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A microwave-assisted fluorescent labeling method for the separation and detection of amphetamine-like designer drugs by capillary electrophoresis

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ABSTRACT

A microwave-assisted fluorescence labeling method for use in CE–LIF (capillary electrophoresis–laser induced fluorescence) is described. Six amphetamine-like designer drugs, namely, *o*-, *m*-, *p*-chloro- and *o*-, *m*-, *p*-fluoro-amphetamine derivatives, were synthesized and used as model compounds. FITC (fluorescein isothiocyanate isomer I) and a blue-laser were used as the fluorescent labeling reagent and excitation source, respectively. When a microwave oven was used, the reaction was complete within ~5 min, while the classical method required at least 20 h (usually, an overnight reaction). A mimic oral fluid sample was obtained by spiking oral fluid from a volunteer with the six standards, and after liquid–liquid extraction and microwave-derivatization, it was possible to process the analytes by CE–LIF within a period of ~10 min; the wavelength of the blue-laser used was 473 nm. For comparison, data obtained using classical methods, including CZE–UV (capillary zone electrophoresis–UV absorbance detection), sweeping-MEKC–UV (micellar electrokinetic chromatography–UV absorbance detection) and LC–Q-TOFMS (liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry) are also reported.

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1. Introduction

CE–LIF (capillary electrophoresis–laser induced fluorescence) is one of the most popular and powerful techniques available for the analysis of biological samples and illicit drugs [1–5]. When an argon ion laser (488 nm) is used as the excitation source, FITC (fluorescein isothiocyanate isomer I) is a nearly perfect fluorescent labeling reagent. Instead of an expensive argon ion laser, the use of a blue LED [6–9] or blue diode laser has recently become popular [10,11]. FITC is yellow-orange in color with an absorption maximum at 495 nm and, upon excitation, it emits a yellow-green color with an emission maximum at 525 nm. However, the overall labeling process in time consuming, when FITC is used as the fluorescent labeling reagent. For this reason, while CE–LIF is a highly sensitive technique, it is not routinely used as a complementary method for rapid screening.

The increasing availability of amphetamine-like designer drugs on the illicit market has become a serious social problem [12–15]. However, even though drug abuse is a serious problem [16,17], little information is available concerning methods for the extraction and detection of these types of compounds. From the point of view of screening and confirming the numbers of clandestine tablets that are probably available on the illicit market, a more rapid screening method would be highly desirable. As of this writing, GC–MS (gas chromatograph–mass spectrometry) [18–21] and LC–MS (liquid chromatograph–mass spectrometry) [22–25] continue to be the officially prescribed methods. However, while data obtained by mass spectrometry constitutes legally acceptable evidence, secondary evidence acquired by means of a different technique would be highly desirable.

In Taiwan in 2011, *p*-chloro-amphetamine was permanently placed in Schedule III and, because of this, methods for its analysis, as well as derivatives thereof, are needed. In this study, we selected *o*-, *m*-, *p*-chloro- and *o*-, *m*-, *p*-fluoro-amphetamines as model compounds. A microwave oven was used to increase the rate of the fluorescent labeling reaction. Results obtained by classical methods, including CE–UV and LC–MS are also reported and the results are compared to data acquired using this method. The analysis of the six designer drugs in a human oral fluid sample was optimized, the detection limits and precision of these methods are discussed and complete data regarding their characteristics are reported herein.

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2. Materials and methods

2.1. Reagents

o-, *m*-, *p*-Chloro- and *o*-, *m*-, *p*-fluoro-amphetamines were generously donated by the Military Police Command, Forensic Science Center, Taiwan. Following the synthesis, the final products were verified by Ann and Alexander Shulgin in their book entitled TiHKAL (Phenethylamines I Have Known and Loved) [26]. Following the synthesis-steps, the final products were verified by NMR, IR and GC/MS. All other chemicals were of analytical grade and were obtained from commercial sources.

2.2. Apparatus

In-house fabricated CE–UV and CE–LIF systems were used this study. The CE set-ups were identical to those used in our previous studies and are abbreviated herein [8,27–29]. The blue laser used (100 mW/473 nm) was purchased from Sinhuang Technology Co., Ltd., Taiwan; a microwave oven (SAMPO, RE-081M1) was obtained from a local shop. The LC–Q-TOFMS system consisted of a Waters 1525 binary HPLC pump, a reversed phase column (Cosmosil 5C18-MS, 5 μm, 25 cm × 4.6 mm i.d.; Nacalai Tesque, Kyoto, Japan) and a mass spectrometer (Micromass Q-TOF).

2.3. Oral fluid extraction procedure

Extraction procedures were referenced and modified from the literature [30–32]. In a typical experiment, a 500-μL aliquot of an oral fluid sample obtained from a human volunteer was placed in a test tube and the solution spiked with the 6 amphetamine derivatives (0.5 mg each). The pH values of the spiked oral fluid samples were adjusted to 9.0 by adding ammonium carbonate (250 μL of 0.2 M). Following this, 1.3 mL of an ethyl acetate–hexane solution (4/1, v/v) were added, followed by gentle mixing for 1 min and centrifugation for 5 min. The upper layer was then collected and evaporated to dryness. The residue was dissolved in 500 μL of methanol and filtered through a 0.45 μm nylon filter for use in the subsequent experiments.

2.4. Fluorescence derivatization

To 150 μL of a solution containing 50 μL of 10 mM sodium tetraborate, 100 μL of a solution of an amphetamine-like designer drug (20 μg/mL each in H_2O or oral fluid) was added. The reaction was initiated by the addition of 50 μL of a FITC solution (500 μg/mL in acetone) to give concentrations of [amphetamine-like designer drugs] equal to 10 μg/mL and [FITC] equal to 125 μg/mL, respectively. The reaction solution was allowed to stand at room temperature in the dark for 24 h or placed in a microwave oven (power, 800 W) for 5 min, at which time, the reaction was complete. The resulting derivative was diluted 1/20 with water, and then used directly for the CE separation.

2.5. Separation conditions for the CZE/UV

The UV absorption maxima for the chloro- and fluoro-amphetamines are at 266 and 262 nm; after fluorescent labeling the observed fluorescence emission maximum was in the range of 543–548 nm. This indicates that these compounds can be detected and identified using either CE or HPLC separation, based on their fluorescence properties.

3. Results and discussion

Fig. 1 shows typical electropherograms for these analytes for various separation modes (frame A, CZE/UV; frame B, sweeping-MEKC/UV modes, respectively). The concentrations of the six samples are 100 μg/mL in frame A. The optimal buffer for their separation consisted of 7.5 mM β-CD, phosphate (NaH_2PO_4, 50 mM/Na_2HPO_4, 100 mM) in a mixture of acetonitrile–methanol–water (12.5:17.5:70, v/v/v) pH 3.1. The applied voltage and current were +15 kV and ~64 μA; total/effective length of the capillary used was 50/41 cm. The order of migration of the six analytes basically followed the mass per charge. As can be seen from the electropherogram, the order of migration is: *o*-fluoro-, *m*-fluoro-, *p*-fluoro-, followed by the *o*-, *m*-, *p*-chloro-derivatives. The inset in Fig. 1A shows the results when no β-CD is used. In this case, the separation was not complete because of the similarity of the chemical structures of the six analytes. Numerous attempts were made to separate and identify them using LC–Q-TOFMS (liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry). The findings show that, when a gradient elution (A, 0.1% formic acid aqueous solution/pH 2.5; B, methanol) was used, the six analytes can be nearly completely separated, except for the *m*- and *p*-chloro-amphetamines, which were incompletely separated. To achieve complete separation of these 2 compounds, a chiral column would be needed. The order of

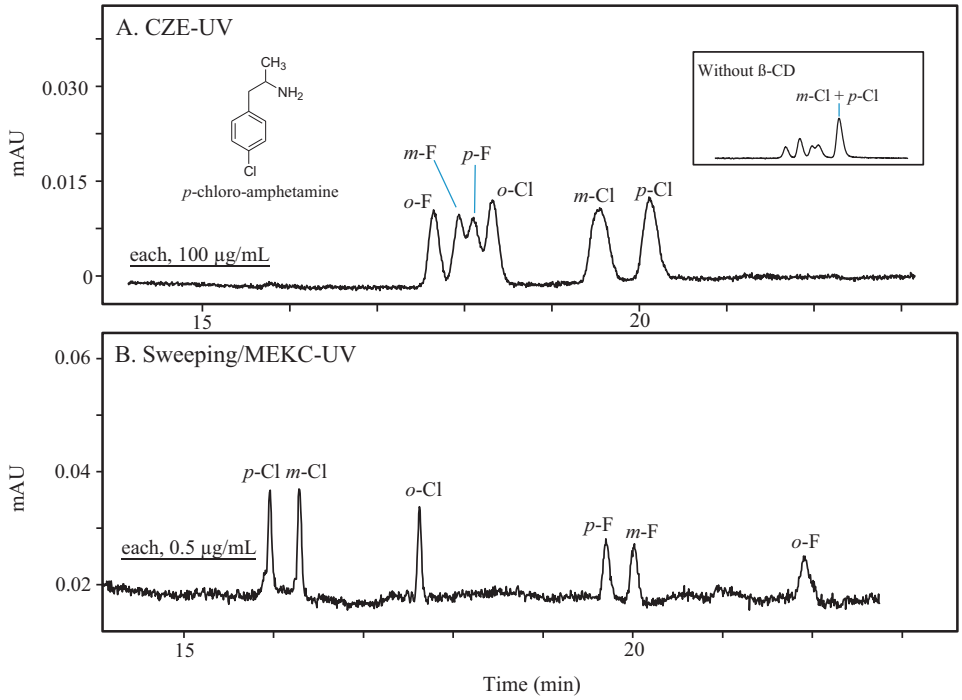

Fig. 1. UV absorption detection. Electropherograms of a mixture of the six amphetamine-like designer drugs by various separation modes (frame A, CZE–UV; frame B, sweeping-MEKC mode, respectively). CE conditions: A, a solution of acetonitrile–methanol–water (12.5:17.5:70, v/v), containing phosphate (NaH_2PO_4, 50 mM/Na_2HPO_4, 100 mM) and 7.5 mM β-CD (inset, non-β-CD solution), pH 3.1. The voltage used was +15 kV (current, ~64 μA); B, a background electrolyte consisted of acetonitrile–methanol–water (5:30:65, v/v), containing 50 mM NaH_2PO_4, 75 mM of SDS. Sample matrix, 50 mM NaH_2PO_4; sample injected length, 14 cm. The voltage used was −22 kV (current, ~−40 μA); total/effective length of the capillary used was 75/61 cm, respectively.

migration is the same as that for CZE and the LODs were determined to be 0.5 and 1.0 μg/mL for the standard solution and the oral fluid extract, respectively.

In the case of the chloro- and fluoro-amphetamine derivatives, intersystem crossing occurs more efficiently by stabilization of the triplet state by intramolecular hydrogen bonding, and as a result, the absorption and fluorescence intensities are weaker, which results in poor detection limits. For such a weakly fluorescent compound, an online sample concentration technique or fluorescence derivatization is recommended. Fig. 1B shows the results obtained when the sweeping-MEKC mode was used. The optimized background electrolyte consisted of 75 mM SDS, and 50 mM NaH_2PO_4 in a solution of acetonitrile–methanol–water (5:30:65, v/v) pH 2.13. The applied voltage and current were −22 kV and ∼−40 μA; total/effective length of the capillary (50 μm, i.d.) used was 75/61 cm (sample injection length, 14 cm). Under these conditions, the LOD can be improved to 0.5 μg/mL. However, when the sweeping-MEKC method was used, the effects of sample injection length and the corresponding signal intensity need to be further investigated. Choosing between a longer sample injection length (higher sensitivity) and better separation efficiency (lower sensitivity) is an important, but difficult choice. Furthermore, because of the numerous unknown matrix effects associated with an authentic oral fluid sample, many unidentified peaks (due to all UV absorbers) appear when the sweeping-MEKC technique was used. In contrast to this, when the CE–LIF method was used, a simple separation buffer can be used and the LOD can be dramatically improved, even when an online sample concentration technique is not used. As shown in the inset in Fig. 2B, the LODs for the six amphetamine-like designer drugs can be improved to 0.05 μg/mL after fluorescent labeling with FITC. Furthermore, FITC only reacts with primary (as in the six analytes) and secondary amines. This characteristic can be useful in terms of avoiding interference by extraneous components in the saliva sample. However, as mentioned above, the classical fluorescent labeling method is time consuming in terms of completing the reaction. In order to shorten the time required for achieving complete fluorescent labeling, a microwave oven was used to increase the rate of the fluorescence derivatization reaction. Fig. 2 shows typical microwave-assisted CE–LIF electropherograms of an extract of human oral fluids. The optimized separation buffer is simple, consisting of only a borate buffer (tetraborate, 10 mM) containing 75 mM SDS (pH 9.5). The applied voltage and current were +17 kV and 24 μA; total/effective length of the capillary (75 μm, i.d.) used was 62/52 cm. Frame (A) shows the results obtained for extracts of a clean oral fluid sample. As can be seen, although some peaks, due to unknown matrix effects, are present, the results are much simpler than the results obtained by LC–Q-TOFMS (data not shown). Frame (B) shows the results for an oral fluid sample obtained by spiking a clean oral fluid sample collected from a human volunteer with the six amphetamine designer drugs (spiked concentration: 0.5 μg/mL, each). As can be seen, even though some background peaks are still present, all of the six analytes can clearly be identified. Thus, this approach can be applied to the rapid and sensitive detection and identification of amphetamine derivatives and related designer drugs in saliva obtained from suspects.

In order to determine the time required to complete the microwave-assisted fluorescence derivatization and the corresponding signal intensity, several different reaction times (0–48 h) were examined. As shown in Fig. 3, when a microwave oven was used, the derivatization was complete within 5 min. However, when the time in the oven was increased to 10 min, the fluorescence intensity decreased. The dashed line shows the intensity changes of FITC. In contrast to this, using the classical procedure, more than 20 h were needed to reach the maximum fluorescence intensity, although the intensity was almost two-fold that for the microwave method. Fig. 4 shows another example (2C-series drugs) obtained using the microwave-assisted fluorescence derivatization procedure. In a previous study, we reported on optimizing the separation and online sample concentration of the 2C-series of phenethylamine designer drugs with CE-fluorescence detection. In that study, LED-induced fluorescence detection was examined by derivatizing the compounds by reacting them with

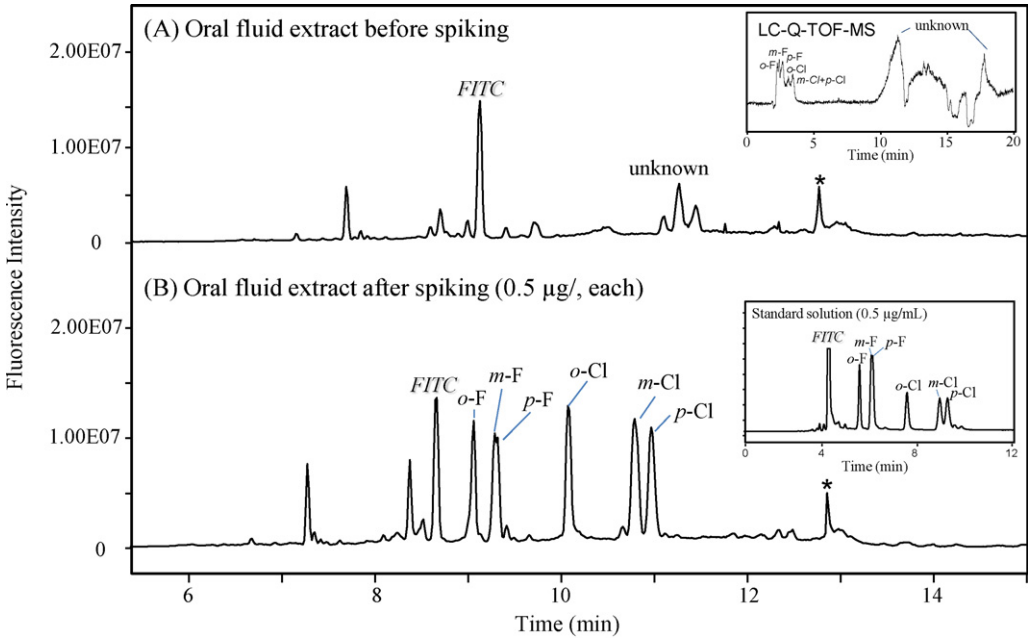

Fig. 2. Microwave-assisted fluorescence derivatization method. Electropherograms of a mixture of the six amphetamine-like designer drugs for various samples: frame A, salvia blank sample; frame B, spiked sample; insets, the result obtained by LC–Q-TOF-MS method and the electropherogram of the six standards, respectively. CE conditions: A, an aqueous solution containing 10 mM tetraborate and 75 mM of SDS, pH 9.5. The voltage used was +17 kV (current, ∼24 μA).

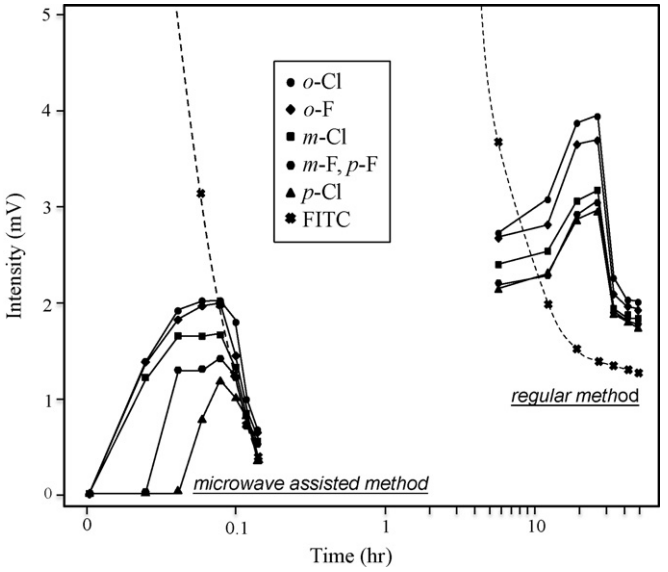

Fig. 3. Comparison of the signal intensity and the time used for fluorescence derivatization (left, the microwave-assisted method; right, a regular method) in CE–LIF. Test sample: *o*-fluoro-, *m*-fluoro-, *p*-fluoro-, *o*-chloro-, *m*-chloro- and *p*-chloro-amphetamine (concentration level, 1 μg/mL, each).

FITC and a blue-LED was used as the fluorescence excitation source. For comparison, we repeated these analyses using the microwave-assisted method. Similar to the amphetamines derivatives examined in this study, the derivatization was complete within 5 min, and the fluorescence intensity was better than the results obtained by the previous method. Thus, we conclude that the microwave-assisted method is very useful and that it does not affect the properties of FITC or the samples.

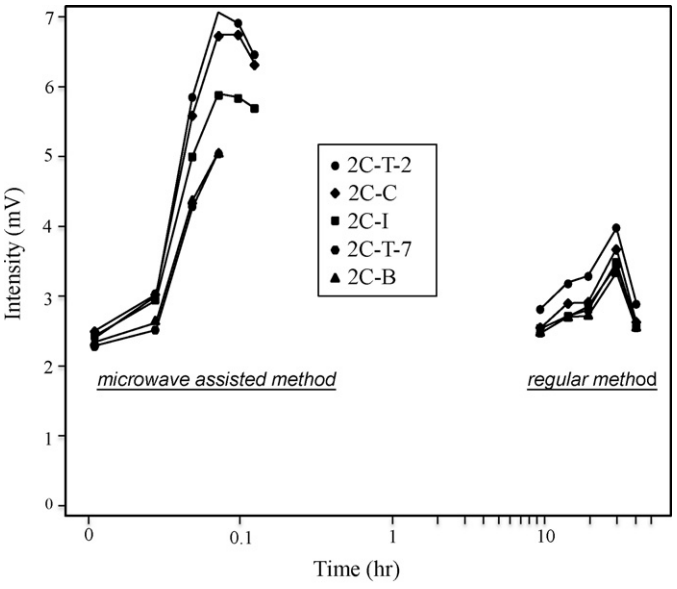

Fig. 4. Comparison of the signal intensity and time used for fluorescence derivatization (left, the microwave-assisted method; right, a regular method) in CE–LIF. Test sample: 2,5-dimethoxy-4-ethylthio-phenethylamine (2C-T-2), 2,5-dimethoxy-4-(*n*)-propylthiophenethylamine (2C-T-7), 4-chloro-2,5-dimethoxyphenethylamine (2C-C), 4-bromo-2,5-dimethoxy-phenethylamine (2C-B), 2,5-dimethoxy-4-iodo-phenethylamine (2C-I) (concentration level, 1 μg/mL, each).

4. Conclusions

In this study, we describe the development of a novel microwave-assisted CE–LIF method. It is suitable for use in the rapid screening of drugs, since it has a high degree of sensitivity and the operating procedure is simple and economical. This method constitutes a sensitive, simple, and economically complementary method for either rapid screening or officially prescribed methods (such as GC/MS or LC/MS) for use in forensic and clinical analysis, as well as in related work.

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