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ASSESSING MUTAGENICITY OF THE DRUG MDMA (ECSTASY) AVAILABLE IN IRAN USING AMES BIOASSAY

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Abstract

An increase in incidence of illegal use of tablets containing 3, 4-methylene dioxy- metamphetamine (MDMA), has recently become a widespread social problem. MDMA most commonly known today by the street name ecstasy (often abbreviated XTC), is a semi-synthetic member of the amphetamine class of psychoactive drugs. The aim of this study was to investigate the genotoxic effects of MDMA on the basis of the results of an *in vitro* Ames test.

In the first step, a sensitive method of gas chromatography-mass spectrometry (GC-MS) was used for simultaneous quantitation of MDMA in the tablets. The Ames test uses one of the strains of the bacterium *Salmonella typhymurium* (TA100*)* that carries mutations in genes involved in histidine synthesis, so that it requires histidine for growth. The variable being tested is the mutagen's ability to cause a reversion, making bacteria grows on a histidine-free medium. When the cultures are exposed to a mutagen, in the absence and presence of a rat liver metabolizing system, some of the bacteria undergo genetic changes due to chemical interactions resulting in reversion of the bacteria to a non-histidine requiring state. The reverted bacteria will then grow in the absence of exogenous histidine. In this test, we used MDMA as a possible mutagen. No increase in bacteria growth in the histidine-free medium was observed. So, no MDMA genotoxicity was observed in this method.

Key words:

3, 4-methylene-dioxy-metamphetamine (MDMA), Ames test, *Salmonella typhymurium (TA100),* gas chromatography-mass spectrometry (GC-MS).

Introduction

3, 4-Methylenedioxymethamphetamine hydrochloride (MDMA) is a hallucinogen with a chemical structure similar to methamphetamine (MA). MDMA was synthesized as an anorexing agent for the first time by Merck Pharmaceuticals in 1912, and a patent for it was obtained. However, it was considered to have a harmful influence on the human body and was not manufactured at that time. MDMA began to be circulated on the market around 1970 and had been prescribed as a therapeutic drug by

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psychiatrists from the beginning of the 1970s to the 1980s.

MDMA had been used as a therapeutic agent for post-traumatic stress disorder (PTSD) mainly in the US until 1985, but the use of this drug thereafter became social problems (1, 2). Finally, MDMA was regulated in the United States in 1985, Japan in 1991, and most countries now strictly regulate its use (3). It produces an energizing effect as well as feelings of euphoria, emotional warmth and distortions in time perception and tactile experiences.

Tablets containing MDMA are sometimes mixed with illegal drugs such as ketamine, and unexpected acute intoxication can thus be caused as a result of drug interactions (4). The toxicity of MDMA has been reported (5, 6, 7, 8, 9). Cases of MDMA tolerance and dependence have also been reported (5).

The Ames *Salmonella*/microsome mutagenicity assay (*Salmonella* test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations. The test employs histidine dependent *Salmonella* strains carrying mutation in various genes in the histidine operon . These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. When the *Salmonella* tester strains are grown on a minimal media agar plate containing a trace of histidine, only those bacteria that revert to histidine independence (*his*C) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner (7). We used TA 100 as the primary tester strain because of its broad spectrum of sensitivity to mutagens and its low rate of spontaneous revertant mutations.

Available Ecstasy tablets supposed containing MDMA and mixed with illegal other drugs such as ketamine, and unexpected toxicity including genotoxicity was considered. So the aim of this study was to confirm the possibility of developing health problems due to MDMA abuse. The genotoxicity of this available drug in Iran has not yet been studied. In this study, the risk of carcinogenesis induced by MDMA was investigated.

Materials and methods

Totally 20 Tablets (4 types, 5 tablets for each type), containing MDMA (ecstasy) were obtained from Khuzestan Campaign against Narcotic Drugs Organization. *Salmonella typhimurium* strain TA100 was purchased from Persian Type Culture Collection (PTCC) organization.

Identification of amphetamine derivatives in the samples with Gas chromatography-mass spectrometry (GC-MS) method

GC-MS is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample.

At first, 5mg of each sample was dissolved in 2ml of distilled water. 150 µL of the solution was added to a clean borosilicate glass tube and spiked with 100 µL internal standard d_5-MDMA (1 mg per mL). The mixture was alkalinized with 750 µL of sodium carbonate 10% (pH=12) and extracted with 2 ml of diethyl ether by vortex. The mixture was given a rest for 2 minutes. The organic layer was evaporated to dryness and reconstituted in 500 µL methanol and 1 µL was injected in split-less mode. Each sample took 20 minutes for analysis. A solvent blank was run between samples (10).

Assessing the presence of histidine in the samples with Thin Layer Cromatography (TLC)

The presence of histidine in samples of ecstasy tablets can raise apparent spontaneous revertant mutation rates, so it was indicated that no histidine is present in samples with a Thin Layer Cromatography (TLC) according to the methods given by Freid and Sherma (11).

Preparation of test compounds for mutagenicity assay

Bacterial Strain Growth

Overnight cultures of *Salmonella* TA100 was grown in LB broth supplemented with histidine and biotin for 48 hours at 37°C to reach the concentration of 1.5×10^8 bacteria per ml.

Genotype confirming

The tester strain was confirmed prior to use for different requirements and characteristics according to the methods given by Maron and Ames (12).

Histidine dependence (*his*)

a loopful of the culture was streaked across a LB agar plate supplemented with an excess of biotin. Because all the *Salmonella* strains are histidine dependent, there should be no growth on the plates.

Biotin dependence (*bio*)

a loopful of the culture was streaked across a LB agar plate supplemented with an excess of histidine. There should be no growth on the plate.

Biotin and histidine dependence (bio;his)

a loopful of the culture was streaked across a LB agar plate supplemented with an excess of biotin and histidine. Growth should be observed with all strains.

rfa marker

a loopful of the culture was streaked across a LB agar plate supplemented with an excess of biotin and histidine. A sterile filter paper disk was placed in the center of the streak and 10 ml of a sterile 0.1% crystal violet solution was applied. The *Salmonella* strain showed a zone of growth inhibition surrounding the disk.

Preparation of Rat-liver S9 Fraction and Mix

For checking the metabolic activation of the test compounds, incubation with S9 fractions were carried out. Male Wistar rats (body weight~200g) were treated with 30mg/kg sodium phenobarbitone (in 0.9% w/v saline) on day one and 60mg/kg on day 2,3 and 4. Five days later, rats were sacrificed by cervical dislocation and livers were collected, homogenized in 0.15 M KCl. The homogenate was centrifuged at 9,000g for 10 min. The supernatant was aliquoted (2 ml portions) and stored at -18°C until used (13). The S9 mix was prepared according to the recipe recommended by Maron and Ames (12) and Mortelmans and Zeiger (14). 0.4 ml per plate of the high S9 mix was used in the experiment.

S9 Mix

The S9 mix composing of 8 mM MgCl, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADPH, 4 mM NADH, 100 mM sodium phosphate (pH 7.4) and 2 mlof S9 was used.

Ames/Salmonella typhimurium mutagenicity Test

Four concentrations of samples containing MDMA (1.17, 1.47, 1.84 and 2.3mg per ml in distilled water) were tested. Ames test was carried out as standard plate incorporation test (15) with *Salmonella typhimurium* strain TA100 with and without *in vitro* microsomal activation (by S9 rat liver homogenate). The assay was performed as follows: molten LB agar containing 0.08 mg per ml histidine and 0.12 mg per ml biotin was poured into plates (with or without 0.4 ml per plate metabolic activation (S9) mix). When the

LB agar has hardened (2–3 min), 0.2 ml of the overnight culture of *Salmonella* TA100 was added to each plate and distributed with a sterile loop on top of the LB agar. 0.2 ml of test samples /or 2.5 mg/plate NaN3 in distilled water as positive control/or distilled water as negative control was administered into a sterile paper disk, and placed on each plate. The plates were incubated in an inverted position for up to 4 days at 37°C. All the experiments were repeated twice in triplicate. All experimental data were expressed as Mean ± SD.

Results and discussion

Identification of amphetamine derivatives in the samples with Gas chromatography-mass spectrometry (GC-MS) method

Full-scan mass spectra of AM, AM-d8, MA, MA-d8, MDA, MDA-d5, MDMA, and MDMA-d5 were obtained from analytical standards that were processed in accordance with the procedure described above. The means of searched amphetamines in samples are presented in table1.

Assessing the presence of histidine in the samples with Thin Layer Cromatography (TLC)

No histidine was detected with TLC method. Therefore, the presence of histidine in samples can not contribute to the spontaneous revertant rate.

Ames/Salmonella typhimurium mutagenicity Test

The results of the bacterial reversion assay with four concentrations of samples are presented in Table 2. According to the EPA and GenPharmTox guidelines, a mutagenic potential of a test item, tested with Ames test, is confirmed if the mutant frequency (expressed as induction factor) is 2.0 or higher (16,17).

A dose effect relationship could underly this conclusion. A possible mutagenic potential is assumed if the IF quotient ranges 1.7 to 1.9 in combination with dose effect relationship. No mutagenic potential is assumed if all IF quotients range 1.0 (and lower) to 1.6. A nonexistent dose effect relationship could underline this conclusion. In our study non of the results of the Ames test (+S9 and –S9) exceeded the critical value 2.0 and all the IF quotients ranged below 1.6. Therefore no mutagenic activity was observed in any of ecstasy tablet samples tested on *Salmonella typhimurium* strain TA100. Also, the statistical significance of genotoxic potentials in any of the samples according to the negative control were not proven ($p > 0.05$). The results of short-term genotoxicity/mutagenicity tests on *Salmonella typhimurium* did not show the presence of genotoxic compounds in samples of ecstasy tablets. The bacterial mutagenicity assays can be carried out in 48 hrs and considered as rapid prescreens for distinguishing between carcinogenic and non-carcinogenic chemicals.

Table 1: Mean percentages of MDMA in samples Determined with GC-MS (N=3)

Sample	3,4-METHYLENEDIOXY METHAMPHETAMINE (MDMA) **(%)**	KETAMIN	AMPHETAMINE
Tablet A	48±5	-------	--------
Tablet B	57.5 ±12	Detectable	--------
Tablet C	37.6	-------	--------
Tablet d	-------	----------	Detectable

Table 2: Results of the Ames test with the strain TA100 of samples of ecstasy tablet expressed as revertants/plate and induction factors (Mean±SD) (N=3)

Sample concentration (mg/ml)	Average numbers of revertants/plate±SD (%)		Average induction Factor (IF)	
	-S9	+S9	-S9	+S9
Concentration (1.17)	74±9	68±14	1.07±0.05	0.97±0.02
Concentration (1.47)	68±10	76±8	0.98±0.01	1.08±0.01
Concentration (1.84)	63±13	68±14	0.91±0.06	0.97±0.02
Concentration (2.3)	69±10	67±9	1±0.01	0.95±0.03
Control + (0.01 NaN3)	363±36	420±42	5.2±2.9	6±3.5
Control – (distilled water)	69±11	70±8	1	1

This test allowing many thousands of compounds in our environment, not previously tested, to be screened for potential hazard.

A good correlation has been observed by several groups, for a number of carcinogenic drugs in their ability to induce mutation in the above strain and the ability to induce a response in animals.

Thus Ames test can easily and quickly assess mutagenic potential of these chemicals. For this initial screening, the tester strain TA100 was used due to its sensitivity to a broad range of mutagens and carcinogens. However, many substances are inactive in the TA100 assay and active against other tester strains, e.g., TA98. Thus, the use of strains in addition to TA100 should be considered in more comprehensive screening programs.

Besides, the presence of impurities in available illegal ecstasy tablets has been reported. As the tablets under investigation were not purified, the impurities present in them could be effective on the obtained results. Since innumerable kinds of illegal ecstasy tablets are available, chemical analysis of each and every tablet is not possible because of the time and cost involved.

The mutagenicity results obtained in this study are similar to those of previous work done by *Yoshioka et al.*, with Micronucleus and Chromosomal Aberration Tests using Chinese Hamster Lung Fibroblast Cell Line (18) who showed that MDMA is not mutagen by itself but it is converted into mutagens via some activation systems.

However, MDMA itself showed negative results in these test systems, but an important point should be considered that MDMA is a secondary amine. Secondary and tertiary amines react with the nitrous acid found in medicines and food additives to form N-nitroso compounds in the stomach. N-Nitroso compounds induce genotoxic activity according to some mutagenicity tests and carcinogenicity (19). Therefore, tablets containing MDMA are considered to react with nitrous acid to form N-nitroso-3, 4-methylenedioxymethamphetamine (N-MDMA) in the stomach. There is widespread MDMA abuse among young people overwhelmingly because tablets containing MDMA can be easily obtained. However, until now, the effects of the N-nitroso compounds of the drug have not been studied extensively regarding drug abuse. the results of this test on *Salmonella typhimurium* did not show the presence of any genotoxic compound in Available ecstasy tablets.

The evaluation of the significance of these findings through clinical follow-up of addicted patients will be continued. Clarification of these mechanistic elements in mutation induction and determining the differences in specificity between bacterial and mammalian systems remains an interesting goal for further investigation.

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References

1. NIDA–NIH–U.S. Department of Health & Human Services. MDMA (Ecstasy): NIDA INFO FACTS, March. 2005. p. 1–3.
2. National Police Agency, Government of Japan. White Paper on Police 2006. 2006. p. 158–165. (Article in Japanese)
3. World Health Organization. Expert Committee on Drug Dependence. Twenty-Second Report. 1985. p. 24–25.
4. Makino Y, Tanaka S, Kurobane S, Nakauchi M, Terasaki T, Ohta S. Profiling of illegal amphetamine-type stimulant tablets in Japan. J Health Science. 2003; 49:129–137.
5. Cami J, Farre M, Mas M. Human pharmacology of 3, 4- methylenedioxymethamphetamine ("Ecstasy"): psychomotor performance and subjective effects. J Clin Psychopharmac. 2000; 20:455–466.
6. Morton J. Ecstasy: pharmacology and neurotoxicity. Curr Opin Pharmacol. 2005; 5: 79–86.
7. McCann J, Choi E, Yamasaki E, Ames B N. Detection of carcinogens in the Salmonella/microsome test:Assay of 300 chemicals. Proc Natl Acad Sci USA. 1975; 72: 5135–39.
8. Cardesa A, Pour P, Althoff J, Mohr U. Vascular tumors in female mice after intraperitoneal injection of dimethylnitrosamine. JNCI. 1973; 51: 201–8.
9. Toth B, Magee PN, Shubik P. Carcinogenesis study with dimethyl-nitrosamine administered orally to adult and subcutaneously to new born BALB/C mice. Cancer Res. 1964; 24: 1712–21.
10. Gudzinowicz BJ, Gudzinowicz MJ. Analysis of drugs and metabolites by gas chromatography-mass spectrometry. New York: Marcel Dekker Inc; 1978. Vol 4.
11. Freid B, Sherma J. Thin layer chromatography. New York: Marcel Dekker Inc; 1982. p230.
12. Maron D, Ames BN. Revised methods for the Salmonella mutagenicity test. Mutat Res. 1983; 113: 173-215.
13. Garner RC, Miller EC, Miller JA. Liver microsomal metabolism of aflatoxin B1 to anreactive derivative toxic to S. typhimurium TA1530. Cancer Res. 1972; 32: 2058-66.
14. Mortelmans K, Zeiger E. The ames Salmonella/ microsome mutagenicity test. Mutat Res. 2000; 455: 29-60.
15. Ames BN, McCann J, Yamasaki E. Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutat Res. 1975; 3: 347-364.
16. United States Environmental Protection Agency. Health Effects Tests Guidelines: OPPTS 870.5265, The Salmonella typhimurium Reverse Mutation Assay EPA712-C-96-219. 1996.
17. Organization for Economic Cooperation and Development (OECD) Guideline for the Testing of Chemicals: Bacteria Reverse Mutation Test Guideline 471, 1997.
18. Yoshioka H, Shimizu H, Toyama Y, Miyakoshi Y, Suzuki Y, Takagi R. Genotoxicity Study of Illegal Drug MDMA and Its Nitroso Derivative N-MDMA by Micronucleus and Chromosomal Aberration Tests using Chinese Hamster Lung Fibroblast Cell Line. Environmental Health and Preventive Medicine. 2007; 12(1): 129–137.
19. Nishimura Y. Recent abuse of 3, 4-methylenedioxymethamphetamine ("Yaoto-wang", "Ecstasy"). Seishin Shinkeigaku Zasshi. 2002; 104: 819.