T. Kaur[1)], S. Dubey[1)], M. Chakraborty[1)], S. Jain[1)], A. Beotra[1)], C. P. Shinde[2)]

Enantiomeric profiling of amphetamine and methamphetamine in sportspersons: a comparison of two chiral derivatization methods

1. National Dope Testing laboratory, Ministry of Youth affairs & Sports, New Delhi, India
2. School of Studies in Chemistry, Jiwaji University, Gwalior, India

Introduction:

The use of Amphetamine (AM) & Methamphetamine (MA) is banned in sports by World Anti Doping Agency (WADA)[1,2] and both are chiral compounds (figure-1) whose *d*-(+) enantiomers exhibit five times greater pharmacological potency than the corresponding *l*-(-) enantiomers. The levo *(l)*-MA is generally present in over the counter decongestants, whereas dextro *(d)*-MA is abused as Central Nervous System (CNS) stimulant. Therefore, it becomes essential to discriminate the illegal use of AM/MA & their analogues from the legitimate use of prescribed AM/MA or their derivatives[3-6]. This study explores the feasibility of two chiral reagents in separation & detection of enantiomers.

Material & Methods:

Chemicals & Reagents: All the reagents & chemicals used were of analytical or HPLC grade. The chiral reagents were purchased from Sigma-Aldrich (*S*-(-)-N-trifluoroacetylprolyl Chloride , TPC) and Fluka, USA, ((+) -α-methoxy-trifluoromethylprolyl Chloride , MTPA). Reference standards of *d*-AM, *dl*-AM, *d*-MA, Diphenylamine (DPA) & 10-methylphenothiazine (NMPZ) were purchased from Sigma-Aldrich, USA. The stock solutions (1 mg/mL) & further dilutions of all the standards were prepared in ethanol. NMPZ & DPA were used as internal standards at the concentration 0.2 mg/mL.

Excretion study :

Single therapeutic dose of l-Selegilline (Selgine-10 mg from Intas Pharma.Ltd. India) & Vicks Inhaler (two nasal inhalations) given to two different healthy human male volunteer as per the approval of the Departmental Ethics committee. The samples were collected for 72 hours at interval of 2-3 hours and kept frozen at -20°c.

Sample Preparation & Instrumental Analysis:

The complete set of samples comprising of excretion study, positive and negative quality controls were divided into two batches. Each batch of samples was extracted using 2 ml tert. butyl methyl ether after alkalinizing urine samples (5ml) with 0.5 ml of 5N KOH. The

organic layer was separated and both the batches were derivatized differently. For derivatising one batch, 50µl of TPC was added and incubated at room temperature for 15 minutes. 3 ml of 0.01M NaOH was added and shaken for 20 minutes. The organic layer was separated, dried under N_2 stream & reconstituted in ethyl acetate. For, the second batch the extract was shaken for 15 minutes after adding 50µl of MTPA and 100µl of 6M NaOH. The organic layer was separated, dried & reconstituted in 100µl of ethyl acetate. Analysis of all the samples was performed on GC-MSD system (Table-1). The analytical methods were validated as per the requirement of WADA ISL (ver 6.0) keeping in view recovery, specificity, matrix interference, limit of detection (LOD) & precision.

Results and Discussion:

The results of both the derivatization methods showed good separation of *d* & *l* forms of MA & AM as their α-methoxy-trifluoromethyl-prolyl products with MTPA and as trifluoroacetyl prolyl derivatives with TPC (figure-1 & 2).

The quantifier ions were m/z 237 (AM-TPC) and m/z 251(MA-TPC) for TPC derivatives whereas, m/z 260 (AM-MTPA) & m/z 274 (MA-MTPA) for MTPA derivatives (figure-3). The TPC derivatives showed two co eluting peaks which were not found in MTPA derivatives and may have origin from the reagent.

The validation results revealed that the recovery percentage and LOD of TPC derivatives (recovery% 80-90%; LOD 100 ng/mL) was better than MTPA derivatives (recovery% 60-85%; LOD 250 ng/mL) for both AM & MA enantiomers.

The excretion study results showed presence of only *l*-MA in the urine samples from the excretion study of Vicks inhaler whereas, selegiline administration samples showed presence of only levo form of both AM & MA, which is in accordance to the reported stereo specific metabolism of *l*-selegiline[7].

Conclusion: It is concluded that TPC is a better method for enantiomeric separation although it showed 5-10% racemization (small peak of *l*-MA & *l*-AM observed in case of *d*-MA & *d*-AM). The present work will be further extended to investigate the cause of racemization.

References:

1. The World anti Doping Code. WADA International Standard for Laboratories Version 5.0.
2. The World Anti Doping Code. The 2009 prohibited list international standard.

3. Kazlauskas R. Lisi A, Trout, Chiral derivatization In: Schanzer W, Geyer H, Gotzmann A, Mareck U. (eds.) Recent Advances in Doping Analysis (6), Cologne, 431-441, 1999.
4. Herráez-Hernández,R., Campíns-Falcó,P., Tortajada-Genaro, L. A., Chiral determination of amphetamine and related compounds using chloroformates for derivatization and high-performance liquid chromatography, *Analyst*, 1998, 123, 2131–2137.
5. Ju-Tsung Liu, Ray H. Liu, Enantiomeric composition of abused amine drugs: chromatographic methods of analysis and data interpretation, J. Biochem. Biophys. Methods 54 (2002) 115–146
6. Ju-Tsung, Main-Ping Sun, Yun-Shun Tsai, Regioisomeric differentiation of mono-methoxy ring-substituted amphetamine and methamphetamine by GC-MS, *Forensic Sci Jour 2003;2:59-68.*
7. Kupiec TC, Chaturvedi AK., Stereochemical determination of selegiline metabolites in postmortem biological specimens**,** J Forensic Sci. 1999 Jan; 44(1):222-6.

Agilent GC-MSD system	
Oven Temperature	**Initial Temp:120$^{\circ C}$; Hold: 1.0 min; Rate: 16/min$^{\circ C}$; Final Temp: 300$^{\circ C}$; Final Hold: 2 min.**
Injector Port	**Temp: 280$^{\circ C}$; Split ratio 11:1; Inj. vol. 2 µl**
Carrier Flow	**Carrier Gas: Helium; Mode: Constant pressure (120 Kpa); Column flow: 1.2 ml/min at 100$^{\circ C}$**
MS Parameters	**Temperature: Ion Source: 230$^{\circ C}$; Analyzer: 150$^{\circ C}$; Interface: 300$^{\circ C}$** **Ionization: Current: 300 µA ; Voltage: 70 eV** **Acquisition: Mode: Scan; Range: 50-550 m/z; Scan Rate: 1.53 scan/sec**
Software	**Chemstation®**
Workstation	**Windows XP SP2**

Table-1: Instrumental conditions for GC-MSD analysis

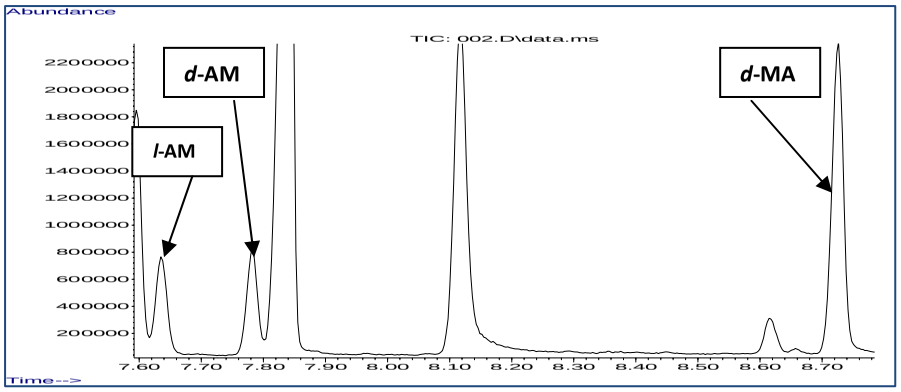

Figure-1 TIC showing TPC derivatives of *l*-AM, *d*-AM *l*-MA & *d*-MA in spiked Urine at 500ng/mL concentration

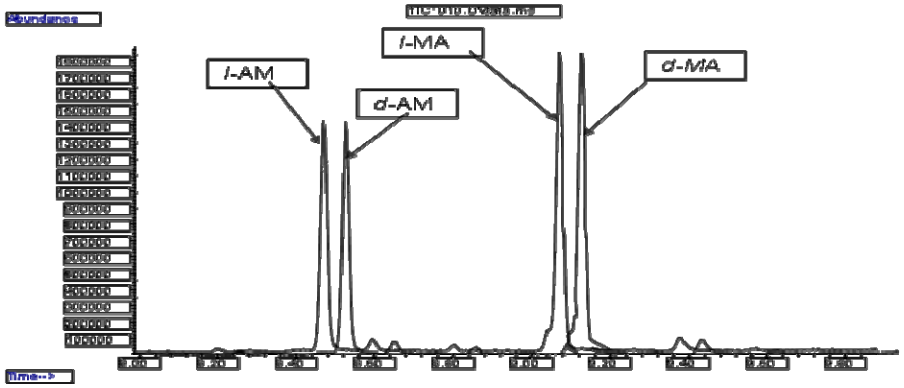

Figure-2: TIC showing MTPA derivatives of *l*-AM, *d*-AM *l*-MA & *d*-MA in spiked urine at 500 ng/mL concentration

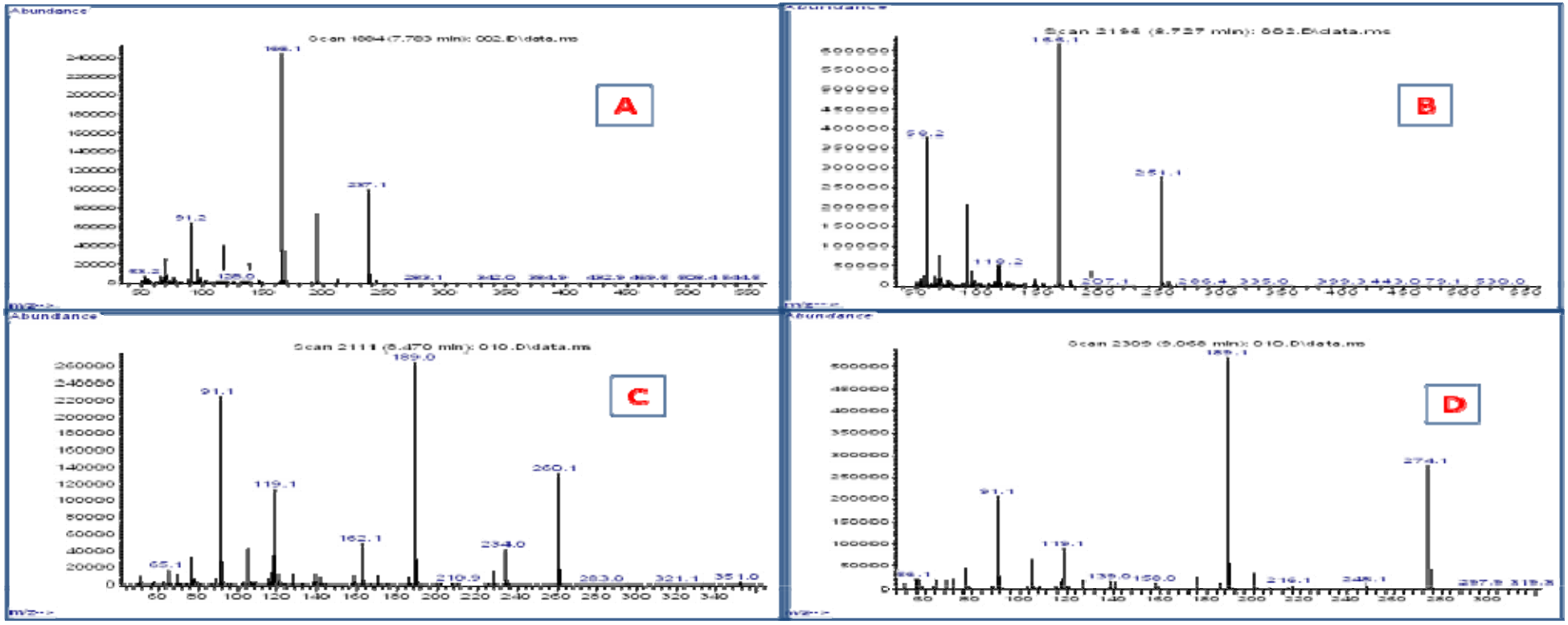

Figure-3 Mass spectra of (A) *d*-AM-TPC, (B) *d*-MA-TPC, (C) *d*-AM-MTPA & (D) *d*-MA-MTPA in spiked urine sample at 500 ng/mL concentration