Chinese Chemical Letters Vol. 15, No. 8, pp 943-946, 2004
http://www.imm.ac.cn/journal/ccl.html

Production of Polyclonal Antibody of Morphine and Determination of Morphine in Urine by Capillary Electrophoresis Immunoassay with Laser-induced Fluorescence Detection

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Abstract: N-Conjugated antigen was synthesized and polyclonal antibody with high specificity was obtained from immunizing animals. With this polyclonal antibody, a rapid and efficient CEIA-LIF method was developed to determine the free morphine in urine of abusers. The detection limit was calculated to be 40 ng/mL. Simulated urine samples were analyzed with good recoveries, which showed the feasibility of its application in specific morphine determination in urine of morphine abusers.

Keywords: Polyclonal antibody, morphine, capillary electrophoresis immunoassay (CEIA), laser-induced fluorescence (LIF), specific.

Drug abuse is becoming more prevalent and widespread throughout the world than ever before[1]. The identification and determination of abused drugs are paid great attention[2~5]. Morphine is one of the most badly abused opiates. It is also the major metabolite of heroin and a minor metabolite of codeine. Many immunoassays have been reported on morphine determination[6,7]. However, in most assays the employed antibody was polyclonal antibody obtained from immunizing animals using 3-conjugated antigen, which has severe cross-reactivity with many morphine derivatives. Using N-conjugated immuno-antigen, polyclonal antibody with high specificity for morphine might be obtained[8]. Combined with CEIA and LIF detection, a rapid, specific and sensitive determination for morphine in urine could be established.

In this paper, N-morphine hapten was synthesized and coupled with carrier protein BSA through a linker of five carbons[8], as shown in **Scheme 1**. The obtained antigen was used to immunize rabbits. The antiserum produced by all three rabbits had similar titer of higher than 2×10^5 and the blood of one of the rabbits was used to produce antibody through purification. N-morphine was also coupled with ovalbumin (OVA) and then reacted with isothiocyano-fluorescein (FITC) to obtain labeled antigen, as shown in **Scheme 2**.

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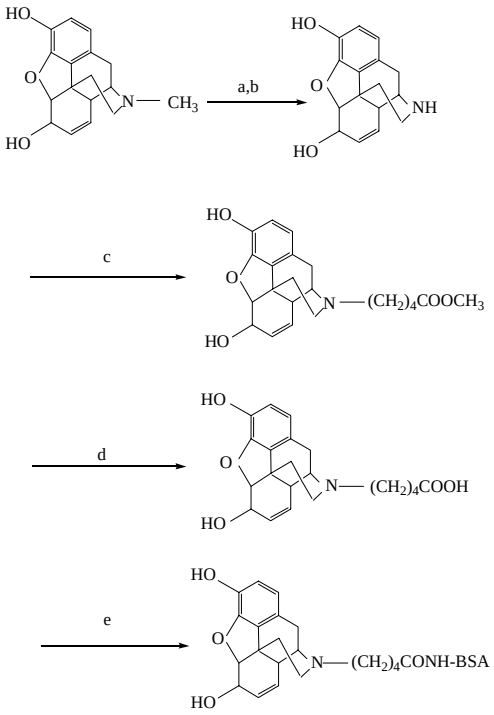
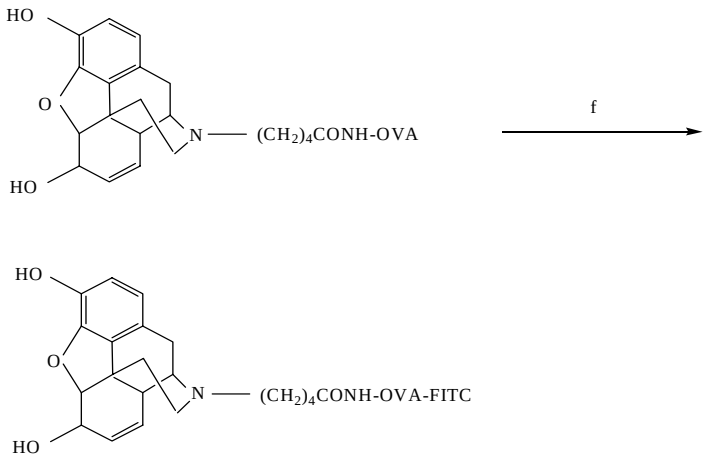
Scheme 1



Reagents and conditions: a. methyl chloroformate and $NaHCO_3$ in $CHCl_3$ refluxed 20 h[9]; b. 80% hydrazine refluxed 63 h[9]; c. methyl 5-bromovalerate and $NaHCO_3$ in dimethylformamide at 115°C refluxed 2 h[8]; d. $NaOH/H_2O$ at 50°C stirred 15 min; e. BSA, N-hydroxy succinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at room temperature for 4 h; f. isothiocyano-fluorescein and Na_2CO_3 (pH>9.0) at room temperature for 4 h.

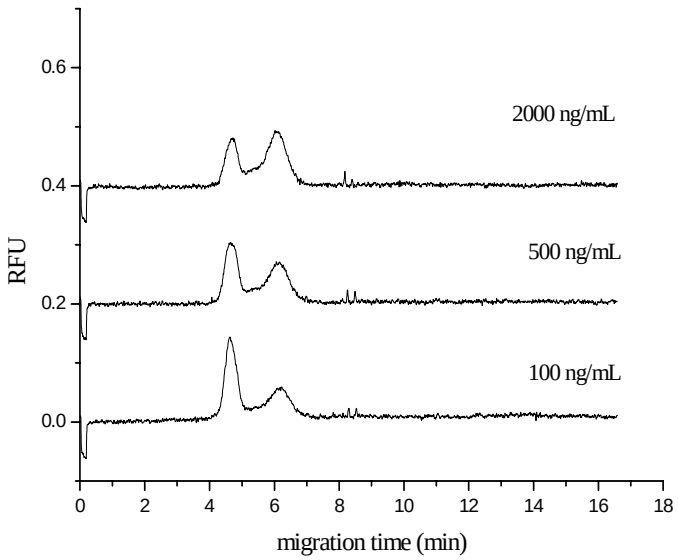
Scheme 2



Competitive immunoassay was conducted later for the determination of morphine. Electrophoretic and immunoassay conditions were both studied. Antibody solution and labeled antigen solution were mixed with a series of morphine solutions. The final concentrations of morphine were 50, 100, 200, 500, 1000, 2000, 5000 ng/mL, respectively. The concentrations of antibody and labeled antigen were 200 μg/mL and 5 μg/mL, respectively. Then the mixtures were incubated at 37°C for 1 hour. The sample was injected for 10 s at the positive end of the capillary by pressure of 0.1 MPa. Then CEIA-LIF analysis was carried out under the following condition: 100 mmol/L tricine buffer solution (pH 8.1), 20 °C and 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 with the running buffer solution for 3 min, successively. Typical electropherograms of morphine immunoassay are shown in **Figure.1**. For quantification, the area ratio of immunocomplex to free labeled antigen (Y) was plotted against the logarithm of morphine concentration (X, ng/mL). The linear working curve was obtained as: $Y=2.35-0.544*X$, with the correlation coefficient $r^2=0.991$. The observed linear range was 50-5000 ng/mL and the limit of detection (LOD) was calculated to be 40 ng/mL based on S/N =2.

The specificity of the polyclonal antibody was examined using both ELISA and CEIA method. No cross-reaction was observed at 10 μg/mL levels for codeine, acetyl codeine, dionin, thebaine and morphine-3-glucuronide. Compared with the polyclonal antibody acquired from immunizing animals using 3-*O*-conjugated hapten, this one obviously has much higher specificity and can discriminate morphine from its major metabolites and many other opiates.

Figure 1 Electropherograms of morphine immunoassay



The application of the method was also assessed. Three simulated urine samples were determined using the established immunoassay. The recoveries were all between 90% and 110% with replicates of 5 each and RSD of less than 3%. The salts and proteins in urine were not observed to have obvious effect on the electrophoretic results due to their low concentrations.

Acknowledgment

This work was financially supported by the National Natural Science Foundation of China (NNSFC) (20075001, 20075003) and the Excellent Young Teachers Program of Ministry of Education, China. Some organic synthesis was completed in the lab of Prof. Jian-Bo Wang and thanks were also given here.

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Received 21 July, 2003