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Short Communication

TOXICOLOGICAL METHODS FOR TRACING DRUG ABUSE: CHROMATOGRAPHIC, SPECTROSCOPIC AND BIOLOGICAL CHARACTERISATION OF ECSTASY DERIVATIVES

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Analysis often reveals variability in the composition of ecstasy pills from pure 3,4-methylenedioxymethamphetamine (MDMA) to mixtures of MDMA derivatives, amphetamine, and other unidentified substances. For a comprehensive toxicological analysis one needs to know all steps to MDMA synthesis which may originate impurities. The aim of this study was to synthesise and determine the chemical-physical and *in vitro* biological properties of a series of MDMA derivatives.
3,4-methylendioxyphenyl-2-nitropropene (MDNP) was obtained by condensation of piperonal with an excess of nitroethane in the presence of ammonium acetate. MDNP was then reduced to methylenedioxyamphetamine (MDA) by $LiAlH_3$. All compounds were analysed using HPLC and spectroscopic technique [Raman, nuclear magnetic resonance (NMR), or infrared (IR)] at all the steps of synthesis. In addition, we assessed the biological potentials of these compounds by measuring *in vitro* their (i) blood cell/whole blood partition coefficient, (ii) binding to plasmatic proteins (Fbp), and (iii) membrane adsorption. Chemical structure was determined with antibody fluorescence polarisation immunoassay (FPIA). This study showed the presence of solid impurities, particularly of a neurotoxic compound of Al^{3+} in the final products. FPIA identified the aminoethane group close to the substituted benzene ring, but did not detect the two major precursors of MDMA: MDNP and piperonal. Raman spectroscopy is an attractive alternative technique to characterise ecstasy pills and it can identify stereoisomeric forms such as *cis*-MDNP and *trans*-MDNP, which exhibit signals at 1650 cm^{-1} and 1300 cm^{-1}, respectively.

KEY WORDS: *IR, MDA, MDMA, MDNP, NMR, Raman, toxicology*

Ecstasy is a synthetic drug used recreationally worldwide, especially by young people, which makes it a major public health concern (1). A recent French study with 1666 ecstasy pills carried out by a non-governmental organisation Rave Mission revealed that the pill composition varied from pure 3,4-methylenedioxymethamphetamine (MDMA) to a mixture of ecstasy derivatives, amphetamine, and 20 % of other substances unidentified by Rapid Product Control (RCP) (2). There are several possible ways to synthesise ecstasy, all of them starting with piperonal to obtain either a ketone (3,4-methylenedioxyphylpropanone) or an amine chemical form (3,4-methylenedioxyamphetamine) (2). Various impurities and by-products were identified in powdered tablets: 3,4-methylenedioxyamphetamine (MDA), MDA or MDMA dimers, substituted pyridines, lead, aluminium, and nitroethane (3,4).

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Some of these compounds are toxic and could be involved in documented cases of fulminant toxic hepatitis and neurotoxic effects (4).

This suggests that a comprehensive toxicological analysis of an ecstasy pill requires expert knowledge of all synthetic steps and identification of impurities. Raman spectroscopy is a powerful technique to characterise ecstasy pills. In addition, it is an *in situ*, non-destructive technique that identifies chemical bonds through intramolecular vibration spectrum (5-7). The technique has proven successful in several published studies of illegal drugs (8,9). In addition, Raman spectroscopy was used to probe photoexcitated phase transition with second irradiation using nanosecond pulsed laser leading for example to colour changes (10). These properties could be exploited to identify organic metals in a sample.

The aim of our study was to first synthesise a series of MDMA derivatives, then to characterise them using HPLC and spectroscopic techniques [Raman, nuclear magnetic resonance (NMR), or infrared (IR)], and finally to establish their biological effects on blood cells, proteins, anisotropic polar membrane, and antibodies.

MATERIALS AND METHODS

Chemicals

Piperonal, lithium aluminium hydride (LiAlH$_3$, 1 mol L^{-1} in tetrahydrofuran), sodium borohydride (NaBH$_4$), ammonium acetate [(NH$_4$(CH$_3$COO)], trifluoroacetic anhydride [(CF$_3$CO)$_2$O], nitroethane (CH$_3$CH$_2$NO$_2$), and other solvents (all of pro analysis grade) were purchased from Acros Organics (Geel, Belgium). All reagents were used without further purification.

Synthesis

The synthesis started with piperonal (11), which was dissolved with excess of nitroethane in the presence of ammonium acetate, leading to 3,4-methylendioxyphenyl-2-nitropropene (MDNP). MDA was obtained by reducing MDNP with LiAlH$_3$. Typically, MDNP was prepared by refluxing a mixture of 25 mmol of piperonal (3.75 g), 133 mmol of nitroethane, 12.5 mmol of ammonium acetate, and 60 mL of toluene. The mixture was heated overnight with continuous removal of water into a Dean-Starck

tube. The solvent was then evaporated in vacuum and the residue re-crystallised from solvent mixture CH$_3$OH:H$_2$O (1:10).

Dropwise we carefully added 1.15 mmol of LiAlH$_3$ to 100 mL of the stirred solution of 0.05 mol L^{-1} MDNP in tetrahydrofuran. The resulting mixture was heated for five hours. LiAlH$_3$ was decomposed by H$_2$O and the solid was removed by filtration. MDA was dissolved in 20 mL of dichloromethane and washed with 500 mL of 1 mol L^{-1} sodium hydroxide solution.

To obtain 3,4-methylendioxyphenyl-2-nitropropane, we added NaBH$_4$ (0.02 mol) to a solution of MDNP (0.01 mol) in methanol (25 mL) by stirring at 5 °C to 10 °C. After allowing the reaction to continue for another hour at room temperature, the mixture was concentrated. Excess of NaBH$_4$ was decomposed with acetic acid and extracted with chloroform.

CHARACTERISATION

HPLC

The precursor and final products were determined by HPLC equipped with a UV detector. The mobile phase consisted of methanol:H$_2$O:trifluoroacetic acid (60:40:0.01). Eluation was in isocrat mode at the flow rate of 1 mL min^{-1}. HPLC separation was performed on a reverse-phase C$_{18}$ column (LiChrospher 60, RP-select B ,125 mm x 4 mm, with 5 μm sized particles).

NMR spectroscopy

^{1}H and ^{13}C NMR spectra were acquired at 250.13 MHz on a Bruker WM250 spectrometer. Chemical shifts are given in ppm *vs.* tetramethylsilane (TMS,^{1}H and ^{13}C) using (CD$_3$)$_2$SO as solvent (d^{1}H=2.62 ppm and d^{13}C=39.6 ppm).

Raman spectroscopy

Spectra were acquired using a Dilor XY1800 triplemate spectrograph coupled to a Princeton Instruments CCD detector. The 647.1 nm line of a Kr$^+$ laser (Coherent Radiation Innova) was used as the excitation source with laser power of 50 mW. Raman signals were collected at room temperature, at 180 °C to the incident laser beam in the 170 cm^{-1} to 2400 cm^{-1} frequency range. Rayleigh scattering was removed using a holographic notch filter.

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Infrared spectroscopy

IR spectra were recorded on KBr discs with a Perkin-Elmer 983 spectrometer. For all the studied compounds we scanned four accumulations between 400 cm^{-1} and 4000 cm^{-1} with 2 cm^{-1} resolution.

Biological characterisation

The biological potentials of compounds were assessed from *in vitro* measurements of their fraction bound to plasma proteins coefficient (F_{bp}), determination of blood cell/whole blood partition coefficient (F_{cb}), membrane adsorption coefficient (F_{ad}), and interaction with specific anti-MDMA antibody. These biological methods were described elsewhere (12).

Determination of F_{bp}

200 µL of plasma spiked with the studied compound (0.1 nmol L^{-1}) was incubated in a water bath at 37 °C for 15 min and placed in an ultrafiltration cell. After centrifugation in an SM24 centrifuge (Dupont Instruments; Wilmington, DE, USA) at 1978 g for 20 min, the ultrafiltrate was analysed using HPLC. The unbound to protein fraction (F_{up}) was determined by calculating the ratio of the concentration in ultrafiltrate and the initial compound concentration in plasma. The fraction bound to plasma proteins was deducted from the following equation: F_{bp}=100 % -F_{up}.

Determination of F_{cb}

20 µL of physiological solution of each compound at concentration of 1 mmol L^{-1} was added to 2 mL fresh blood taken on EDTA tripotassium salt (K$_3$EDTA). After incubation for 15 min at 37 °C, the blood sample was centrifuged (at 2000 g for 15 min) and 200 µL plasma were taken to determine the concentration in the ultrafiltrate (see determining F_{bp} above). Calculations were performed using the following equation:

$$F_{cb}= [C_{bt}-(C_{uf}/F_{up})]/[C_{bt} h]$$

The free fraction, which is neither bound to blood cells nor to proteins, was determined using the following equation:

$$F_{fr}= (1-F_{cb})(1-F_{bp}),$$

where F_{cb} is the blood cell/whole blood partition coefficient; C_{bt} the concentration in total blood (0.01 mmol L^{-1}); C_{uf} the concentration in ultrafiltrate; F_{up} the unbound-to-protein fraction coefficient; h is haematocrit; F_{bp} binding to plasma proteins coefficient, and F_{fr} the free fraction coefficient.

Determination of F_{ad}

200 µL of physiological solution of each compound (1 µmol L^{-1}) were centrifuged through permeable anisotropic membrane (Amicon) at 1978 g for 20 min (SM24 centrifuge, Dupont Instruments; Wilmington, DE, USA) and the ultrafiltrate was analysed using HPLC. The coefficient of membrane adsorption was estimated using the following equation:

$$F_{ad}= [C_{is}-C_{su}]/C_{is},$$

where F_{ad} is the membrane adsorption coefficient; C_{is} the concentration in initial physiological solution; and C_{su} the concentration in the ultrafiltrate.

Interaction with specific anti-MDMA antibody

Physiological solution of each compound (0.01 mmol L^{-1}) was analysed using an automated FPIA system (Abbott AxSYM®, USA).

RESULTS AND DISCUSSION

In this study, we have described the synthesis route starting from piperonal and ending with 3,4-methylenedioxyamphetamine (MDA) (11). We have also reported the synthesis of 3,4 methylendioxyphenyl-2-nitropropane, which is not considered precursor of MDMA. We used 3,4 methylendioxyphenyl-2-nitropropane to study its interaction with specific anti-MDMA antibody.

MDNP and MDA were obtained with high chemical purity. The synthesis reactions were followed by HPLC determination of retention times of the obtained products. Piperonal, MDA and MDNP exhibited high lipophilicity (Figure 1). The retention
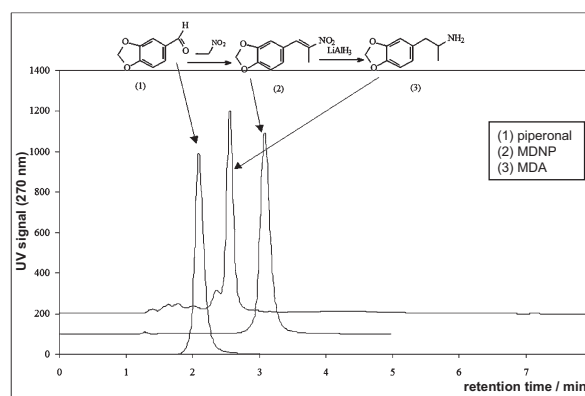

Figure 1 *HPLC patterns of piperonal and synthesised MDNP and MDA*

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times of piperonal, MDA, and MDNP were 2 min, 2.5 min, and 3.1 min, respectively, and correlated to their lipophilicity (13).

IR and NMR spectroscopies showed a clear profile of piperonal, MDNP, and MDA. NMR detected the following signals:

^{1}H NMR: d=1.22 (d, J=6.2 Hz, 3 H, CH_3), 2.71 (dd, J=13.4 and 8.8 Hz, ^{1}H, CH_2), 3.07 (dd, J=13.4 and 5.2 Hz, ^{1}H, CH_2), 3.43 (m, ^{1}H, CH), 6.10 (s, 2 H, CH_2), 6.80 (dd, J=7.8 and 1.3 Hz, ^{1}H, C_4H), 6.97 (d, J=1.3 Hz, ^{1}H, C_6H), 6.97 (d, J=7.8 Hz, ^{1}H, C_3H).

^{13}C NMR: 17.6 (CH_3), 39.7 (CH_2), 48.2 (CH), 101.0 (CH_2 dioxole), 108.4 (C_3H), 109.6 (C_6H), 122.5 (C_4H), 130.6 (C_5), 146.1 (C_1), 147.5 (C_2).

IR (Figure 2) revealed signals related to benzene ring (absorption at 1603 cm^{-1}, 1505 cm^{-1}, 1680 cm^{-1}, and 1489 cm^{-1}), to ether oxide bridge at 1489 cm^{-1}, and to MDMA derivatives.

For piperonal, the band located at 1677 cm^{-1} revealed the presence of aldehyde, for MDNP the band at 1510 cm^{-1} indicated a nitro compound in position *alpha* of an unsaturated bond, and MDA was characterised by bands at 3358 cm^{-1} and 1634 cm^{-1} pointing to amines, and by the disappearance of the nitro signals.

Figure 2 shows the vibrational modes of piperonal, MDNP, and MDA plotted in the range of 250 cm^{-1} to 1750 cm^{-1}. While piperonal and MDNP showed high quality Raman spectra (Figure 3), MDA showed a spectrum with a rather large background, possibly due to a fluorescence in the system at either 632 nm or 541 nm (Figure 4). Milhazes et al. (9) obtained MDA spectrum at 541 nm.

Table 1 lists the biological characteristics of the ecstasy derivatives and impurities in the final product. Of solid impurities found, a neurotoxic compound of Al^{3+} is of particular interest (4). Save for F_{bp}, all other biological parameters seem to be proportional to their retention time determined by HPLC and to their lipophilicity. Retention times for piperonal was 2 min, for MDA and 2.5 min, and for MDNP 3.1 min and corresponded to F_{ad} and F_{bp}.

Table 1 shows that the percent of free compounds in plasma is less than 40 % of total blood concentration: F_{fr}=23 % for piperonal, F_{fr}=27 % for MDA, and F_{fr}=38 % for MDNP.

FPIA antibody recognised the aminoethane group close to the substituted benzene ring, but did not detect the two major precursors of MDMA, that is, MDNP and piperonal (Figure 5). This limitation must be taken into consideration, as detection of all ecstasy
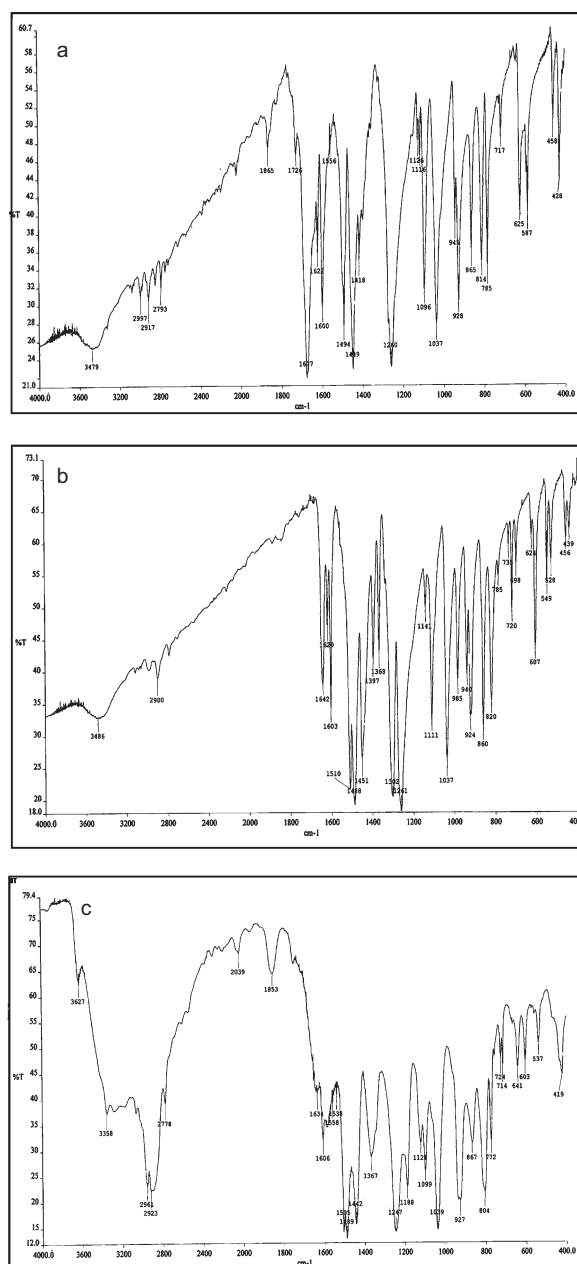

Figure 2 *Infrared spectra of (a) piperonal, (b) MDNP, and (c) MDA*

derivatives is required to optimise performance of detection methods.

CONCLUSION

This study has detected solid impurities and by-products in the synthesised products: MDA, unreacted piperonal, MDNP, and aluminium. While HPLC, Raman, and IR spectroscopy identified ecstasy derivatives, FPIA did not detect MDNP and piperonal.
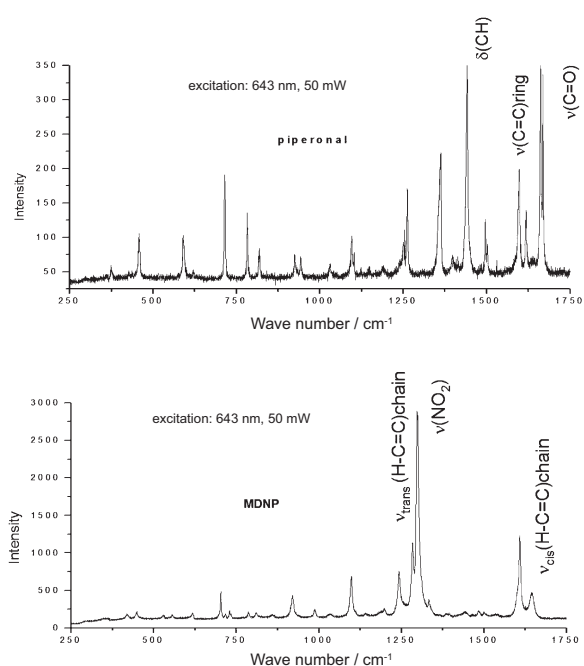
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Figure 4 *Raman spectrum of MDA*

Figure 3 *Raman spectra of piperonal and MDNP*
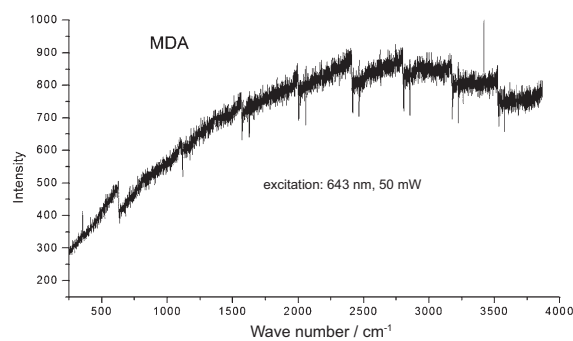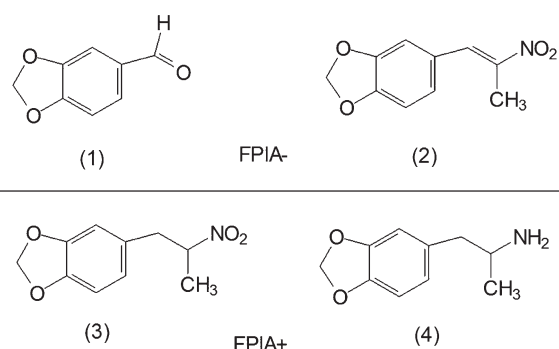

Figure 5 *Molecules tested in vitro with fluorescence polarization immunoassay (FPIA). (1) piperonal; (2) MDNP; (3) 3,4-methylendioxyphenyl-2-nitropropane; and (4) MDA.*

Raman spectroscopy has turned out to be a particularly interesting alternative technique for characterising ecstasy pills, as it can determine stereoisomeric forms of compound such as *cis*-MDNP and *trans*-MDNP at 1650 cm^{-1} and 1300 cm^{-1}, respectively.

Our findings call for further study of Raman spectroscopy to better characterise other drug derivatives.

Acknowledgements

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Table 1 *Biological characteristics of ecstasy derivatives and impurities in the final product*

	F_{bp} / %	F_{cb} / %	F_{fr} / %	F_{ad} / %	Derivatives and impurities	FPIA
Piperonal	63	38	23	47		Negative
MDNP	88	68	38	90	Piperonal[(s)],Toluene[(l)], NH$_4$Ac[(s)],EtNO$_2$[(l)]	Negative
MDA	71	30	27	63	MDNP [(s)] , THF [(l)], Al^{3+} [(s)]	Positive

(l) liquid; (s) solid
MDNP: 3,4-methylendioxyphenyl-2-nitropropene; MDA: 3,4-methylenedioxyamphetamine; FPIA: fluorescence polarization immunoassay; F_{bp}: blood cell/whole blood partition coefficient; F_{fr}: free fraction coefficient; F_{cb}: binding to plasmatic proteins coefficient; F_{ad}: membrane adsorption coefficient; THF: tetrahydrofuran; NH$_4$Ac: ammonium acetate; EtNO$_2$: nitroethane

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Sažetak

TOKSIKOLOŠKE METODE OTKRIVANJA OPOJNIH DROGA U TRAGOVIMA: KROMATOGRAFSKA, SPEKTROSKOPSKA I BIOLOŠKA KARAKTERIZACIJA DERIVATA ECSTASYJA

Analize često otkriju neujednačenost sastava tableta ecstasyja od čistoga 3,4-metilendioksimetamfetamina (MDMA) do mješavina njegovih derivata, amfetamina i drugih neutvrđenih tvari. Stoga je za kvalitetnu toksikološku analizu potreban uvid u sve korake sinteze MDMA, s obzirom na to da se ondje vjerojatno kriju izvori nečistoće (prekursori, katalizatori). Cilj ovog ispitivanja bio je sintetizirati derivate MDMA te napraviti njihovu kemijsko-fizikalnu i biološku *in vitro* karakterizaciju.

3,4-metilendioksifenil-2-nitropropen (MDNP) dobiven je kondenzacijom piperonala u suvišku nitroetana uz dodatak amonijeva acetata. Njegovom redukcijom s pomoću LiAlH$_3$ dobiven je 3,4-metilendioksiamfetamin (MDA). Svi spojevi iz pojedinih koraka sinteze karakterizirani su s pomoću tekućinske kromatografije visoke djelotvornosti (HPLC) i spektroskopskih tehnika [Ramanove spektroskopije, nuklearne magnetske rezonancije (NMR-a) te infracrvene spektroskopije (IR-a)]. Usto je ocijenjen i njihov biološki učinak *in vitro* mjerenjem (i) koeficijenta raspodjele krvna stanica/puna krv, (ii) vezanja za bjelančevine u plazmi (Fbp) te (iii) adsorpcije na membranu. Kemijska je struktura utvrđena s pomoću fluorescentnoga polarizacijskog imunokemijskog testa (FPIA). Analiza je u konačnim proizvodima utvrdila prisutnost krutih nečistoća, napose spojeva neurotoksičnog aluminija (Al^{3+}). FPIA je prepoznao aminoetansku skupinu blizu supstituiranoga benzenskog prstena, ali ne i dva glavna prekursora za MDMA: MDNP i piperonal. Posebno je zanimljiva Ramanova spektroskopija budući da (i) pruža privlačnu alternativu za karakterizaciju sastava tableta ecstasyja te (ii) može otkriti stereoizomerne *cis*/*trans*-oblike spoja poput *cis*-MDNP-a odnosno *trans*-MDNP-a, čiji se signal vidi na 1650 cm^{-1} odnosno 1300 cm^{-1}.

KLJUČNE RIJEČI: *IR, MDA, MDMA, MDNP, NMR, Ramanova spektroskopija, toksikologija*

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