The bioactivity of 2,5-dimethoxy-4-ethylthiophenethylamine (2C-T-2) and its detection in rat urine by capillary electrophoresis combined with an on-line sample concentration technique

Yu-Chih Chiua, Shiu-Huey Choub, Ju-Tsung Liuc, Cheng-Huang Linab,∗

a Department of Chemistry, National Taiwan Normal University, 88 Sec. 4, Tingchow Road, Taipei, Taiwan
b Department of Life Science, Fu-Jen University, 510 Chung Cheng Road, Hsinchuang, Taipei, Hsien 24205, Taiwan
c Forensic Science Center, Command of the Army Force of Military Police, Department of Defense, Taipei, Taiwan

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Abstract

The bioactivity of 2,5-dimethoxy-4-ethylthiophenethylamine (2C-T-2) on nitric oxide (NO) production and the proliferation of spleen and thymus lymphocytes to mitogen stimulation in mice are reported for the first time. NO production by T and B lymphocytes in spleen and T cells in the thymus of mice decreased after the oral administration of 2C-T-2. This indicates that 2C-T-2 intake may perturb both neural and immune activity since a decrease in NO production is indicative of a weakened defense function. 2C-T-2 (the parent drug) in rat urine samples was detected by means of capillary electrophoresis/UV absorbance combined with an on-line sample concentration technique. When the CZE and MEKC modes were employed, the detection limit was found to be 4.5 and 5.0 ng/mL (at a 92.1% confidence level); whereas when on-line sample concentration methods, including stacking and sweeping-micellar electrophoretic chromatography were used, the detection limits were improved to 19.2 and 9.1 ng/mL, respectively. In an analysis of some actual samples from animal experiments, three male rats were administered 20 mg/kg of body weight of 2C-T-2 by intra-peritoneal injection. The first- and second-day urine fractions were collected after the administration, for use in the analysis. As a result, 2.9 μg/mL and 0.25 μg/mL of 2C-T-2, respectively, were detected after ingestion of the doses.

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1. Introduction

β-Phenethylamine is a natural biochemical found in both plants and animals. The increased availability of β-phenethylamine derivatives in the illicit market represents a serious social problem. 2,5-Dimethoxy-4-ethylthiophenethylamine (2C-T-2), a ring-substituted phenethylamine, was first synthesized by Shulgin et al., the synthetic procedures were also reported in their monograph [1]. Although the dosages (12–25 mg) of 2C-T-2 were generally mentioned in this publication, little information is available concerning its bioactivity, detection and metabolic fate. Nitric oxide (NO) has been identified as an important molecule that is involved in the regulation of biological activities in the vascular, neural, and immune system [2]. It has been reported that psychostimulants have immunomodulatory effects [3,4]. In this work, the bioactivity of 2C-T-2 was investigated by NO production and the proliferation of spleen and thymus lymphocytes in response to mitogen stimulation in mice. In order to understand the effects of 2C-T-2 bioactivity in animals, a detailed knowledge of the metabolic pathways associated with 2C-T-2 in the animals is required. GC/MS remains the official method of analysis and is useful for the examining the metabolic fate of a compound such as 2C-T-2. However, it is necessary to derivatize phenethylamines prior...
to their injection into a GC system and these processes are
time consuming. Hence, a rapid and complementary method
would be highly desirable. Capillary electrophoresis (CE)
represents a rapidly growing separation technique that might
well meet this need, since it is now well established and is
a widely used analytical method in many fields, such as bio-
science, pharmaceutical, environmental studies, food science
and forensic research. A series of reports on on-line sample
concentration techniques recently appeared, concerning the
so called “stacking” and “sweeping” technique [5–16]. A
dramatic increase in sensitivity can be obtained using these
techniques. In our previous studies, four metabolites of
2C-T-2 were detected and the metabolic pathway in the rat
was outlined, based on a gas chromatography–selected ion
monitoring–mass spectrometry (GC–SIM–MS) method for the
analysis of in vivo urine samples from rats [17]. In this
study, we report on a simple and specific method for the
detection of 2C-T-2 in urine samples using UV absorption
combined with on-line sample concentration techniques. The
extraction procedures used for the urine samples and several
electrophoretic parameters, including buffer conditions,
SDS concentration, and the injection length required for
the separation were optimized. These data are reported
herein.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used were of analytical grade. Ethyl ac-
etate, methanol, diethylether, 1,4-dimethoxybenzene, chloro-
sulfonic acid, zinc, sulfuric acid, ethyl bromide, potassium
hydroxide, N-methylformamide, nitromethane, ammonium
acetate anhydrous, sodium dodecyl sulfate (SDS), sodium
tetraborate, methanol and phosphoric acid were all pur-
chased from Yakuri Pure Chemicals Co. Ltd. (Osaka, Japan)
and J.T. Baker (NJ, USA), respectively. The complete culture media used for all
assays consisted of RPMI-1640 or Dulbecco’s modified eagle
medium (DMEM) supplemented with l-glutamine (2 mM),
penicillin (100 U/mL), streptomycin (100 μg/mL), Hepes
(10 mM) (Gibco, Life Technologies, USA) and 10% fetal
bovine serum (HyClone, USA). The mitogens Concanavalin
A (Con A), lipopolysaccharide (LPS) from Escherichia
coli, heparin (100 U/mL) and MTT (3-[4,5-dimethylthiazol-
zy]) 2,5-diphenyltetrazolium bromide) were purchased from Sigma (St. Louis, MO, USA). Thymocytes and splenocytes
were collected after removal of cell debris and
clumps. Splenocytes and thymocytes were enumerated and
then diluted to 5 × 10⁶/mL with complete culture medium.

2.2. Animals

A BALB/c strain of inbred male mice (10–12 weeks) or
Wistar strain rats (220–250 g) were purchased from Labo-
ratory animal center in National Taiwan University College
of Medicine (Taipei, Taiwan). Upon arrival, the mice and
rats were caged in a separate colony room where a 12-h
light–dark cycle was maintained through artificial illumina-
tion. The mice received free access to both food and water
throughout the experiment, except as noted, and a 2-week
acclimation period prior to experimental manipulation. The
animals were also handled on a bidaily basis to prevent the
hyperactivity that occurs when they are left untouched for a
prolonged period.

2.3. Drug administration and urine sampling

For urine collection, Wistar rats were housed in a
metabolic study cage one week before the 2C-T-2 adminis-
tration. Blank urine samples were collected 24 h before drug
administration. An intra-peritoneal injection (10 mg/kg) was
then performed. All urine samples were collected over a 24 h
period and stored at −20 °C until used. Nitric oxide and
the proliferation analysis of immune tissues, mice were ran-
domly assigned to a control or an experimental group (n =
3–4 mice/group). 2C-T-2 (10 mg/kg) was fed to the test an-
imals by gavage. Control mice received a sterile phosphate
buffer saline solution (PBS). The experimental groups of an-
imals were fed a solution of 2C-T-2. The 2C-T-2 fed group of
animals were sacrificed two hours after gavage. Each subject
was anesthesized with carbon oxide gas. One ml of blood was
harvested by a heart puncture. After the blood was collected,
the mice were rapidly sacrificed by cervical dislocation and
immune tissues were then harvested.

2.4. Spleen and thymus sample preparation

Tissue and blood were collected as described previously
[18]. After sacrifice, the animal was placed on its back. Whole
blood (1 mL) was collected from the heart of each animal by
using a 22-gauge needle into a heparinized syringe. Serum
was separated and transferred 1.5 mL microcentrifuged tubes
and stored at −20 °C until used. Each spleen and thymus
was dissociated into a single-cell suspension by grinding the
spleen between the frosted ends of two glass slides. A single-
cell suspension was collected after removal of cell debris and
clumps. Splenocytes and thymocytes were enumerated and
then diluted to 5 × 10⁶/mL with complete culture medium.

2.5. Mitogen proliferation assay

A mitogen proliferation assay was performed with thymo-
cytes and splenocytes to assess lymphocyte proliferation as

Nitric oxide (NO) assays were prepared in flat-bottomed tis-
sue culture plates (Nalge Nunc, USA).
dition of excess K$_2$CO$_3$. The free bases were then extracted into 2 mL of a hexane/CH$_2$Cl$_2$ (3:1, v/v) solution by stirring the suspension for 1 min. After centrifugation, the upper layer (1.5 mL) was collected and evaporated to dryness. The residue was dissolved in 50 μL of matrix (water/acetonitrile, 85/15) for the subsequent sweeping-MEKC separation.

2.8. Apparatus and instrumental techniques

The apparatus used is identical to that described previously [17]. Briefly, a nuclear magnetic resonance spectrometer (NMR; Bruker, Avance 500 MHz), an infrared (IR; Perkin-Elmer, FT-IR Paragon 500) spectrometer and a gas chromatograph (GC 6890 Hewlett-Packard, Avondale, PA, USA) equipped with a mass spectrometer (Hewlett-Packard 5973 mass selective detector) were used for the identification of the products produced during the different synthesis processes and for the final product. The CE set-up was fabricated in-house [21] where a high-voltage power supply (model RR30-2R, 0–30 kV, 0–2 mA, reversible, Gamma, FL, USA) was used to drive the electrophoresis and 50 or 75 μm i.d. fused silica capillary columns (J&W Scientific, CA, USA) were used for the separation. A UV detector (CE-971 UV; Jasco, Japan) was used for the determination of analytes and the wavelength used for the detection was 290 nm. A laboratory-built operational amplifier was used for signal amplification, which contained a single integrated LM1458 circuit chip. The analog signal was converted to a digital signal by an A/D converter (ADAM-4012 module, Advantech Co. Ltd., Taiwan). Electropherograms were collected with a data acquisition system connected to a personal computer.

2.9. Statistical analysis

A Student’s $t$-test was used to assess the effect of 2C-T-2 on NO production in T cell proliferation in activated lymphocytes. In the experiment to characterize the effects of 2C-T-2, an ANOVA comparison was used to assess significance of the differences among the experimental and control groups. The level of significance for the $t$-test was set at a value of $P$ less than or equal to 0.05.

3. Results and discussion

3.1. Synthesis

The synthetic procedures for 2C-T-2 have been previously described in the literature [1]. Following these processes, the final product was identified by NMR, IR and verified by GC–MS. As a result, specific fragments derived from the product were elucidated with high degrees of certainty. The chemical structure of 2C-T-2 is shown in the inset in Fig. 3 (frame A).

3.2. Bioactivity

In the proliferation assays, nitric oxide after mitogen stimulation was measured as an indicator of functional potential. NO production was measured by converting NO to the more easily detectable form, nitrite. The results of the nitrite assays from supernatants of thymocytes stimulated with Con A or LPS, and thymocytes stimulated with Con A and 2C-T-2, were expressed as the mean nitrite concentration of triplicate wells and analyzed as repeated-measures ANOVA using the no-nitrogen control and all concentrations of a given mitogen as controls. The level of significance for the $t$-test was set at a value of $P$ less than or equal to 0.05.

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Fig. 1. The effect of 2C-T on nitric oxide production by splenocytes and thymocytes incubated with the optimal concentration of mitogens Con A and LPS as indicated in (A)–(C). Nitrite was measured as an indicator of nitric oxide production in the supernatant of mitogen-induced blastogenesis cultures. Mice were orally administered either (0.9%) saline or 2C-T (10 mg/kg). The results are expressed as the mean (± S.E.) MTT optical absorbency at 570 nm; n = 3/group.

In contrast to the increase in NO production which then induces a decline in neural chemical events and behavioral changes [23,24]. In contrast to the increase in NO production which then induces a decline in neural chemical events and behavioral changes [23,24]. In contrast to the increase in NO production which then induces a decline in neural chemical events and behavioral changes [23,24]. In contrast to the increase in NO production which then induces a decline in neural chemical events and behavioral changes [23,24]. In contrast to the increase in NO production which then induces a decline in neural chemical events and behavioral changes [23,24]. In contrast to the increase in NO production which then induces a decline in neural chemical events and behavioral changes [23,24].
Fig. 3. CE electropherograms (frames, A–D) obtained using different CE separation modes (CZE, MEKC, stacking and sweeping-MEKC, respectively). CE conditions: (A) buffer consisting of 75 mM H$_3$PO$_4$, 12.6 mM Na$_2$HPO$_4$ and 13.25 mM NaH$_2$PO$_4$ in a mixed water–acetonitrile solution (95:5, v/v); applied voltage 15 kV; currents, 40–50 $\mu$A; (B) buffer consisting of 25 mM SDS, 3 mM HBO$_3$ and 25 mM NaHPO$_4$·7H$_2$O in a mixed water–acetonitrile solution (70:30, v/v); applied voltage 15 kV; current, 70–80 $\mu$A; (C) buffer consisting of 75 mM H$_3$PO$_4$, 12.6 mM Na$_2$HPO$_4$ and 13.25 mM NaH$_2$PO$_4$ in a mixed water–acetonitrile solution (95:5, v/v); matrix, 0.75 mM H$_3$PO$_4$, 0.126 mM Na$_2$HPO$_4$ and 0.1325 mM NaH$_2$PO$_4$ in a mixed water–acetonitrile solution (95:5, v/v); applied voltage 15 kV; currents, 10–50 $\mu$A; (D) buffer consisting of 120 mM SDS and 30 mM H$_3$PO$_4$ in a mixed water–acetonitrile–methanol solution (65:15:20, v/v/v); matrix, 30 mM H$_3$PO$_4$ in a mixed water–acetonitrile solution (85:15, v/v); applied voltage $-20$ kV; currents, $-20$ to $-14$ $\mu$A. UV absorption, 290 nm.

CZE and MEKC modes (normal injection methods), a ∼50- and ∼600-fold improvement was realized when the on-line sample concentration techniques (stacking, sweeping-MEKC, respectively) were applied. Table 1 summarizes these results as well as the equation used in constructing the calibration curves, the coefficient of variation values, the limit of detection and the plate numbers for 2C-T-2 for the CZE, MEKC, stacking and sweeping-MEKC modes, respectively, for the above experiment. When CZE and MEKC were used, the detection limits were 4.5 and 5.0 $\mu$g/mL (at the 92.1% confidence level); whereas when on-line sample concentration techniques were used, including stacking and sweeping-MEKC, the detection limits were improved to 19.2 and 9.1 ng/mL, respectively. To investigate the effects of injection length and the corresponding signal intensity when the stacking and sweeping-MEKC techniques were used under exactly the same experimental conditions, various column lengths (stacking: 12, 16, 22, 33 cm; sweeping-MEKC: 12, 18, 24, 30 and 36 cm) for the sample solution were injected into the capillary and the results were plotted, as shown in Fig. 4 (frames, A and B). Basically the signal intensity (peak area) increased with increasing injection length. The insets in frames A and B show the relationship between the length of the sample injection and plate number. Although reasonable plate numbers (∼10$^5$) can be obtained

<table>
<thead>
<tr>
<th>Method</th>
<th>CZE</th>
<th>MEKC</th>
<th>Stacking</th>
<th>Sweeping-MEKC</th>
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</thead>
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<td>Capillary i.d.</td>
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<td>75 μm i.d.</td>
<td>75 μm i.d.</td>
<td>50 μm i.d.</td>
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<tr>
<td>Effective/total length (cm)</td>
<td>7285</td>
<td>4780</td>
<td>7285</td>
<td>105/118</td>
</tr>
<tr>
<td>Injected length (cm)</td>
<td>0.3</td>
<td>0.3</td>
<td>16.4</td>
<td>30.3</td>
</tr>
<tr>
<td>Concentration ranges</td>
<td>$4.1 \times 10^{-4}$ to $4.1 \times 10^{-3}$ M</td>
<td>$4.1 \times 10^{-4}$ to $2.1 \times 10^{-3}$ M</td>
<td>$2.1 \times 10^{-6}$ to $6.5 \times 10^{-4}$ M</td>
<td>$8.0 \times 10^{-5}$ M (912 ng/mL)</td>
</tr>
<tr>
<td>Equation of line</td>
<td>$y = 0.049$</td>
<td>$y = 0.020$</td>
<td>$y = 0.066$</td>
<td>$y = 0.088$</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>$r^2 = 0.994$</td>
<td>$r^2 = 0.993$</td>
<td>$r^2 = 0.999$</td>
<td>$r^2 = 0.999$</td>
</tr>
<tr>
<td>LOD (at the 92.1% CL)</td>
<td>2.0 $\times 10^{-5}$ M (4.5 μg/mL)</td>
<td>2.1 $\times 10^{-5}$ M (5.0 μg/mL)</td>
<td>3.8 $\times 10^{-3}$ M (912 ng/mL)</td>
<td>3.3 $\times 10^{-2}$ to $1.1 \times 10^6$</td>
</tr>
<tr>
<td>Plate number (N)</td>
<td>$1.5 \times 10^3$ to $1.2 \times 10^4$</td>
<td>$7.2 \times 10^3$ to $5.3 \times 10^4$</td>
<td>$1.2 \times 10^2$ to $6.6 \times 10^4$</td>
<td>$3.3 \times 10^4$ to $1.1 \times 10^6$</td>
</tr>
</tbody>
</table>

* UV absorption at 290 nm.
Fig. 5. (A) Electropherogram a, urine extract (after ingestion of 2C-T-2 for 24 h) from the 2C-T-2 dosed mouse; electropherogram a, spiked with 1.0 g/mL of 2C-T-2 before extraction as a single-point standard addition. The inset shows the CE electropherogram of a control mouse (blank urine sample extract). (B) Electropherograms for the second day of urine collection (electropherograms a and b, before and after 1.0 g/mL of 2C-T-2 standard addition).

In all of these conditions, the sweeping-MEKC mode provides better detection sensitivity and better separation efficiency. For this reason, the sweeping-MEKC mode was used in subsequent experiments.

Fig. 5 shows typical CE electropherograms of urine sample extracts from the 2C-T-2 test mouse (frame A, the first day of urine collection after the ingestion of the 2C-T-2; frame B, the second day of urine collection) by applying the sweeping-MEKC technique. Herein, the background solution used in the sweeping-MEKC mode consisted of 120 mM SDS and 30 mM H₃PO₄ in a mixed water–acetonitrile–methanol solution (65:15:20, v/v/v). The sample extracts were dissolved in the matrix solution which consisted of 30 mM H₃PO₄ in a mixed water–acetonitrile solution (85:15, v/v) resulting a non-micelle buffer. Hydrodynamic injection was achieved by raising the reservoir 45 cm relative to the exit reservoir for 1200 s. Using this procedure, 24 cm column lengths of sample solution were injected into the capillary. Electropherograms a and b (in frame A) show the separation results obtained before and after a single-point standard addition (1 µg/mL of 2C-T-2) for the first day urine sample extracts, respectively. For convenience, the indicated peak (marked as (*) in frames A–D) was selected for comparison. As shown in electropherogram a, the 2C-T-2 parent drug (arrow mark) appears to the right of the indicated peak. In order to determine if this peak (arrow mark) is 2C-T-2 or not, we spiked 1 µg/mL of a 2C-T-2 standard (before extraction) and the peak indeed increased, as shown in the electropherogram b. We assigned this peak as the 2C-T-2 parent drug and its concentration was determined to be 2.9 g/mL. In order to confirm that this peak appeared only for the 2C-T-2 test mouse, a urine extract from a control mouse (without 2C-T-2 dosage) was examined. No peak was detected having this migration time, as shown in the inset in frame A (broken arrow). Using the same procedure, frame B shows CE electropherograms (c, before spiking; d, 1 µg/mL of 2C-T-2 spiking) for the second day urine sample extracts from the same 2C-T-2 test mouse. A minor peak is visible in the electropherogram c (arrow peak). We assigned this peak as the 2C-T-2 parent drug and its concentration was determined to be 0.25 µg/mL. Thus, we conclude that the 2C-T-2 parent drug can be detected in urine samples of this test mouse at levels of 2.9 and 0.25 µg/mL, respectively, from the first day and the second day urine collections. Using the same experimental procedures, including
the liquid-liquid extraction procedures, with the CE running buffer and the sweeping-MEKC mode, a spiked urine sample from a human volunteer was also tested. The spiked 2C-T-2 (1 µg/mL) peak was clearly and easily detected (as shown in the inset). Fig. 6 show the results obtained from sample extracts from the male volunteer (electropherogram a, blank urine extract; electropherogram b, after spiking with 1 µg/mL 2C-T-2 std.) by means of the sweeping-MEKC method. The CE conditions were the same as those described above. For a comparison of the separation efficiency, an indicated peak (marked as (*) in electropherograms a and b) was selected for evaluation. In electropherogram a, the arrow marked indicated the expected migration time for 2C-T-2, but it did not show up in the blank urine sample extract. This indicates that no peaks exist in the extract that could overlap with the 2C-T-2 analyte. Using a standard addition method, the results were compared and the findings show that a peak (marked spiking peak in electropherogram b) clearly appeared. Thus, by applying this approach, to humans, if the relationship between 2C-T-2 concentration in the urine and various dosing times can be determined, it would be of use in determining the quantity of 2C-T-2 ingested, after a period of days, using the method developed here. Furthermore, a blood sample extract from the 2C-T-2 test mouse (6 h after ingestion) also was examined. However, the 2C-T-2 parent drug was not detectable, either by applying the sweeping-MEKC technique or by GC/MS.

4. Conclusions

The bioactivity of 2C-T-2 by an assays for NO production and the proliferation of spleen and thymus lymphocytes to mitogen stimulation was investigated. The decline in NO production is indicative of a weakened defense function and this may be the reason for why 2C-T-2 is classified as a drug of abuse. The detection of 2C-T-2 in urine was successful using capillary electrophoresis/UV absorbance combined with an on-line sample concentration technique. This method provides a sensitive, accurate, rapid, simple, and economic complementary method to GC/MS for use in forensic and clinical analysis, as well as in related work.

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