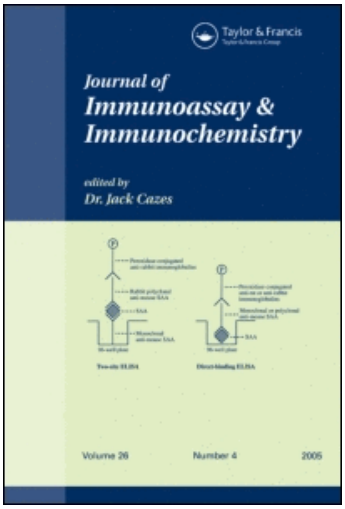

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Determination of Morphine by Capillary Zone Electrophoresis Immunoassay Combined with Laser-Induced Fluorescence Detection

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ABSTRACT

A competitive immunoassay for detecting morphine in bio-samples was established by capillary zone electrophoresis combined with laser-induced fluorescence detection (CZE-LIF). The antigen of morphine was labeled with isothiocyano-fluorescein (FITC) and then incubated with morphine monoclonal antibody and samples. The linear range was 50–1000 ng/mL, which was suitable for clinical and forensic applications. The detection limit can reach 40 ng/mL, based on $S/N = 2$. The recoveries of morphine from serum were satisfactory.

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Key Words: Capillary electrophoresis immunoassay (CEIA); Laser induced fluorescence (LIF); Morphine.

INTRODUCTION

With the wide use of drugs and the continuous invention of new drugs, drug abuse has become more and more seriously a social problem.[1] The identification and determination of abused drugs are attracting greater attention.[2–5] Currently, the most widely used methods for determining abused drugs are chromatographic methods, which are characterized with high speed, simple apparatus, and capability of screening multiple analytes simultaneously when combined with MS detection.[6–10] However, sample pretreatments, such as extraction, are always needed for those methods. In many cases, derivatization is also required.[11] Immunoassay has been applied in analyzing complicated systems because of its high specificity and sensitivity.[12–16] However, traditional immunoassay would take a long time and involves tedious procedures which have restricted its wide use.[17]

Capillary electrophoresis (CE) has many priorities compared with other analytical techniques: high resolution, increased speed, and extremely low volume consumption for samples and reagents. Moreover, CE has many separation modes, such as capillary zone electrophoresis (CZE) and micellar electrophoresis capillary chromatography (MECC).[18–20] CE, combined with immunoassay (CEIA), has been proved to be a powerful technique for the separation and analysis of abused drugs.[21–23] It greatly improves the analytical speed and simplifies the overall analysis procedures. Furthermore, with the application of LIF detection, the detection limit can be lowered over a large range.[24]

Morphine is one of most severely abused opiates. It is also the major metabolite of heroin and a minor metabolite of codeine. The determination of morphine undoubtedly has important meaning. For example, testing for morphine in body fluids and tissues is now a common means of identifying heroin abusers. The mean concentrations of morphine in serum and urine of abusers were demonstrated to be around the $\mu g/mL$ level.[25] While the detection limit is not the key problem, how to carry out a quick and efficient assay is the most important. Different techniques have been studied for morphine determination. Our group has developed a temperature controlled phase separation immunoassay of morphine,[17] in which thermally reversible hydrogel conjugated with antibody was used as the phase separation reagent. We[26] also applied the thermally reversible hydrogel solution as the electrophoresis running buffer for the analysis of morphine. Colbert et al.[27] developed a fluoroimmunoassay for the specific detection of morphine in urine

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using fluorescein-labeled morphine as the tracer. HPLC and GC techniques were widely used, but sample pretreatments and stacking were always required.[25,28]

In this study, a competitive immunoassay, using capillary zone electrophoresis with laser-induced fluorescence detection (CZE-LIF), was established. Based on the standard curve, the morphine from serum was determined directly.

EXPERIMENTAL

Reagents

Isothiocyano-fluorescein (FITC) isomer I and bovine serum album (BSA) were products of Sigma (St. Louis, MO). Morphine monoclonal antibody (McAb-MP) was a product of Fitzgerald (Concord, MA). Morphine hydrochloride was obtained from Beijing Center of Controlled Chemicals (Beijing, China). All other reagents were of analytical grade.

Apparatus

A Beckmann P/ACE 5000 system and a Beckmann GS-15R high-rate centrifuge were used in this study. Ultra-filtration tubes, 0.5 mL, (cut off Mw 3000) were purchased from Millipore (Bedford, MA).

Synthesis and Purification of Labeled Antigen (MP-3-BSA-FITC)

The antigen morphine-3-BSA (MP-3-BSA) was synthesized according to a procedure given in a previous report.[29] For the labeling of the antigen, 4 mg of MP-3-BSA was dissolved in 1 mL phosphate buffer solution (PBS, 0.01 mol/L, pH 7.4). 0.04 mg of FITC was dissolved in 100 μL carbonate buffer solution (0.5 mol/L, pH 9.5). With continuous stirring, the FITC solution was slowly dropped into the antigen solution. The mixture was stirred slowly for an additional 4 hr at room temperature. The reaction should be carried out under airtight conditions to ensure that the pH was above 9.0; bubbles should also be avoided. After the completion of the reaction, ultra-filtration was carried out (1 hr, 10,000 rpm) to remove the unreacted labeling reagent with an ultra-filtration tube. The labeled antigen on the filter was recovered by adding 400 μL of PBS in the filter cup of the ultra-filtration tube, followed by mixing for 1 min with a vortex mixer. The concentration of the

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labeled antigen was then determined from the absorbance at 260 and 280 nm by the following formula:[30]

$$C\left(\frac{mg}{mL}\right) = 1.45A_{280} - 0.74A_{260}$$

The labeling rate (F/P) of the labeled antigen was determined by the following formula:[31]

$$\frac{F}{P} = 2.8 \times \left(\frac{A_{495}}{A_{280}} - 0.35A_{495}\right)$$

where A_{495} and A_{280} are the absorbances of purified labeled antigen at 495 and 280 nm, respectively.

Competitive Immunoassay of Morphine By CZE-LIF

Antibody solution and labeled antigen solution were mixed with a series of morphine solutions of different final concentrations: 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL. The concentration of antibody and labeled antigen were optimized at 50 and 67.5 μg/mL, respectively. Then, the mixtures were incubated at 37°C for 1 hr. Each sample was injected for 10 s at the positive electrode, by pressure at 15 psi. Then CZE-LIF analysis was carried out under the following conditions: 100 mmol/L Tris–Borate buffer solution (pH 8.6), 25 kV applied voltage. Between runs, the capillary was rinsed with NaOH solution (0.2 mol/L) for 3 min, with distilled water for 2 min, and then with the running buffer solution for 3 min. Each sample was determined five times at the same concentration. With the system free of morphine as the reference, the change of peak area (abbreviated as RFU) ratio (free labeled antigen to immunocomplex) with the change of morphine concentration in samples was determined and used for the quantification. A standard curve was obtained.

Two simulated serous samples were determined by the method above and the recoveries were calculated.

RESULTS AND DISCUSSION

The Application of Ultrafiltration

Ultra-filtration is one of the techniques which can easily separate different molecules based on their different molecular sizes. It uses the size exclusion effect of the filter apertures and has the advantage of high speed

and high efficiency, and also can achieve sample stacking when compared with other traditional techniques such as dialysis and column separation. A cutoff Mw 3000 ultra-filtration cup was used here because the antigen has a molecular weight of about 73,000 while the FITC has a molecular weight of only 380. A cutoff Mw 10,000 ultra-filtration tube was also tested, but some of the protein (about 10% according to the peak area) was found in the filtrate. This was because the molecular weights of some protein fragments were below 10,000. Similar phenomena were not found when the cutoff 3000 tubes were used.

Optimization of Electrophoresis Conditions

Parameters that affect the speed and quality of separation were studied, including buffer pH and concentration.

Three background electrolyte (BGE) buffer solutions of different pH: pH 8.0, 8.6, and 9.3 were studied in this work. The electrophoresis results are shown in Fig. 1. When pH 9.3 buffer was used, the migration time was shortened because the ratio of charge to mass increased, but the two peaks of labeled antigen and immunocomplex were not well separated. When the pH was lower to 8.6, the migration time was significantly prolonged. So, pH 8.6 was chosen as the optimized pH.

Then a series of buffer solutions of different concentrations were prepared: 25, 50, and 100 mmol/L. The electrophoresis results are shown in Fig. 2. When the buffer concentration was decreased, the two peaks were not well separated, although the migration time was shortened. When the concentration was 25 mmol/L, the two peaks became one peak and serious adsorption was observed. So, 100 mmol/L was chosen as the optimized buffer concentration.

The optimization assumed that other parameters, such as column temperature, column length, etc., were held constant.

Optimization of Immunoassay Conditions

In the competitive immunoassay, the concentration of antibody and labeled antigen should be optimized so that they can compete efficiently. The optimization was carried out according to the following three steps:

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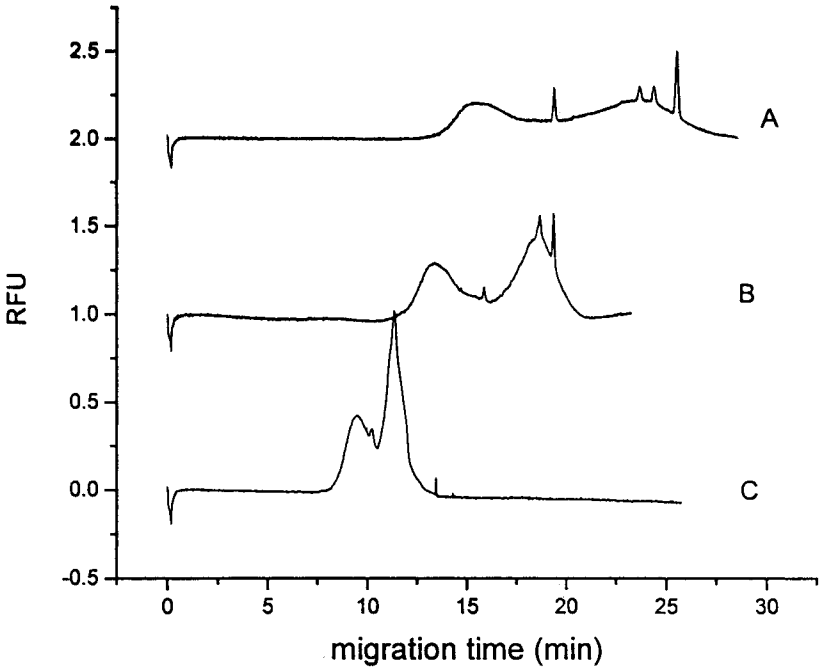

Figure 1. Optimization of the pH of the buffer solution. A. pH 8.0; B. pH 8.6; C. pH 9.3. Applied voltage was 25 kV. Buffer concentration was 100 mmol/L.

Primary Optimization of Labeled Antigen Concentration

The labeled antigen was diluted with PBS. The change of peak area with the change of labeled antigen concentration is shown in Fig. 3. A dilution number of 40 (corresponding to a concentration of 67.5 μg/mL) was chosen as the primary concentration of labeled antigen in view of obvious change of signal with the change of concentration and appropriate signal intensity.

Optimization of Antibody Concentration

The antibody was diluted with PBS containing a fixed concentration of labeled antigen. The mixture was incubated at 37°C; then, separation was carried out. The change of immunocomplex peak area with the change of antibody concentration is shown in Fig. 4.

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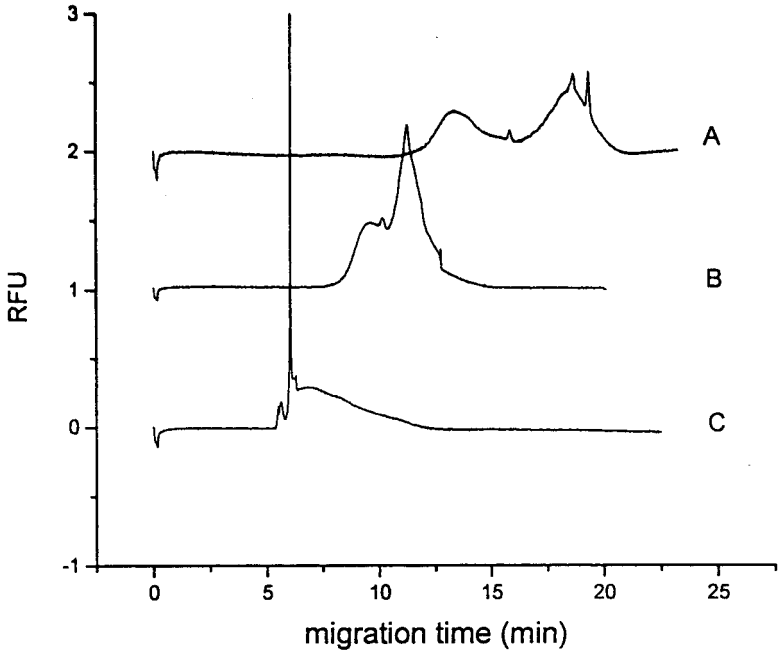

Figure 2. Optimization of the buffer concentration. A. 100 mmol/L; B. 50 mmol/L; C. 25 mmol/L. Applied voltage was 25 kV. Buffer pH was 8.6.

A dilution number of 80 (corresponding to a concentration of 50 μg/mL) was chosen as the optimized concentration of antibody in view of obvious change of signal with the change of concentration.

Further Optimization of Labeled Antigen Concentration

The labeled antigen was diluted with PBS containing a fixed concentration of antibody. The mixture was incubated at 37°C; then, CZE was carried out. The change of immunocomplex peak area with the change of labeled antigen concentration is shown in Fig. 5.

Because enough binding sites of antibody must be involved to ensure effective competition between labeled antigen and native antigen, a dilution number of 40 (corresponding to a concentration of 67.5 μg/mL) was chosen as the optimized concentration of labeled antigen.

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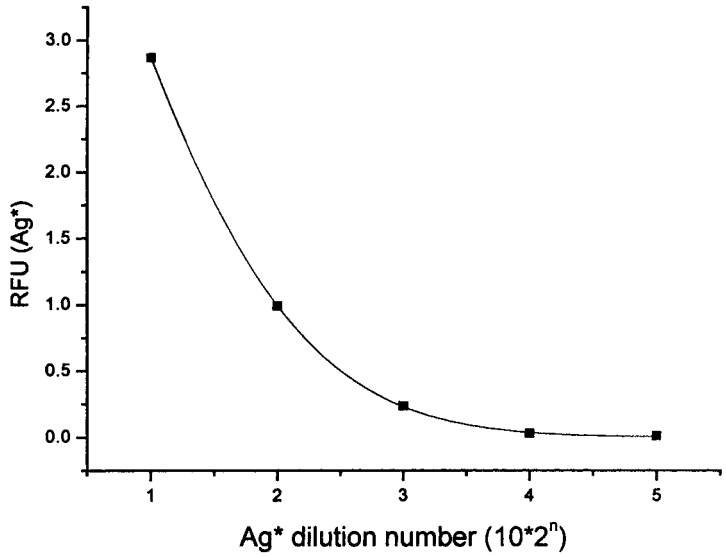

Figure 3. Primary optimization of labeled antigen concentration.

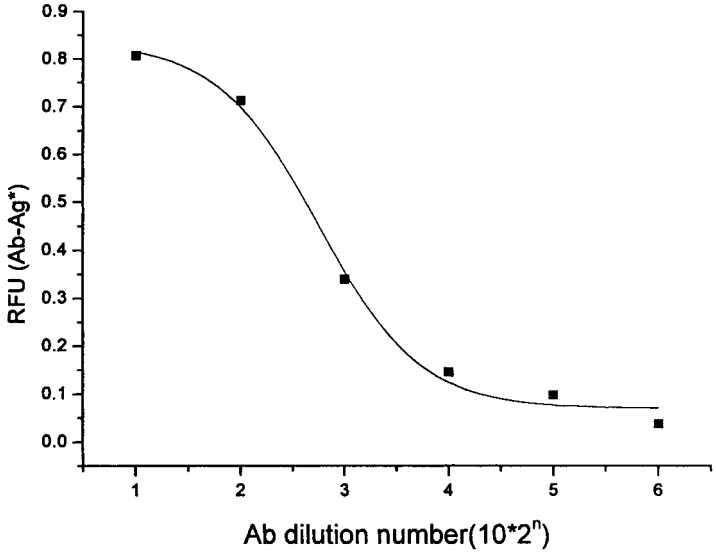

Figure 4. Optimization of antibody concentration.

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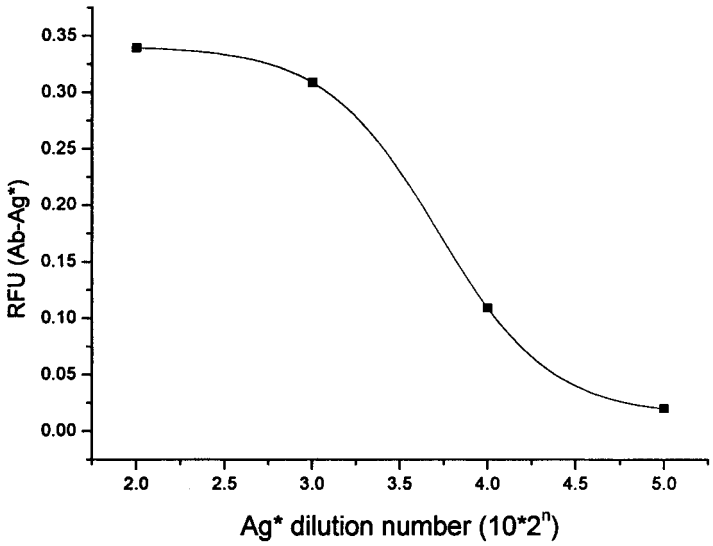

Figure 5. Further optimization of labeled antigen concentration.

Determination of Morphine

The electropherogram of immunoassay is shown in Fig. 6. With the increase of morphine concentration, the increase of the labeled antigen peak and the decrease of immunocomplex peak were observed. Both peaks were not very well shaped. This was the result of nonuniformity of the protein molecules and heterogeneous labeling. Furthermore, there may be slight adsorption between protein and the silica capillary wall, which also affects the shapes of the peaks.

The standard curve obtained is shown in Fig. 7. The corresponding standard equation is

$$\frac{\mathrm{RFU(Ag^*)}}{\mathrm{RFU(Ab-Ag^*)}} = 1.077 + 0.00349 * \mathrm{C(Ag)(ng\,mL^{-1})},$$

where C(Ag) is the concentration of morphine in the samples. The correlation coefficient (R) was 0.998. The linear range was 50–1000 ng/mL, which is suitable for clinical applications. The detection limit can reach 40 ng/mL based on S/N = 2.

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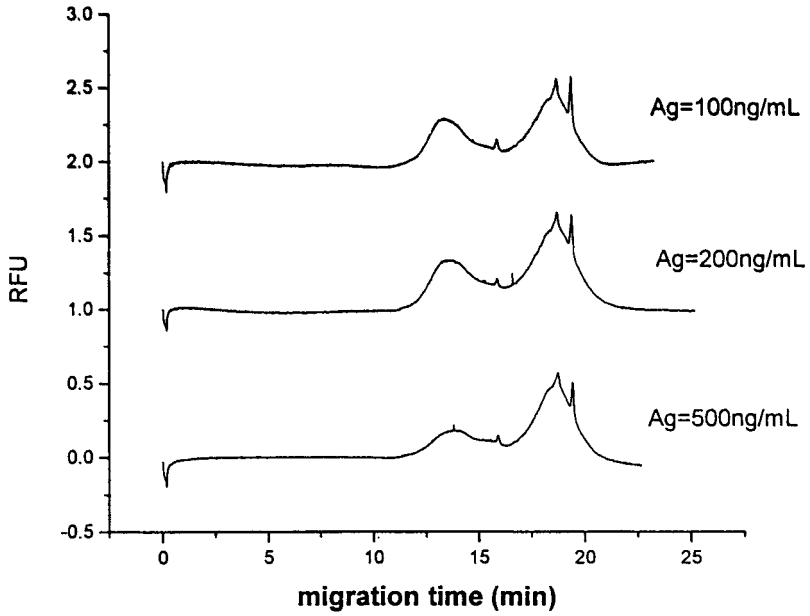

Figure 6. The CZE-LIF immunoassay of morphine with applied voltage 25 kV and 100 mmol/L Tris-Borate buffer solution (pH 8.6).

Application of the Analysis

Two simulated samples were prepared by adding various volumes of standard morphine solutions (1 mg/mL) to serous solution diluted 10 times. The high protein and salt concentrations in the serum did not have an obvious effect on the analysis. The final concentration of morphine was 80 and 400 ng/mL, respectively. The recoveries were determined and the results are shown in Table 1.

CONCLUSION

Table 2 shows the results of some previous work. Compared with the previous reports, our detection limit is lower than traditional immunoassay methods. However, it is higher than chromatographic methods. This is because sample pretreatments and stacking were applied in chromatographic methods. We reported CE immunoassay of morphine using thermally reversible hydrogel solution as a separation buffer in a previous work.[26] The

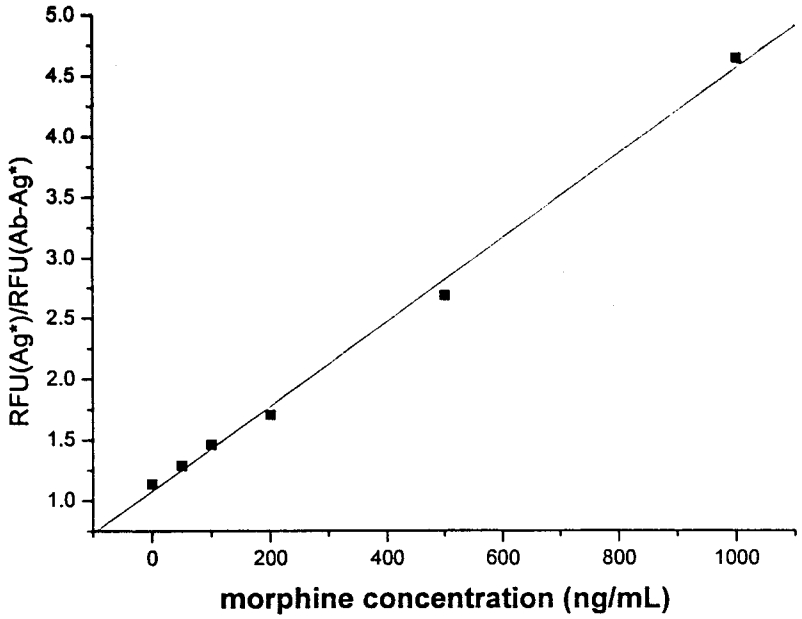

Figure 7. The standard curve for morphine determination.

polyclonal antibody was obtained by immunizing animals and was conjugated to the hydrogel. The addition of hydrogel can improve peak shape and reduce adsorption of protein on the silica capillary wall. However, in our study, the compared polyclonal antibody showed serious cross-reaction with many other opiates and the monoclonal antibody provided good specificity. It would also lose some activity when the polyclonal antibody was conjugated with the polymer and the polyclonal antibody may also exhibit interaction with proteins.[32] For these reasons, monoclonal antibody was used in this work. Furthermore, although the peaks were not very well shaped, the recoveries of simulated samples have proven it to have no significant effect on our quantitation.

Table 1. The recoveries of morphine from serum.

Morphine added (ng/mL)	Replicates	Morphine measured (ng/mL)	Recovery (%)	RSD (%)
80	6	65.5	82	2.6
400	6	405.6	101	2.3

Table 2. Comparison of several methods for morphine determination.

Methods of morphine determination	Detection limit (ng/mL)	References
Thermally reversible hydrogel immunoassay	60	[17]
Fluoroimmunoassay	1000	[28]
HPLC with two-step solid-phase extraction	0.25	[25]
GC-MS	20	[29]
CZE-LIF	40	[a]

[a]The method used in this work.

Our method has been proven to be rapid and efficient. It can be employed to detect morphine in serum. When the antibody and labeled antigen involve other specific reagents, the method can also be used as a useful one for the clinical analysis of other abused drugs.

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