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Preparation and Application of an Immunoaffinity Column for Direct Extraction of Morphine and its Analogs from Opium

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Abstract: A rapid, simple and accurate method using an immunoaffinity column (IAC) and capillary electrophoresis (CE) for the analysis of the major alkaloids in opium is developed. The IAC was synthesized by coupling specific morphine polyclonal antibodies to CNBr-actived Sepharose 4B. The IAC showed high selectivity and obvious enrichment to morphine, codeine, dionin and thebaine. The extraction solution was analyzed by CE with β-cyclodextrin as an additive. Recoveries of the four alkaloids from PBS were between 93%-105% with RSD value less than 5.0%. The result showed that this method was practical for the determination of morphine analogs in opium.

Keywords: Immunoaffinity column (IAC), capillary electrophoresis (CE), morphine, opium.

The analysis of analogs in opium plays an important role in clinical investigation, forensic toxicology and pharmacokinetic research. Morphine and its analogs, such as codeine, dionin and thebaine are the major alkaloids to legal or illegal purpose. The quantitative determination of these compounds is typically performed by immunological method and chromatographic techniques such as capillary electrophoresis immunoassay (CEIA)[1], high-performance liquid chromatography (HPLC)[2]，gas chromatography-mass spectrometry (GC-MS)[3] and liquid chromatography-mass spectrometry (LC-MS)[4]. However, conventional separation methods require highly absolute specimen for injection, which demands strictly pre-treating for samples and operation process is complicated, furthermore, instrumental cost is relatively expensive. Due to the appealing advantages of high resolution, small injection volume and inexpensive column, capillary electrophoresis (CE) has been developed and widely used with various modes[5-7] for the separation of small molecules to biological macromolecules.

Since the high specificity of immunolgical system, some affinity systems have been introduced in the pretreatment of special samples from complicated matrix. Antibodies coupled Sepharose columns[8] have been used for estrogens and bisphenol A extraction from biological matrices in our group[9] and has achieved satisfactory result.

The present study describes the methodological optimization and validation of immunoaffinity column in combination with CZE method for the rapid and simultaneous

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determination of four alkaloids in opium, based on the addition of β-cyclodextrin to the running buffer in order to enhance the separation power of this system.

Experimental

Generation of IAC

The specificity of morphine polyclonal antibody has been detected by competitive ELISA. The cross reactivities of codeine, dionin and thebaine to antibody are all above 90%. This result indicated that this antibody could bind with several analytes, which can be applied for the determination of different related analogs. The coupling efficiency of the immobilized polyclonal antibodies to CNBr-actived Sepharose 4B was tested 96% by UV.

The capacity of Immunoaffinity Column

After equilibrating the IAC with 0.01 mol/L PBS, solution containing morphine, codeine, dionin and thebaine was added onto the IAC continuously, eluent was collected with different tubes, each aliquot was 200 μL. Then the aliquots were analyzed by CE. Their eluated curves of morphine is showed in **Figure 1**. The retention behaviors of other three analytes is similar to morphine. The results showed that the retention behaviors of morphine in different concentration had a little difference. The breakthrough volumes were 2.8 mL, 2.2 mL and 2.0 mL, respectively. The breakthrough volume for lower concentration of morphine was larger than that of in higher concentration. This may be caused by the dynamics of antibody-antigen interaction which was relatively slow and antigen had not enough time to interact with antibody in higher concentration. Considering the content of analytes in actual specimens is very low, we chose 2.5 mL as loading volume.

Figure 1 Elution profiles for the continuous loading of morphine in 0.01 mol/L PBS

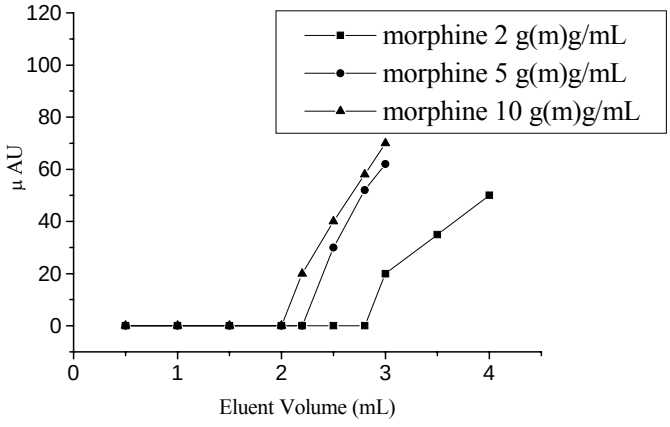
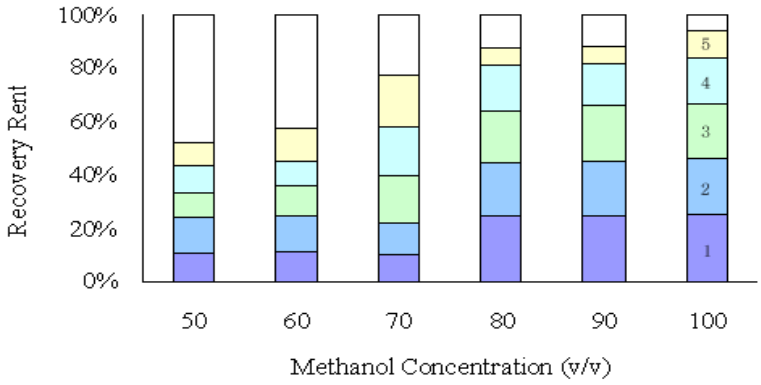


Figure 2 Elution profile of morphine with different percentages of methanol



Selection of elution solvent

Different concentration of methanol were studied as eluent for purification. The results showed that the analytes recoveries were increased with the increase of methanol concentration from 50% to 100%. The elution result for morphine is shown in **Figure 2**. It can be seen that for five consecutive elutions, 100% methanol as eluent made the recovery approach to 94%. The same results were acquired for other three related analogs. So 100% methanol was chose as elution solvent. After preconditioning of samples through the IAC, target analytes were extracted from samples matrix.

Recoveries of four analogs in PBS

To determine the practical feasibility of the IAC, the recoveries of four analogs were investigated. The results were showed that in PBS matrix, all four recoveries are between 93% and 105% with RSD values less than 5.0% (**Table 1,** R'=recovery, R=RSD).

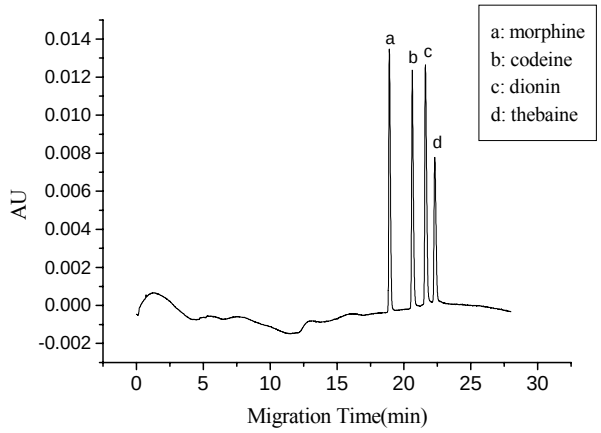
Table 1 Recoveries of four alkaloids in PBS matrix

concentration	morphine		codeine		dionin		Thebaine	
	R' (%, n=5)	R (%, n=5)	R'(%, n=5)	R (%, n=5)	R' (%, n=5)	R (%, n=5)	R' (%, n=5)	R (%, n=5)
1 μg/mL	94.3	4.9	96.68	2.2	95.07	3.2	94.65	3.8
2 μg./mL	102.14	3.8	93.84	3.7	104.34	1.9	94.91	2.5

Separation of four analogs by CZE

The structures of the four alkaloids are similar to each other and separation was achieved by CZE. The β-cyclodextrin was reported to enhance separation efficiency in the field of forensic analysis[10-11]. The baseline separation of a standard mixture containing morphine, codeine, dionin and thebaine was achieved in optimized condition using 15 mmol/L β-cyclodextrin added to 0.1 mol/L phosphate solution as running buffer. A typical electropherogram was showen in **Figure 3** (separation voltage: 20 kV; sample injection: 10 s; temperature: 20℃).

Figure 3 Electropherogram of four alkaoids (10.0 μg/mL each) with β-CD as an additive



Study for determination of the four structurally related alkaloids in real sample is under investigation.

Acknowledgments

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