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PAPER

A rapid method for the in-field analysis of amphetamines employing the Agilent Bioanalyzer

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This paper reports the first analysis of small molecules on the Agilent bio-analyser. The Bioanalyzer is a commercial lab-on-a-chip instrument designed for the analysis of DNA and proteins. We demonstrate that the instrument is suitable for analyses beyond its design specifications. Amphetamine, methamphetamine and pseudoephedrine were separated with a 50 mM borate and 50 mM sodium dodecyl sulfate (SDS) buffer at pH 9.66. The analytes were derivatised with fluorescein isothiocyanate (FITC) in 3 minutes with a heating block set at 90 °C, reducing the typical time of 12 hours required for amine-labelling. Analytes were detected by LED-induced fluorescence ($\lambda_{ex} = 525$ nm and $\lambda_{em} = 470$ nm). Furthermore, five amphetamine analogues were baseline separated within 1 minute. An average limit of detection of 0.6 μg mL^{-1} and limit of quantification of 2.2 μg mL^{-1} were obtained for all analytes. These rapid analyses in conjunction with a fast and reliable derivatisation method with FITC demonstrate its potential use for the in-field analysis of samples of forensic significance.

Introduction

Amphetamines are a group of psycho-stimulants which may cause anxiety, hallucination, violence and antisocial behaviour.[1] The relatively simple methods of manufacture contribute to the popularity and availability of amphetamines in Asia, North America and Oceania. Global seizures of amphetamine type stimulants (ATS) have risen significantly over the last two decades. Since 1990, a 10-fold increase in amphetamine seizures has been reported, with over 50 tonnes seized in 2007.[2] This significant increase has generated a need for a fast and highly automated method for the in-field identification of ATS.

The analysis of clandestine preparations of amphetamines is usually performed in the laboratory using gas chromatography-mass spectrometry (GC-MS),[3–6] liquid chromatography (LC)[7–9] and capillary electrophoresis (CE).[10–12] Capillary electrophoresis has been reported for the analysis of ATS in various matrices including blood, urine and oral fluids.[13–19]

In-field, presumptive colour tests and ion mobility spectroscopy (IMS) are the most commonly employed techniques for the detection of ATS. However, both of these methods lack sensitivity and frequently result in false positives from interferences.[20,21] Lab-on-a-chip (LOC) devices perform extremely fast, cost-effective separations and are also portable.[22] LOC is an attractive alternative for the rapid analysis of forensic samples including DNA, explosives and illicit drugs.[23–26]

Fluorescence is the most common method of choice for analyte detection in LOC applications. Fluorescein isothiocyanate (FITC) is a common fluorescent labelling reagent for amines with excellent quantum efficiency and high stability of its derivatives.[27] The reaction of FITC with proteins and amino acids was first reported in 1969 by Maeda *et al.*[27] and has been used to label amphetamines and other amine-containing compounds.[28,29] Typical derivatisation times with FITC have been up to 12 hours, with recent attempts to shorten the reaction time. Dominguez-Vega *et al.*[30] described an accelerated FITC derivatisation of the amino acid ornithine. The reaction time was reduced from 16 hours to 10 minutes by the use of an ultrasonic probe. Zhou *et al.*[31] reported a microwave-assisted derivatisation procedure for amino acids with FITC which took approximately 3 minutes. The signal intensity associated with the microwave procedure was improved in comparison to the procedures at room temperature and 100 °C in a water-bath.[31]

Ramseier *et al.*[28] reported the separation and detection of amphetamine analogues in human urine. The separation run time was 10 minutes, involving a 24 hour labelling process with FITC. This study utilised a laboratory constructed device and an argon ion laser with excitation at 488 nm. Alternative amino-reactive fluorophores such as *o*-phthaldialdehyde (OPA), dichlorotrizinylaminofluorescein (DTAF) and nitro benzofurazane derivatives have also been used to label amphetamines.[32–35]

The Bioanalyzer 2100 is a commercial LOC system, fitted with both LED-Induced Fluorescence (LED-IF) and Laser Induced

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Table 1 Structures of the target amphetamines and amphetamine analogues

Name	Abbreviation	Structure
2-(4-Methoxyphenyl)ethylamine	2-4-MPEA	
2-Bromo-*N*-methylbenzenemethanamine	BMBA	
2-Methoxy-*N*-methylaniline	2-MMA	
5-Aminomethyl-7-chloro-1,3-benzodioxol-hydrochloride	AM-C-BD	
2-Methoxyphenethylamine	MPEA	
Pseudoephedrine	PSE	
Amphetamine	AMP	
Methamphetamine	MA	

Fluorescence (LIF) detection systems. This device is compact and portable, with a mass of 10 kg. The Bioanalyzer is robust and can withstand operating temperatures of between 5 and 40 °C.[36]

This paper demonstrates the separation and detection of Amphetamine (AMP), Methamphetamine (MA) and Pseudoephedrine (PSE) and selected analogues with the Bioanalyzer. The ATS were rapidly derivatised with FITC by a temperature-accelerated procedure using a dry heating block. The derivatised ATS and analogues were separated by micellar electrokinetic chromatography (MEKC) employing a standard commercial glass microchip.

Experimental

Apparatus

All experiments were performed on an Agilent 2100 Bioanalyzer using the Agilent 2100 Expert software (Agilent technologies, Waldbronn, Germany). Detection was by LED-IF (λ_{ex} 525 nm, λ_{em} 470 nm). All separations were performed using standard DNA 500 microchips obtained from Agilent Technologies (Forest Hill, Australia). The chips were fabricated from soda lime glass. The microchannels which interconnect 12 sample wells have a depth of 10 μm and width of 50 μm. The separation channel length was 15 mm.

Chemicals

Sodium hydroxide, sodium dodecyl sulfate (SDS), nile blue chloride, sodium tetraborate and FITC (>90%) were purchased from Sigma Aldrich (Sydney, Australia). Individual standards of 2-(4-methoxyphenyl)ethylamine, 2-bromo-*N*-methylbenzenemethanamine, 2-methoxy-*N*-methylaniline, 5-aminomethyl-7-chloro-1,3-benzodioxol-hydrochloride and 2-methoxyphenethylamine were also purchased from Sigma Aldrich (Sydney, Australia) as concentrated solids or solutions (in methanol). Pseudoephedrine hydrochloride, dexamphetamine sulfate and methamphetamine hydrochloride were obtained from the National Measurement Institute (Sydney, Australia). Table 1 lists the chemical structures of the target compounds.

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Electrolyte preparation

Electrolytes and sample stock solutions were prepared in ultra--pure water (Arium 611, Sartorius AG, Goettingen, Germany). The separation electrolyte consisted of 50 mM sodium tetraborate buffer at a pH of 9.66 with the addition of 50 mM SDS. The electrolyte was mixed, sonicated for 5 minutes and filtered through a 0.20 μm syringe filter prior to injection (Milipore, Billerica, MA, USA). All electrolyte solutions and stock solutions of amphetamine analogues were kept in the refrigerator at 4 °C and fresh solutions were prepared daily. A concentration of 1 μM nile blue dye was diluted in the running buffer and primed through the microchannels prior to analysis for laser focussing.

Analyte preparation

FITC stock solution. A 10 mM stock solution of FITC was prepared in analytical reagent grade acetone and stored in a plastic bottle wrapped in aluminium foil at −18 °C.

Amphetamine analogues and ATS standards. Individual stock solutions of all analogues and ATS standards were prepared *via* dilution in ultra-pure water to a final concentration of 1000 μg mL^{-1}. Derivatisation of stock solutions was performed with FITC; 100 μL of analogue was diluted in 100 μL of 10 mM sodium tetraborate buffer (pH 9.66) and labelled for 3 minutes at 90 °C after the addition of 100 μL of 10 mM FITC. A dry heater block (Ratek-DBH30D—www.ratek.com.au) was employed. Labelling was carried out in 1.7 mL graduated microtubes wrapped in aluminium foil with the lid closed. Following derivatisation, the labelled analogues were diluted to 10 μg mL^{-1} in the running buffer for analysis.

Results and discussion

Labelling with FITC

FITC has been widely used as a derivatisation reagent due to its excellent quantum efficiency, high molar absorptivity and stability of products.[28,36] Experiments were performed to simplify the FITC derivatisation procedure using a dry heating block. Initially, experiments were conducted using a water-bath as performed by Zhou *et al.*,[31] however a dry heating block was chosen due to its reduced complexity. Whilst the heating block used is portable (*W* 283 × *D* 265 × *H* 100 mm, 4.5 kg and 60 samples capacity), a wide range of more compact heating blocks are also available which would further enhance the portability.

Fig. 1 summarises the influence of temperature on the peak height for derivatised PSE with a 5 minute reaction time. Error bars were estimated from the standard deviation for the PSE fluorescence intensity from triplicate derivatisation of PSE at each temperature and triplicate injections on a single chip. *i.e.* a total of 3 chips were used for each temperature experiment. The response increased linearly with increasing temperature. As expected there was also a concomitant decrease in the signal of FITC. Temperatures beyond 100 °C were not investigated as the reaction mixture boiled. The optimal temperature was chosen as 90 °C; a compromise between sensitivity, high PSE and low FITC fluorescence intensities and reproducibility.

Fig. 2 illustrates the influence of reaction time upon the signal intensity at the optimum temperature of 90 °C. As before, error bars were calculated from the standard deviations of triplicate time experiments and triplicate injections on separate chips. The results show a sharp increase in the signal intensity of PSE up until 180 seconds where it reached a plateau. As expected, the FITC signal intensity gradually decreased over time, again confirming the consumption of FITC. The optimum time was 180 seconds determined by maximum PSE signal intensity, minimum FITC signal intensity and lowest fluorescence signal variability.

The dry heating block procedure was compared against microwave and temperature assisted derivatisation methods described by Dominguez-Vega and Zhou *et al.*[30,31] The separation profile of a 24 hour standard procedure at room temperature is shown in Fig. 3a. The 3 minute microwave programme consisted of alternating 30 second periods of microwave irradiation.

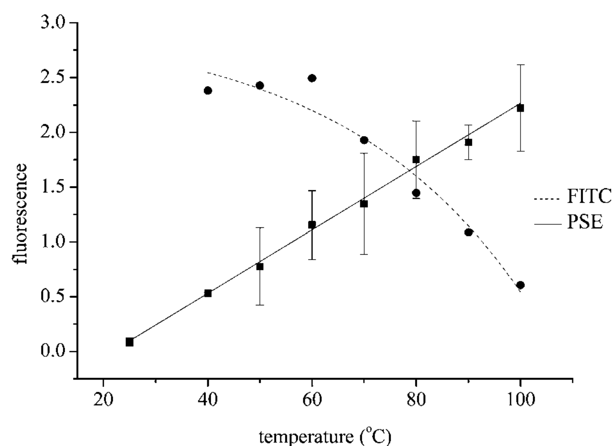

Fig. 1 The influence of temperature on the fluorescence intensity of derivatised PSE (5 minute reaction time). Error bars represent the corresponding standard deviations of the peak heights for each temperature experiment (n = 3), and triplicate injection of derivatised PSE. Each experiment was performed on a separate chip.

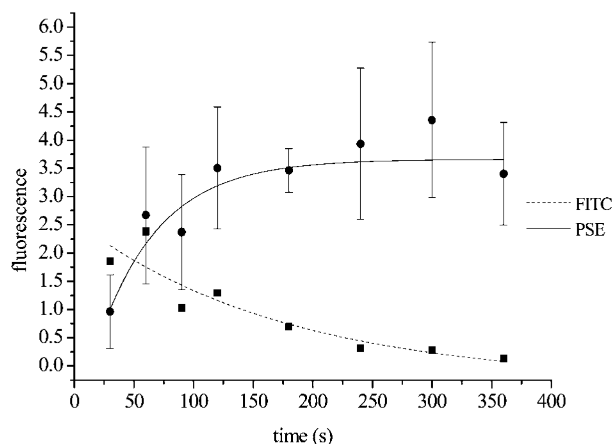

Fig. 2 The influence of time on the fluorescence intensity of derivatised PSE for a reaction performed at 90 °C. Error bars represent the corresponding standard deviations of the derivatisation time periods for each temperature experiment (n = 3), and triplicate injection of derivatised PSE. Each experiment was performed on a separate chip.

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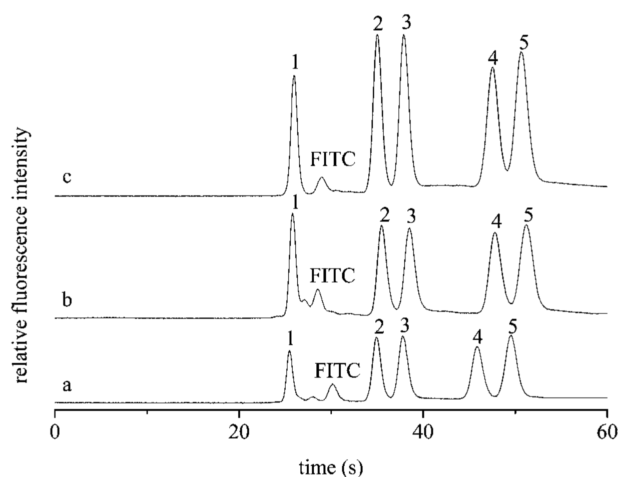

Fig. 3 Separation profiles of the fluorescence intensity for amphetamine analogues (20 μg mL^{-1}): (a) 24 hours at room temperature, (b) 3 minute microwave program—30 second periods alternating between microwave application and resting, (c) 3 minutes at 90 °C. Separation conditions as in Fig. 4. (1) 2-4-MPEA, (2) BMBA, (3) 2-MMA, (4) AM-C-BD and (5) MPEA.

A microwave irradiation power of 250 watts was applied to a 300 μL mixture contained in a closed 1.7 mL centrifuge vial. With the application of microwave irradiation for 3 minutes (Fig. 3b), a similar fluorescence intensity was observed. When the reaction was performed using a dry heating block set at 90 °C, the fluorescence signal intensity was approximately doubled (Fig. 3c). The temperature-accelerated method was chosen for optimisation due to its simplicity and greater suitability for in-field analysis. A heating block was chosen due to its increased feasibility for laboratory and in-field analysis.

Electrolyte optimisation

Sodium tetraborate was chosen as a suitable buffer given the average pK_a for the amphetamine analogues was 8.7. Optimisation experiments considered electrolyte pH, and the concentration of borate and SDS. At lower pH, the peaks broadened, decreasing the efficiency of the separation. The separation efficiency increased with increasing borate and SDS concentration up to a maximum concentration of 50 mM of each. Therefore the best separation buffer comprised 50 mM sodium tetraborate and 50 mM SDS at pH 9.66. The influence of hydroxyethyl-cellulose, methanol and acetonitrile was also investigated with the objective of improving the separation by slowing down the electroosmotic flow (EOF). However, there was no significant enhancement of separation and the baseline noise increased.

MEKC separation of labelled analogues and ATS

Fig. 4 shows the separation of five amphetamine analogues employing 50 mM sodium tetraborate and 50 mM SDS. These analogues were chosen due to their physical and chemical similarity to AMP and MA, as well as to highlight the potential of the Bioanalyzer lab-on-a-chip device for the analysis of ATS and precursors.

Fig. 5 shows the separation of pseudoephedrine, amphetamine, and methamphetamine. These compounds were treated separately to simulate a typical clandestine laboratory analysis such as the

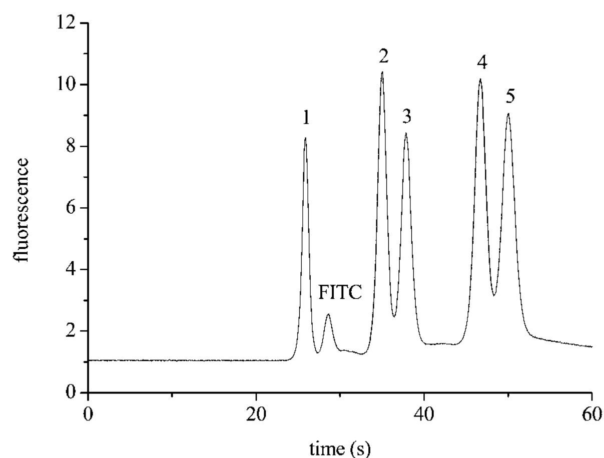

Fig. 4 Electropherogram of 5 amphetamine analogues (30 μg mL^{-1}) using LED-IF (λ_{em} 470, λ_{em} 525). Conditions: 50 mM SDS + 50 mM sodium tetraborate, pH 9.66; 25 °C; injection time 2 seconds; injection voltage 1.5 kV; separation voltage 1.5 kV; (1) 2-4-MPEA, (2) BMBA, (3) 2-MMA, (4) AM-C-BD and (5) MPEA.

rapid identification of the presence of amphetamine or methamphetamine and its potential synthesis route, *i.e.* the manufacture of methamphetamine from pseudoephedrine as a starting material. In both cases all compounds were resolved in less than 1 minute, with sufficient resolution for identification. The method was evaluated in terms of sensitivity and linearity for all amphetamine analogues. These results are summarised in Table 2.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated for each compound (n = 6) based on 3 times and 10 times the signal-to-noise ratio, respectively. Average LOD and LOQ values were 0.6 μg mL^{-1} and 2.2 μg mL^{-1}, respectively. This is consistent with detection limits of 0.5 μg mL^{-1} for 3,4-methylenedioxy methamphetamine (MDMA) for a CE-LIF method reported by Fang *et al.*[26]

The linearity of each analyte was determined in the concentration range of 1 to 50 μg mL^{-1} (n = 6), producing correlation co-efficients (R^2) that ranged from 0.960 to 0.995.

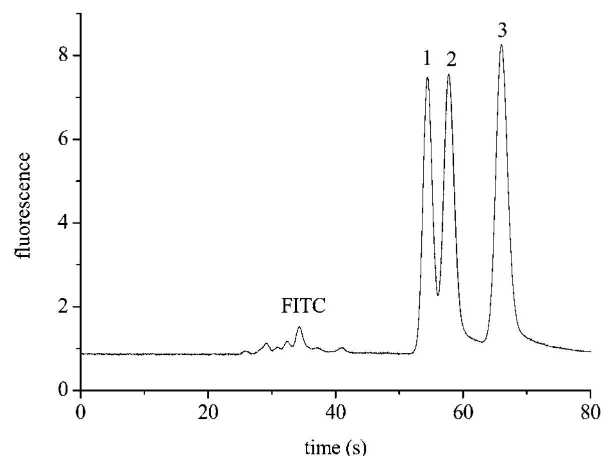

Fig. 5 Electropherogram showing the optimised separation of 3 ATS standards (30 μg mL^{-1}) using LED-IF (λ_{em} 470, λ_{em} 525). Conditions: 50 mM SDS + 50 mM sodium tetraborate, pH 9.66; 25 °C; injection time 2 seconds; injection voltage 1.5 kV; separation voltage 1.5 kV; (1) PSE, (2) AMP and (3) MA.

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Table 2 Analytical performance data for amphetamine analogues and ATS

Analyte	Sensitivity limits ($n = 6$, μg mL^{-1}) LOD[a]	LOQ[a]	Calibration R^2 ($n = 6$, 1–50 μg mL^{-1})
2-4-MPEA	0.6	2.0	0.988
BMBA	0.7	2.3	0.991
2-MMA	0.7	2.3	0.989
AM-C-BD	0.9	3.0	0.983
MPEA	0.8	2.8	0.965
PSE	0.5	1.8	0.995
AMP	0.4	1.5	0.990
MA	0.4	1.5	0.960

[a] LOD and LOQ calculations were determined using 3 times and 10 times the signal-to-noise ratio, respectively ($n = 6$).

Table 3 Separation and detection reproducibility for amphetamine analogues and ATS

Analyte	Average migration time ± standard deviation[a]/s	Adjusted migration times % RSD[a] Within-chip	Between-chip
2-4-MPEA	26 ± 0.5	1.9	2.8
BMBA	34.9 ± 0.6	1.8	2.7
2-MMA	37.7 ± 0.8	2.1	3.5
AM-C-BD	47.6 ± 2.1	4.4	4.9
MPEA	50.5 ± 1.9	3.7	4.7
PSE	55.3 ± 2.3	2.3	4.1
AMP	58.4 ± 2.1	2.1	3.6
MA	67.2 ± 3.2	2.5	4.8

[a] Calculated from repeated injections of a 30 μg mL^{-1} standard mixture.

The repeatability of the migration times was evaluated for each analogue (30 μg mL^{-1}) from repeat injections both within-chip ($n = 6$) and between-chip ($n = 12$). The corresponding relative standard deviations (RSDs) for all analytes ranged from 1.8 to 4.4 and 2.7 to 4.9, respectively (Table 3).

Conclusions

A fast and reliable method for the derivatisation of amphetamine-type stimulants with FITC and subsequent analysis *via* LED-IF using a portable lab-on-a-chip device was demonstrated.

The rapid derivatisation reduced the typical 12 hours derivatisation of the analytes to 3 minutes. The procedure employed a portable dry heating block, set at 90 °C for the labelling of amphetamines.

The separation of AMP, MA and PSE was complete within 1 minute. Limits of detection and limits of quantification were similar to reported CE-LIF methods ranging from 0.4 to 0.9 μg mL^{-1} and 1.5 to 3.0 μg mL^{-1}, respectively.

The derivatisation procedure with FITC was robust and reliable and was suitable for the in-field detection of amphetamines and other ATS.

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