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Immunological analysis of methamphetamine antibody and its use for the detection of methamphetamine by capillary electrophoresis with laser-induced fluorescence

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Abstract

An accurate, simple and rapid immunoassay is demonstrated for the detection of methamphetamine in urine by capillary electrophoresis (CE) with laser-induced fluorescence (LIF). An aminobutyl derivative of methamphetamine was conjugated with proteins, and used as an immunogen to produce antibodies for the assay. The methamphetamine derivative was also labeled with fluorescein isothiocyanate (FITC) to compete with free methamphetamine in the sample for the antibody binding site. Levels of free and antibody-bound FITC-labeled methamphetamine were monitored by performing CE–LIF using an untreated fused-silica column. This competitive immunoassay used antiserum instead of purified antibody or antibody fragment, yet was found to have good precision with a sensitivity of lower than 20 ng/ml. Various antibodies were also screened, and cross-reactivity of anti-MA antibody with methamphetamine analogues were also investigated. The results indicate that CE–LIF-based immunoassay is a powerful tool for the screening and characterization of antibody and may have possible applications in the detection of abused drugs in urine.

Keywords: Urine; Methamphetamine

1. Introduction

Methamphetamine (MA), a controlled drug, has potent sympathomimetic and stimulant effects on the central nervous system (CNS). MA abuse has become a serious problem, particularly in Asia. It is excreted rapidly in urine, and approximately 40% of the initial dose is eliminated in an unchanged form of MA within the first 24 h [1]. Various screening methods to test for MA in urine samples have been developed including those which use gas chromatography (GC) [2–4] or immunoassays [5–7]. GC–MS gives confirmatory results but the apparatus is expensive. It also requires great expertise and a considerable amount of time to prepare and analyze the sample. Immunoassay is often used as the initial screening method as it is relatively rapid and simple. However, to achieve better sensitivity and specificity, the selection and purification of the antibody and labeled tracer is a critical but often complicated step [8].

Capillary electrophoresis (CE) is a convenient technique for the analysis of various therapeutic drugs [9] or abused drugs [10], for which the

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separation of analytes is based on differences in their electrophoretic mobility. The molecules to be separated migrate in an electric field at velocities according to their molecular size and net charge. Besides being a powerful separation tool, CE has a number of favorable operating characteristics; for example, its instrumentation is small scale, high speed and easy to use. CE-based immunoassay has also been applied to some drugs [11–13] and biological hormones, such as insulin [14,15] and human growth hormone [16], using laser-induced fluorescence (LIF) detection. The application of CE to protein samples is more difficult due to the adsorption of sample components to the capillary wall, which affect the reproducibility of the assay. Various approaches to overcome the problems have been tried such as using a coated capillary column [12,15], micellar electrokinetic chromatography [17], or buffers of extreme pH or high salt. CE–LIF using purified antibody or antibody fragments has also been successfully performed on protein samples [1,12,15,16].

We report here a simple and rapid CE–LIF immunoassay that can be used to detect methamphetamine in urine with good precision and accuracy. This CE–LIF method uses antiserum, not purified antibody or antibody fragments, in an untreated fused-silica column and is shown to be an effective method to screen and characterize antibodies.

2. Experimental

2.1. Chemicals and reagents

MA, benzphetamine and amphetamine were obtained from the Korean National Institute of Health. Phenylpropanolamine, ephedrine and methyl ephedrine, the drugs used for the cross-reactivity study, were purchased from Sigma (St. Louis, MO, USA). *N*-(4-Bromobutyl)phthalimide, hydrazine hydrate, Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), 1-ethyl-3-(3-methyl-aminopropyl)carbodiimide HCl (EDC) and fluorescein isothiocyanate (FITC) were also purchased from Sigma. Bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH), used to prepare the MA immunogens, were purchased from Pierce (Rockford, IL, USA). Twenty-five chromatofolios AL TLC sheets (silica gel 60 F_{254}) were purchased from Merck (Darmstadt, Germany). CE capillary regenerator solution A (a less than one percent of sodium hydroxide solution) was purchased from Beckman (Fullerton, CA, USA) and the syringe filter (0.45 μm) used for the filtration of CE reagents was purchased from Whatman (Clifton, NJ, USA). All other chemicals used were of analytical grade, and the solutions were made in deionized water using Milli-Q water purification system (Millipore, Bedford, MA, USA). Urine from a healthy man who did not take any medicine for a week was used as blank urine and was also used to prepare of MA standard solution and control samples.

2.2. Apparatus

For CE–LIF application, a P/ACE 5000 system fitted with an argon-ion laser light source was used (Beckman, Fullerton, CA, USA). Excitation was at 488 nm and detection at 520 nm. CE separation was performed on an untreated fused-silica column (50 cm×75 μm I.D.) which was assembled in the P/ACE cartridge format.

2.3. Preparation of MA-immunogen and MA-antisera

To prepare MA-antisera, MA was derivatized to *N*-(4-aminobutyl)methamphetamine and further conjugated with BSA or KLH as described previously [7,8]. Three kinds of polyclonal antisera were developed by immunizing goats with the different MA conjugates. We used one kind of MA-KLH and two kinds of MA-BSA immunogens, one with a low molar ratio of MA to BSA [MA-BSA(L)] and the other with a high ratio [MA-BSA(H)], as reported previously [8].

2.4. Preparation of fluorescein-labeled MA tracer

N-(4-Aminobutyl)methamphetamine was labeled with fluorescein to compete with free MA in the sample for antibody binding sites. The purity of the FITC-labeled MA derivative (MA-FITC) was checked as described previously [18] and was further confirmed by CE.

2.5. Immunoassay

To construct the standard curve, a stock solution of MA·HCl in distilled water (1 mg/ml) was diluted with blank urine in concentrations of 0, 50, 100, 200, 500 and 1000 ng/ml. For the cross-reactivity study, the cross-reactant standards of benzphetamine, amphetamine, phenylpropanolamine, ephedrine and methylephedrine were also prepared with blank urine in concentrations of 200 ng/ml. Two kinds of control samples, one with low and the other with high concentrations of MA were prepared to test the accuracy and inter- and intra-precision of the immunoassay.

In small vials with 15-μl aliquots of antiserum, 50 μl of 3 n*M* MA-FITC and 135 μl of separation buffer were added. To each was added 2 μl of standard solution or sample. The antiserum having a high concentration of specific antibody was diluted appropriately with phosphate buffered saline (PBS) to yield maximum binding with the MA tracer, where approximately 60% of the total amount of tracer bound to the antibody in the antiserum at zero concentration of MA.

2.6. Electrophoretic conditions

Electrophoresis was performed at 25°C using 50 m*M* borate buffer (pH 8.7) containing 20 m*M* LiCl. The samples were pressure injected for 5 s at the negative electrode. The applied voltage was 25 kV and the current was 92 μA. Between runs, the column was prerinsed for 2 min with separation buffer and postrinsed for 2 min with sequential washing solutions of water, CE capillary regenerator solution A and water, respectively. The samples and reagents used for CE–LIF were filtered through the 0.45-μm syringe filter.

3. Results and discussion

The technique of using CE to study antibody–antigen reactions has been applied to various drugs, and its sensitivity was greatly improved with the use of a fluorescence labeled tracer [12–14]. It is very important to use pure tracer in order to obtain a sensitive immunoassay because an impure tracer is prone to increase nonspecific binding, which results in an elevated detection limit. The purity of MA-FITC was checked by CE–LIF (Fig. 1A). There was only a single MA-FITC peak, which indicated the absence of any other detectable impurity.

In our CE–LIF-based immunoassay system, the free FITC-labeled tracer was well separated from the antibody-bound fraction, and the response of antibody-bound tracer was dependent on the characteristics of each antiserum. We obtained three kinds of goat anti-MA antisera: anti-MA-BSA(L), anti-MA-BSA(H) and anti-MA-KLH. The amount of specific antibody in those antisera and in normal goat serum was examined (Fig. 1B–E). As the amount of specific antibody increased, the height of the free MA-FITC peak decreased. In the electropherogram of normal goat serum (Fig. 1B), no antibody-bound MA-FITC peak was detected, indicating the absence of any MA specific antibody. The antibody responses from the anti-MA-BSA(L) and anti-MA-BSA(H) antisera were too weak to use in the immunoassay (Fig. 1C,D). On the other hand, anti-MA-KLH antisera proved to have high amounts of MA-specific antibody, corroborated by the low peak for free MA-FITC (Fig. 1E).

Free MA-FITC migrated a little more slowly in the various antisera and normal goat serum samples than in the sample without serum. The migration time of free MA-FITC seems to be affected by the presence of blood proteins and other components. We found that the migration time for the tracer was constant throughout the assay for each antiserum, and that retard time with serum addition was restored when diluted serum was used.

Anti-MA-KLH antiserum was diluted five times with PBS to optimize the sensitivity of the MA immunoassay. Fig. 2 shows that the responses of free and bound MA-FITC tracers changed according to differing MA concentrations in the sample. Though the peaks of antibody-bound MA-FITC were broader than those of free MA-FITC, the change in area ratios of the two peaks was consistent with the MA concentration. We constructed the standard curve for MA by plotting the relative area ratios over the maximum area ratio (the area ratio of bound to free tracer at zero concentration of MA) against the MA concentrations without an internal standard. The standard curve constructed, the relative area ratio vs.

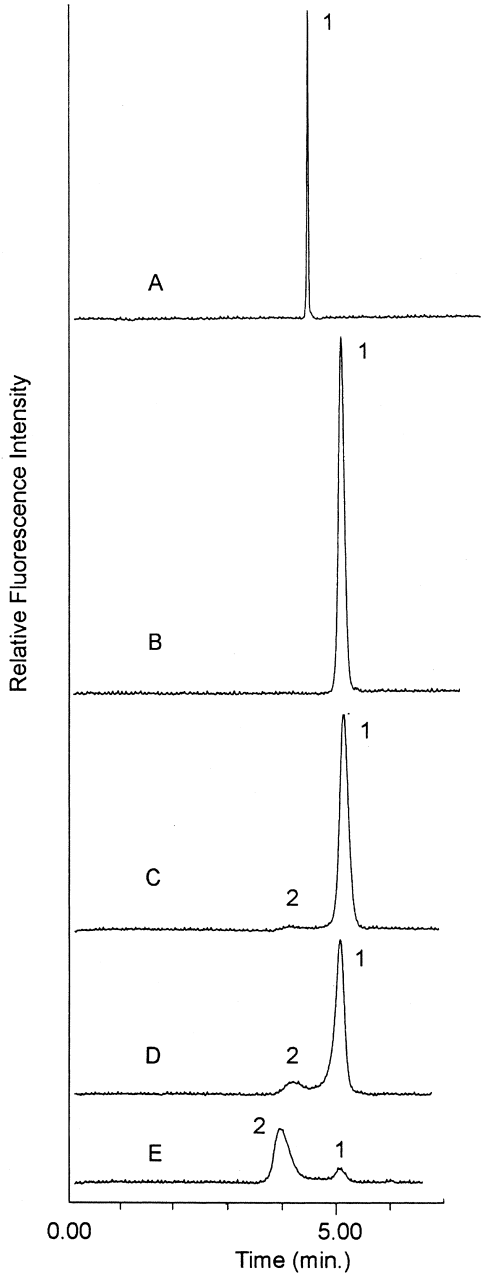

Fig. 1. CE–LIF of purified fluorecein-labeled methamphetamine (MA-FITC), and screening of various antisera. Conditions: untreated fused-silica capillary, 50 cm×75 μm I.D.; applied potential, 25 kV/92 μA; buffer, 50 m*M* borate, 20 m*M* LiCl at pH 8.7. Peaks: 1=free MA-FITC; 2=antibody-bound MA-FITC. Reaction mixtures were composed of MA-FITC (50 μl of 3 n*M*) and antiserum (15 μl) in 200 μl of separating buffer. (A) Without antiserum; (B) normal goat serum; (C) goat anti-MA-BSA(L) antiserum; (D) goat anti-MA-BSA(H) antiserum; (E) goat anti-MA-KLH antiserum.

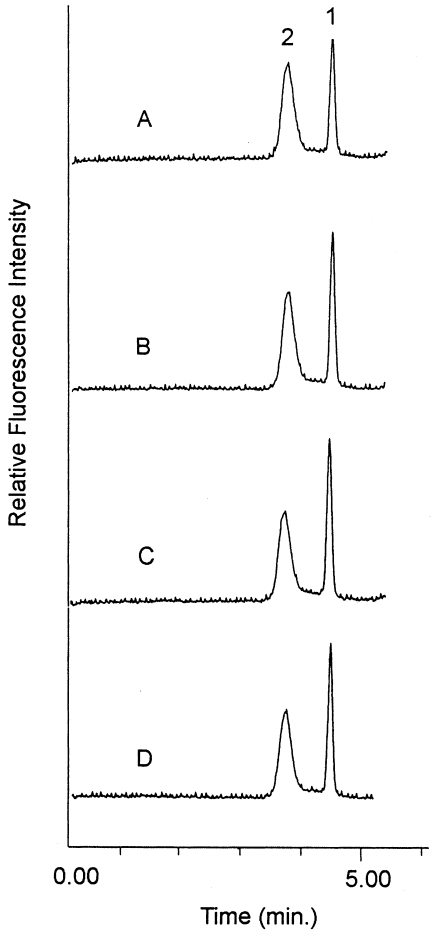

Fig. 2. CE–LIF immunoassay profiles with anti-MA-KLH antiserum (five times diluted). Conditions as in Fig. 1. Peaks: 1=free MA-FITC; 2=antibody-bound MA-FITC. (A) MA zero ng/ml; (B) MA 200 ng/ml; (C) MA 1000 ng/ml; (D) benzphetamine 200 ng/ml.

log concentration, had a good linearity (r=0.999) within a range from 50 ng/ml to 1000 ng/ml of MA. The detection limit, the concentration of MA equivalent to the mean area ratio of six replicates of the zero concentration sample plus two standard deviations, was 19.0 ng/ml with a C.V. of 4.1% in the MA standard curve.

Intra- and inter-assay precision was studied by including control samples with low (100 ng/ml) and high (400 ng/ml) concentrations of MA in every assay. The intra-assay study gave mean value results (n=5) of 100.2 and 389.9 ng/ml for the low and

high concentrations with C.V.s of 5.6% and 4.0%, respectively. The results of the inter-assay precision study were mean values ($n=4$) of 102.1 and 402.5 ng/ml of MA with C.V.s of 5.2% and 5.7%, respectively. Recoveries were 100.2% and 97.5% for the low and high concentrations, respectively.

The specificity of anti-MA-KLH antiserum was studied by comparing its response to cross-reactant (200 ng/ml) with that to MA (200 ng/ml) (Fig. 2D). Table 1 shows the cross-reactivity results for anti-MA-KLH antiserum when using CE–LIF as the assay system compared with the cross-reactivities obtained when using ELISA [8]. The antibodies 1, 2 and 3 used with ELISA all originated from the same anti-MA-KLH antiserum that was used in the CE–LIF immunoassay even though they were purified using different ligands in the immunoaffinity columns. In ELISA, the immobilized MA-OVA competed with free MA in the sample for the antibody binding site in the same manner as MA-FITC in the CE–LIF assay. The two assay systems containing different competitors showed different degrees of cross-reactivities, even though the same antibodies were used. In the CE–LIF system, the antibody had a far greater affinity for benzphetamine than MA. A similar phenomenon was observed when using an FPIA system in a homogeneous immunoassay as described in another work [18]. On the other hand, antibodies used in the ELISA system showed relatively strong cross-reactivities to methylephedrine only. This is probably due to the difference in binding affinity between the antibody and the labeled MA in free form in solution (CE–LIF system) versus in immobilized form on solid-phase (ELISA system). Besides the circumstances under which antigen–antibody binding was performed, the type of tracer used may affect the cross-reactivity even more greatly. Several reports show that different tracers cause exactly the same antibody to reveal different cross-reactivity patterns in different assay systems [19,20] or in the same assay system [21]. Maybe the affinity of the antibody to the MA-FITC tracer is weaker than its affinity to benzphetamine, whereas the affinity of the antibody to the MA-OVA is not. MA-FITC seems to be a good competitor with benzphetamine when using anti-MA-KLH antibody.

In the ELISA system reported previously [8], the MA concentration yielding 70% of the maximum response was too high (about 10 μg/ml). We recalculated the detection limit for ELISA in the same way as described in this report for CE–LIF, but its sensitivity was still inferior to CE–LIF. The binding affinity between the antibody and competitor could affect the immunoassay sensitivity. Using a weak binding competitor was better than using a strong one and gave a lower detection limit [22]. In ELISA, the MA-OVA competitor was similar to MA-KLH, the immunogen of the antibody concerned. This

Table 1
The cross-reactivity results of anti-MA antibodies in various assay systems

	Relative cross-reactivity (%)			
Antibody used[a]:	Antiserum	Antibody 1[b]	Antibody 2[b]	Antibody 3[b]
Antibody purification method: (ligand of immunoaffinity column)	Not purified	MA-BSA	MA-OVA	Protein G
Assay system:	CE–LIF	ELISA	ELISA	ELISA
Competing ligand:	MA-FITC	MA-OVA	MA-OVA	MA-OVA
Compound				
Methamphetamine	100	100	100	100
Benzphetamine	700	nd[c]	12	5
Amphetamine	nd	nd	nd	nd
Phenyl propanolamine	nd	nd	nd	nd
Ephedrine	nd	nd	nd	nd
Methylephedrine	nd	67	30	55

[a] The antibodies of both assay systems originated from the same antiserum prepared from immunizing goats using MA-KLH as the immunogen.
[b] ELISA data were cited from previous work [8].
[c] nd: Not detectable (less than 1%).

similarity might cause the antibody to have a stronger affinity for MA-OVA than the MA-FITC competitor used in CE–LIF. This could be the main reason why the detection limit for the CE assay is better than that for ELISA. These results suggest that the immunoassay system and the character of the competitor or tracer can affect the sensitivity and specificity of the antibody to a great degree.

In this present work, we developed an accurate, simple and sensitive immunoassay for the detection of methamphetamine in spiked urine using CE–LIF with an untreated fused-silica column, a simple separating buffer system, and antiserum itself. This method can be used for the screening and characterization of antibody with strong advantages. One feasible application of this work would be for routine screening of MA in urine.

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