol/L).

Chromatographic conditions: The GC comprised with HP-5890 gas chromatograph and a HP-7673 automatic sampler. Deliver the derivatized amino acids by automatic injection (split injection port 1:20) over a glass wool liner to the WCOT fused-silica CP

chirasil-L-val (*N*-propionyl-L-valine *tert*-butylamide polysiloxane) capillary column (25 by 0.25 mm internal diameter; 0.12 µm film thickness). Helium was used as carrier gas (1 mL/min), with automatic pressure adaptation. The temperature program started at 80 °C for 3 min, increased at 3 °C/min to 190 °C, and held at 190 °C for 5 min. The injector and detector temperatures were set at 220 °C.

Mass spectrometric conditions: In the quadrupole HP-5989B mass spectrometer, the eluted derivatized amino acids were ionized by negative chemical ionization using 5% ammonia in methane .The ion source and the quadrupole temperatures are set at 250 °C and 150 °C, respectively, according to the manufacturer's protocol. The MS runs in the selected ion monitoring mode (SIM). The appropriate ion sets were selected, using the following characteristic mass fragments (*m*/*z*) of the *N*(*O*) pentafluoropropionyl-2-propanol esters of the amino acids: serine (*m*/*z*255), ^{13}C-serine (*m*/*z* 256), glycine (*m*/*z* 243), and $^{13}C_2$-glycine (*m*/*z* 245). GC-MS control and data processing were performed with HP G1034C and G1710BA MS.

LC-MS analysis Sample preparation procedure: CSF samples were thawed at room temperature and derivatized. [12, 13] Add 50 µL internal standard solution (600.5 µmol/L 3-^{13}C-DL-serine and 61 µmol/L 1,2-$^{13}C_2$-glycin**e** in 0.1 mol/L HCl) to 100 µL CSF. On drying in a nitrogen stream, the residues were derivatized with 50 µL 0.5% Marfey's reagent (wt/vol in acetone) and100 µL 0.125 mol/L disodiumtetraborate, 10H_2O for 30 min at 40 °C. The reaction was stopped with 25 µL 4 mol/L HCl. Dilute the resulting solution (1:10) with eluent buffer (250 mg ammonium formiate in 1 L milliQ-water; pH adjusted to 4.6 with formic acid) and subject 10 µL to LC-MS. For each analytical run, prepare a 6-point calibration curve with aqueous solutions of L-serine (0–100 µmol/L), D-serine, and glycine (0–20 µmol/L).

Chromatographic conditions: Resolve the derivatized amino acids on an Alliance 2795 HPLC system (Waters), with separation on an Atlantis dC18 analytical column (3 µm, 3.9 by 150 mm) (Waters), using a linear gradient of 100% mobile phase A (250 mg ammonium formiate in 1 L milliQ-water; pH adjusted to 4.6 with formic acid) to 50% mobile phase A and B (acetonitrile) in 15 min. The flow rate was 0.3 mL/min.

Mass spectrometric conditions: A Quattro Ultima triple quadrupole mass spectrometer was used in the negative electron spray ionization (ESI) mode. The following mass spectrometer settings were used: capillary voltage 3.0 kV, cone voltage 40 V, cone gas flow 185 l/h, desolvation gas flow 677 l/h, collision gas pressure 1.33e–3, and source temperature 150 °C. The appropriate ion sets were selected, using the following characteristic mass fragments (*m*/*z*) of the dinitrophenyl-L-alanine-amides of the amino acids: serine (*m*/*z* 356.1), ^{13}C-serine (*m*/*z* 357.1), glycine (*m*/*z*326.1), and $^{13}C_2$-glycine (*m*/*z* 328.1). Masslynx software, which included Quanlynx (Waters), was used for instrument control, data acquisition, and data processing.

Validation studies: For both analysis techniques, perform validation [14], using CSF samples and the lowest standards of the calibration curves to assess the limit of detection (LOD, concentration at a signal-to-noise ratio of 3, n = 10) and limit of quantification (LOQ, concentration at a signal-to-noise ratio of 10, n = 10). Use quality control samples to assess within-run (n = 10 for QC1a–c, QC2, and QC3) and between-run (n = 10 for QC1a–c, QC2, and Q3) imprecision. Analyze recovery in QC3 and QC4 samples (n = 10), assess linearity and range of detection in CSF (LOQ to the highest concentration of the calibration curve (r^2 > 0.99) + 10%) for D-serine, L-serine, and glycine, and compare the results with the 2 new analysis techniques (n = 68). Stability of samples prepared for LC-MS analysis was assessed up to 13 days.

GC-MS and LC-MS analysis techniques: Both techniques yield baseline separation of amino acid enantiomers (Fig. 1). GC-MS sample preparation comprises approximately 6–8 h, LC-MS sample preparation takes 60 min. Elution time was similar (GC-MS 6–12 min; LC-MS 12–14 min). The chromatographs represent patient CSF samples, with the internal standard peaks (higher peaks) and endogenous peaks (lower peaks) overlain in the same figure. The *x* axis shows the retention time in minutes, the *y* axis the abundance in counts (A) or percentage (B; 100% in this case representing 5.89e5 counts).

Conclusion: The LC-MS technique has several advantages. LC-MS sample preparation was definitely less laborious and time-consuming, and theoretically can be automated and performed in 96-well plates. Separation is achieved on a nonchiral column, concomitantly applicable for a wide variety of polar compounds. Furthermore, the relatively short LC-MS derivatization reaction time in alkaline solution at 40°C might facilitate simultaneous determination of a variety

of chiral amino acids, including glutamate and aspartate. These dicarboxylic amino acids are important excitatory amino acids in human physiology and pathology [15] but are difficult to quantify accurately [16] because glutamine and asparagine, present in high concentrations in CSF, are very prone to conversion to glutamate and aspartate, respectively. However, in laboratories without LC-MS, theGC-MS method is a very suitable alternative.

Protein Engineering of Toluene Monooxygenases for Synthesis of Chiral Sulfoxides: Toluene monooxygenases (TMOs) have been shown to catalyze regioselective hydroxylation of substituted benzenes and phenols. TMOs are also capable of performing enantioselective oxidation reactions of aromatic sulfides. Chiral sulfoxides are of major importance in organic chemistry, as they are efficient chiral auxiliaries that lead to important asymmetric transformations. In addition, chiral sulfoxides possess a wide range of biological activities from flavor and aroma precursors to antimicrobial properties [17,18]. The world's best-selling antiulcer drug, (*S*)-omeprazole, is a chiral sulfoxide [19]. The enantioselective oxidation of a prochiral sulfide is undoubtedly the most direct and economical approach for the synthesis of optically pure sulfoxides [20]. Enzymes used for obtaining chiral sulfoxides are toluene dioxygenase (TDO) from Pseudomonas putida F1 and naphthalene dioxygenase (NDO) from Pseudomonas sp. strain NCIB 9816-4 [21]. For some alkyl aryl sulfides, the selectivity of these two enzymes was complementary (e.g., TDO favoring the *R*-enantiomer and NDO favoring the *S*-enantiomer). In addition to the aforementioned well-studied enzymes, various fungi and yeasts also oxidize sulfides into chiral sulfoxides with various degrees of selectivity. TMOs have been shown to be effective bioremediating agents (capable of degrading trichloroethylene) as well as useful biocatalysts [22]. The advantages of TMOs are their high selectivity and their use of molecular oxygen as an inexpensive and safe oxidizing agent rather than H_2O_2. The most extensively studied monooxygenases are toluene ortho-monooxygenase (TOM) of Burkholderia cepacia G4, which hydroxylates toluene at the ortho position to formo-cresol [23]; toluene o-xylene monooxygenase (ToMO) of Pseudomonas stutzeri OX1, with a relaxed regiospecificity, producing a mixture of three isomers from toluene hydroxylation and toluene para-monooxygenase (TpMO) of Ralstonia pickettii PKO1 and toluene 4-monooxygenase (T4MO) of Pseudomonas mendocina KR1, both para-hydroxylating enzymes producing primarily p-cresol from toluene.

Chemicals: Thioanisole (99%), methyl p-tolyl sulfide (99%), methyl phenyl sulfoxide (97%), and methyl p-tolyl sulfoxide (98%).

Bacterial strains and growth conditions: Escherichia coli TG1 with the plasmid constructs was routinely cultivated at 37°C with shaking at 250 rpm on a TU-400 incubator shaker in Luria-Bertani (LB) medium supplemented with kanamycin at 100 µg/ml to maintain the plasmids. To stably and constitutively express the TMO genes from the same promoter, the expression vectors pBS(Kan)TOM, pBS(Kan)TpMO, pBS(Kan)ToMO [24], and pBS(Kan)T4MO were constructed. All experiments were conducted by diluting overnight cells to an optical density (OD) at 600 nm of 0.1 and growing them to an OD of 1.3. The exponentially grown cells were centrifuged at 8,000 × *g* for 10 min at 25°C in a Sigma 4K15 centrifuge and resuspended in potassium phosphate buffer (PB; 100 mM, pH 7.0). Expression of TMOs (wild-type [WT] and protein variants) by pBS(Kan) vectors within E. coli strains produce blue or brown cells on agar plates and in broth cultures. The color is indicative of indigoid compounds formed by oxidation of indole from tryptophan.

Protein analysis and molecular techniques: Protein samples of cells grown with and without 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were analyzed on standard 12% Laemmli discontinuous sodium dodecyl sulfate-polyacrylamide gels. Plasmid DNA was isolated using a Midi kit and DNA fragments were isolated from agarose gels using the RBC extraction kit. E. coli strains were transformed by electroporation using a Micro-Pulser instrument with the program Ec1 (1.8 kV, 1 pulse for a 0.1-cm cuvette).

Screening method: Screening for mutants with improved activity was performed by growing them in 5 ml of LB medium supplemented with kanamycin (100 µg/ml). The cultures were grown for 20 h at 37°C with shaking at 250 rpm on a TU-400 incubator shaker and then cells were harvested at 8,000 × *g* for 10 min at 25°C using a Sigma 4K15 centrifuge. The cell pellets were resuspended with 2.5 ml PB, pH 7, 0.1 M, to a final OD at 600 nm of 4. The substrate thioanisole was added to 1 ml of cells in PB to a final concentration of 0.5 mM substrate (from a 50 mM stock solution in ethanol), and the biotransformation was carried out for 3 h in a 16-ml glass vial with shaking at 600 rpm using a Vibramax 100 shaker at room temperature. The reaction was stopped by adding 1 ml of ethyl acetate (1:1 volume) followed by vigorous vortexing. Phase separation was facilitated by a short centrifugation step (8,000 × *g* for 30 s), and the content of the organic phase was measured using a 6890N gas chromatograph (GC). The criteria for better performance were higher reaction rates and higher enantiomeric excess (EE)

than the Wild Type enzyme. In order to ensure the probability (99%) that all 64 possible outcomes from the single-site random mutagenesis had been sampled, 292 colonies needed to be screened.

Analytical methods: Conversion of sulfides to sulfoxides was determined by GC with a 6890N GC using a 30-m × 0.32-mm × 0.25-µm capillary column packed with γ-cyclodextrin trifluoroacetyl and a flame ionization detector. The temperature for thioanisole was programmed at T_1 = 110°C; dT/dt = 10°C/min, T_2 = 130°C; dT/dt = 20°C/min, T_3 = 160°C, 13 min; split ratio, 1:3. Under these conditions, the retention times were 3.89 min for thioanisole, 10.35 min for (*R*)-methyl phenyl sulfoxide, and 14.15 min for (*S*)-methyl phenyl sulfoxide. For determination of methyl p-tolyl sulfide conversion, the temperature was programmed as follows: T_1 = 110°C; dT/dt = 10°C/min, T_2 = 130°C; dT/dt = 20°C/min, T_3 = 160°C, 17.5 min; split ratio, 1:3. Under these conditions, the retention times were 4.45 min for methyl p-tolyl-sulfide, 12.56 min for *(*R)-methyl *p*-tolyl sulfoxide, and 13.46 min for (*S*)-methyl *p*-tolyl sulfoxide. The concentrations of the reactants were determined from calibration curves obtained with commercial standards. The identity of the sulfides and sulfoxides was confirmed by GC-mass spectrometry using a 6890N GC equipped with a capillary column (30 m × 0.32 mm × 0.25 µm) filled with HP-5 [(5%-phenyl)-methylpolysiloxane] and an HP-5975 mass spectrum detector. As the HP-5 capillary column is not chiral, the *R* and *S* sulfoxides were seen as one peak. For determination of thioanisole and methyl *p*-tolyl-sulfide conversion, the temperature was programmed at T_1 = 90°C; dT/dt = 10°C/min, T_2= 190°C; split ratio, 1:10.

Evaluation of four WT TMOs: Four Wild Type TMOs (TOM, ToMO, TpMO, and T4MO) were evaluated for their ability to oxidize two substrates, thioanisole and methyl *p*-tolyl sulfide, at a concentration of 1 mM. The results indicate that TOM had the highest activity rate for both substrates: 1.6 ± 0.2 nmol/min/mg protein for thioanisole and 0.5 ± 0.05 nmol/min/mg protein for methyl *p*-tolyl sulfide. TOM transformes thioanisole to the corresponding sulfoxides 2.5 times faster than T4MO, which had the second highest rate. ToMO exhibits a low transformation rate, while TpMO was a very poor catalyst (16 times lower than TOM). The transformation rate of methyl *p*-tolyl sulfide was dramatically lower for TOM and T4MO in comparison with thioanisole, while ToMO and TpMO had no activity at all (Fig.2A). The enantiospecificity of the Wild Type enzymes vary from 14% Enantiomeric excess for ToMO to 91% Enantiomeric excess for TpMO (Fig. 2B), with TOM exhibiting only moderate preference for the (*S*)-sulfoxide (51% EE). The specificity of TOM was remarkably reduced in the sulfoxidation of methyl *p*-tolyl sulfide to a mere 11% Enantiomeric excess, whereas T4MO completely changes its selectivity toward the *R*-enantiomer. In prolonged experiments a small peak of sulfone was detected by GC-mass spectrometry, indicating further oxidation of the sulfoxide by the enzymes. However, within the short time limits of the experiments, oxidation of sulfoxides to sulfones was not detectable. TOM showes the highest oxidation rate for both substrates and T4MO exhibits high enantioselectivity

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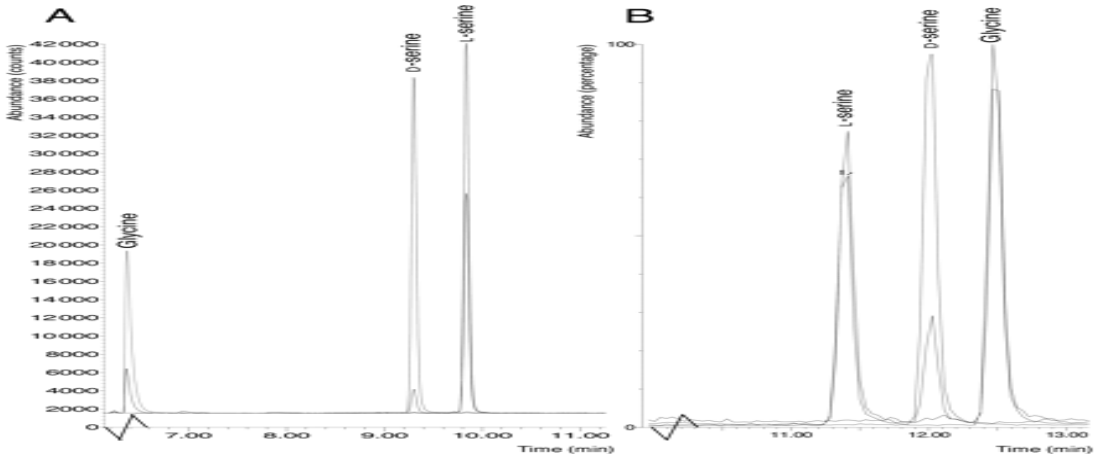

Figure 1 Chromatographs of D-serine, L-serine, and glycine using GC-MS (A) and LC-MS (B)

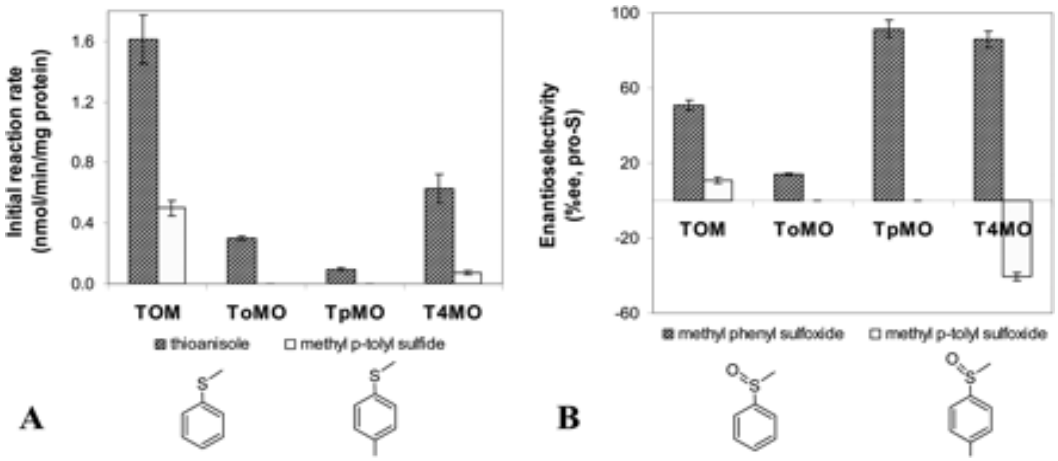

FIG 2: Initial sulfoxidation rate (A) and enantioselectivity (B) of TG1 cells expressing WT TMOs. Initial substrate concentration was 1Mm. Results represent an average of atleast two independent experiments with the absolute measured error being less than 10%.

REFERENCES

1. Berna MJ, Ackermann BL. J Chromatogr B Analyt Technol Biomed Life Sci, 2007; 846:359-63.
2. Bruckner H, Haasmann S, Friedrich A. Amino Acids, 1994; 6:211
3. Canada K A. S Iwashita, H Shim, TK Wood. J Bacteriol, 2002; 184:344-9.
4. Corrigan JJ. Science (Wash DC), 1969; 164: 142-9.
5. Cotton H, T Elebring, M Larsson, L Li, H Sorensen, S von Unge. Tetrahedron Asymmetry, 2000; 11:3819-25.
6. Danysz W, Parsons AC. Pharmacol Rev, 1998;50:597-664.
7. Fernandez I, N Khiar. Chem Rev, 2003; 103:3651-705.
8. Fuchs SA, Dorland L, de Sain-van der, Velden MG, Hendriks M, Klomp LW, Berger R, de Koning TJ. Ann Neurol 2006; 60: 476-80.
9. Goodlett DR, et al. J Chromatogr A, 1995; 707: 33-244.
10. Grimley J. Pharma challenged Chem Eng News, 2006; 84:10-17.
11. Guidelines for a User Laboratory to Evaluate and Select a Kit for Its Own Use. European Committee for Clinical Laboratory Standards, part 1: Quantitative Tests.ECCLS document 1986;:3.
12. Hashimoto A, Nishikawa T, Hayashi T, Fujii N, Harada K, Oka T, Takahashi K. FEBS Lett, 1992;296:33-6.
13. Hashimoto K, Engberg G, Shimizu E, Nordin C, Lindstrom LH, Iyo M. Prog Neuropsychopharmacol Biol Psychiatry, 2005;29:767-9
14. Hashimoto K, et al. Arch Gen Psychiatry, 2003;60:572-6.
15. Holland HL. Nat Prod Rep, 2001, 18:171-81.
16. Lee K, JM Brand, DT Gibson. Biochem Biophys Res Commun, 1995; 212:9-15.
17. McClay K, BG Fox, RJ Steffan. Appl Environ Microbiol, 2000; 66:1877-82.
18. Mothet JP, Parent AT, Wolosker H, Brady RO, Jr, Linden DJ, Ferris CD. Proc Natl Acad Sci USA, 2000;97: 26-4931.
19. Rui L, YM Kwon, A Fishman, KF Reardon, TK Wood. Appl Environ Microbiol, 2004; 70:3246-52.
20. Sasabe J, Chiba T, Yamada M, Okamoto K, Nishimoto I, Matsuoka M, Aiso S. EMBO J, 2007; 26: 4149-59.
21. Schmid DG, der Mulbe FD, Fleckenstein B, Weinschenk T, Jung G. Anal Chem, 2001;73:6008-13.
22. Sethuraman R, et al. Clin Chem, 2007; 53: 1489-94.
23. Thomas RJ. J Am Geriatr Soc, 1995; 43: 1279-89.
24. Vardar G, TK Wood. Appl Microbiol Biotechnol, 2005; 68:510-7.
25. Wolosker H, Blackshaw S, Snyder SH. Proc Natl Acad Sci USA, 1999; 96: 13409-14.