Feasibility Studies for Simultaneous Immunochemical Multianalyte Drug Assay by Capillary Electrophoresis with Laser-Induced Fluorescence

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We present a method for the simultaneous quantitation of multiple drug analytes in urine, based on combining immunochemical binding with capillary electrophoretic separation. Two fluorescent drug–cyanine (Cy) dye conjugates were prepared as competing species for the immunoassay. Morphine was derivatized with Cy5 ($\lambda_{\text{max}} = 652$ nm, $\epsilon = 215 \text{,}000 \text{ mol}^{-1} \text{cm}^{-1} \text{L}$), phenacyclidine (PCP) with Cy5.5 ($\lambda_{\text{max}} = 675$ nm, $\epsilon = 200 \text{,}000 \text{ mol}^{-1} \text{cm}^{-1} \text{L}$). The high-efficiency resolving power of the capillary electrophoresis system (20 µm × 27 cm column) separated the individual labeled drugs, and the antigen–antibody complexes were detected by laser-induced fluorescence (laser: 10 mW He-Ne at 632.8 nm) with Cy5 diacid as internal standard. Simultaneous competitive immunoassay of morphine and PCP in urine showed that the free labeled-drug peak areas were proportional to the concentrations of the drug species present in the urine sample. This immunoassay can be performed routinely and reproducibly in <5 min with analytical detection limits of 4 nmol/L for PCP and 40 nmol/L for morphine.

**Indexing Terms:** abused drugs/screening/urine/morphine/phenacyclidine

To develop sensitive analytical methods that would provide substantially lower detection limits than conventional ultraviolet/visible absorbance detection of urine analytes, we focused on competitive immunoassays with fluorescent-labeled antigen. In most practical immunoassays, either the free or the bound species must be measured in the presence of numerous potentially interfering substances. Because the species of interest will often be at a relatively low concentration, the label and detection method used must provide good sensitivity. Furthermore, the assay technique must include a step that effectively separates the antigen–antibody complex from the free antibody.

The free and antibody-bound labeled antigen can be readily separated and detected by capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection. For effective separation of the antigen or antibody from the antigen–antibody complex, we chose to use highly negatively charged cyanine dyes, Cy5 and Cy5.5 (I, 2), as the fluorescence probes for LIF detection and as the charge-carriers for electrophoretic separation.

Received April 19, 1994; accepted June 6, 1994.

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1 Advanced Technology Center, Beckman Instruments Inc., 2500 Harbor Blvd. D-20A, Fullerton, CA 92634.
2 Nonstandard abbreviations: CE, capillary electrophoresis; DMSO, dimethyl sulfoxide; LIF, laser-induced fluorescence; PBS, phosphate-buffered saline; and PCP, phenacyclidine.

We report here the synthesis of fluorescent-labeled morphine and phenacyclidine (PCP) and the functional utility of these derivatives in CE/LIF for simultaneous immunoassay of PCP and morphine, two of the five classes of drugs of abuse for which guidelines for detecting and reporting have been established by the National Institute on Drug Abuse. Morphine is a major metabolite found in the urine of heroin and codeine users. PCP is excreted in urine as the unchanged drug and some oxidized metabolites.

Until recently, products used to screen for drugs of abuse could detect only one drug at a time so that separate tests had to be conducted to screen for all five drug classes. A system for qualitative screening of all five drug classes has been developed, based on formation of agglutinated complexes (3). The CE/LIF technique we describe is quantitative. Although we report on screening for two drugs of abuse as a representative example, in principle this methodology can be used for simultaneous determination of several analytes, the number being limited only by the effectiveness of separation of the labeled antigens in the CE system.

**Materials and Methods**

**Materials**

Cy5 and Cy5.5 (I, 2) were purchased from Biological Detection Systems, Pittsburgh, PA. Boric acid, morphine, normorphine, and PCP were obtained from Sigma Chemical Co., St. Louis, MO. The PCP derivative, 2'-aminoethylaminosuccinyl-3-amino PCP, was synthesized by Josephine Michael of Beckman Instruments. Sheep antisera to morphine and PCP were from Biodesign International, Kennebunkport, ME.

Borate buffer, 200 mmol/L, pH 10.2, was filtered through 0.45 µm (pore size) filters before use. Drug standards in methanol were obtained from Sigma Chemical Co. Antibodies were diluted in phosphate-buffered saline (PBS) consisting of, per liter, 0.1 g of sodium azide, 75 mmol of sodium chloride, and 20 mmol of potassium phosphate at pH 7.0, and containing 500 mg/L human IgG. Stock solutions of drug conjugates (0.1 µmol/L) in equivolume solutions of water and ethylene glycol were stored at $-20^\circ$C.

**Synthesis of Cy5- and Cy5.5-Labeled Drugs**

**Morphine.** A solution of normorphine (see Fig. 1), 40 nmol in 10 &muL of dimethyl sulfoxide (DMSO), was mixed with a freshly prepared solution of 40 nmol of Cy5 in 40 &muL of 50 mmol/L sodium bicarbonate buffer, pH 8.0. After standing in the dark at room temperature for 1 h, the mixture was passed through a Poros II R/H (PerSep-tive Biosystems, Cambridge, MA) chromatographic col-
umn, eluted with a gradient of 10 mmol/L phosphate (pH 7.0):methanol (70:30 by vol for 2 min, followed by a linear gradient to 20:80 in 8 min) at 1.5 mL/min. We collected the product that eluted at 4.5 min and found it to be immunologically reactive by CE/LIF. The product was rechromatographed with a shallower methanol gradient (60:40 phosphate:methanol for 1 min, and then a linear gradient to 35:65 in 7 min). The homogeneity of the final product was monitored by HPLC and CE/LIF. The concentration of the purified product was calculated from its absorptivity at 650 nm ($\varepsilon = 215 \ 000 \ mol^{-1} \ cm^{-1} \ L$).

**PCP.** A solution of 60 nmol of 2'-aminoethylaminosuccinyl-3-amino PCP (see Fig. 1) in 60 µL of acetonitrile:DMSO (4:1 by vol) was added to a freshly prepared solution of 80 nmol of Cy5.5 in 40 µL of 50 mmol/L phosphate buffer, pH 7.5, and vortex-mixed after standing for 2 h in the dark, the mixture was chromatographed through the Poros R/Hi column with a linear gradient of 10 mmol/L phosphate (pH 7.5) and methanol as eluting solvent. In the first purification, the composition was 65:35 (by vol) for 1 min, changing to 20:80 in 5 min and to all methanol in 1 min. The major product, which eluted at 5.5 min, was collected and then concentrated. In the second purification, we used a phosphate buffer:methanol gradient of 60:40 for 1 min, changing to 50:50 in 5 min and to all methanol in 2 min; the purified product eluted at 5.3 min. The concentration of the purified conjugate was determined from its absorbance at 675 nm ($\varepsilon = 200 \ 000 \ mol^{-1} \ cm^{-1} \ L$). The purity of the conjugate was monitored by HPLC and CE/LIF.

**Imunoassay Protocols**

*Titration of the cyanine-labeled drug conjugates with antiserum.* A starting reaction solution of 50 µL of PBS containing 500 mg/L human IgG, 10 nmol/L each of Cy5-morphine and Cy5.5-PCP, and 2 nmol/L Cy5 diacid was prepared for the titration experiments. We electro-phoresed this solution itself, but also after adding 5 µL of 50-fold-diluted antiserum to morphine, 8 µL of 100-fold-diluted antiserum to PCP, or both. The immunoreaction mixtures were analyzed immediately after mixing the antibody with the labeled antigens in an automated CE system equipped with LIF detection (see below). The results showed that such amounts of antisera were adequate for nearly quantitative capture of the labeled antigens; thus, we adjusted accordingly the antisera amounts used in the competitive immunoassay.

*Simultaneous immunoassay of morphine and PCP.* A 50-µL solution of morphine:PCP calibrator (10:1 molar ratio) in drug-free urine was mixed with 10 µL of 0.1 µmol/L Cy5-morphine and 5 µL of 0.1 µmol/L Cy5.5-PCP. The mixtures were analyzed immediately after addition of 10 µL of antiserum to morphine (50-fold dilution) and 4 µL of antiserum to PCP (100-fold dilution) and mixing.

**Instrumentation**

For CE/LIF applications, a P/ACE 2100 system equipped with a laser-induced fluorescence detector (Beckman Instruments, Fullerton, CA) was used with P/ACE system software controlled by an IBM PS/2 Model 56 SX. The capillary column for CE/LIF was
typically 27 cm long (20 cm to detector window) × 20 μm (i.d.) (Polymeric Technologies, Phoenix, AZ) and was assembled in the P/ACE cartridge format with an ellipsoidal mirror to collect fluorescence. A 10-mW red helium–neon laser emitting at 632.8 nm was purchased from Melles Griot (Irvine, CA). A laser headcoupler to a standard SMA-905 fiber connector to the P/ACE system with LIF detector was a product of OZ Optics, Ontario, Canada. The fluorescence signal was collected through a narrow-band filter of 690 ± 9 nm (Oriel, Stratford, CT), and the laser beam was rejected by a notch filter at 633 nm (Barr Associates, Westford, MA). Samples were injected by pressure (~3.5 kPa) for 20 s. The electrophoretic conditions are given with the electropherograms shown later. Between runs, the capillary was pressure-rinsed with 1.0 mol/L sodium hydroxide and water (12 s each, at 103.5 kPa) and then equilibrated with electrophoresis buffer (200 mmol/L borate, pH 10.2).

Results and Discussion

The study of antibody–antigen reactions by CE was demonstrated by Nielsen et al. (4). In that study, the antigen and antibody molecules were of comparable size, and the peak due to the appreciably larger antibody–antigen complex could be clearly separated from the peaks for the free antibody and the free antigen. The measurements were made with ultraviolet absorbance, with protein concentrations at ~40 μmol/L, and in the absence of significant concentrations of potentially interfering substances.

CE/LIF has a typical detection limit of 1 pmol/L, making the system ideal for higher-sensitivity immunoassay (5–7). For a competitive immunoassay, a labeled antigen (Ag*) or antibody must be well separated from the antigen–antibody complex. The amount of free labeled antigen is directly proportional to the antigen present in the sample.

\[ Ag + Ag^* + Ab = Ag-Ab + Ag^*-Ab + Ag^* \]

The morphine and PCP derivatives used for labeling, Cy5-labeled morphine and Cy5.5-labeled PCP (Fig. 1), both carry net negative charges of 3 to 4, depending on the buffer pH. The relative electrophoretic mobilities of Cy5-morphine and Cy5.5-PCP are shown in Fig. 2A. Cy5 diacid in the mixture acts as a reference peak. Addition of antibody to morphine results in the disappearance of the Cy5–morphine conjugate peak at 3.5 min and the formation of a new broad peak eluting at 2.05 min, the Cy5-morphine–antibody complex (Fig. 2B). The antibody (150 kDa) is so much larger than the Cy5-morphine (820 Da) that the antibody–Cy5-morphine complex migrates at essentially the same position as the antibody. The broad peak indicates the heterogeneity of the antibodies in the antisera. Similarly, the addition of antibody to PCP results in the formation of the antibody–Cy5.5-PCP complex (2.1 min) at the expense of the Cy5.5-PCP peak at 3.2 min (Fig. 2C). Addition of antibody to both conjugates results in the disappearance of both free conjugate peaks and the formation of a rather skewed and broad antibody–antigen complex peak (Fig. 2D); this peak appears to be an aggregate of the peaks in Fig. 2B and C, without the free labeled-drug peaks.

The concentrations of most of the frequently abused drugs and their metabolites in urine are in the range 1 μmol/L to 1 nmol/L. These drugs and their metabolites can be functionalized and labeled with cyanine dye as the competing species for immunoassays. The fluor-labeled drug species may be chemically modified to yield a specific charge-to-mass ratio. Such charge modulation of the labeled drug species, along with an internal standard, should make possible simultaneous screening of several drugs in a single sample. Thus, for free drugs a, b in the sample, in the presence of added fluorescent-labeled drugs a*, b* and antibodies to a and b, ABa and ABb, respectively,

\[ a + b + a^* + b^* + ABa + ABb = (a-ABa) + (a^*-ABa) + (b-ABb) + (b^*-ABb) \]

In competitive immunoassays of a and b, the competing species a* and b* are chemically well-defined synthetic reagents. Each labeled species has a specific charge-to-mass ratio and can be predictably separated by CE and detected by LIF. The number of drug species in a sample that can be analyzed by this method in one assay is limited only by the synthetic design of the labeled charge-modified drugs and the resolving power of the CE.

For the analysis of morphine and PCP, the urine drug calibrator or sample containing one or more of the drugs is mixed with a threshold amount of reagent containing a mixture of Cy5-morphine and Cy5.5-PCP and then with antisera in amounts previously determined by ti-
tration of the labeled morphine and PCP. In CE/LIF of the resulting immunoreaction mixture, the amounts of the free Cy5-morphine and Cy5.5-PCP are proportional to the amount of each drug species present in the urine sample (Fig. 3, A–D). An internal standard, Cy5 diacid, added to each sample mixture corrects relative migration times and yields quantitative information on the concentration of each drug.

We have demonstrated the utility of CE-LIF for quantitative immunoassay. The modulation of the electrophoretic mobility of a cyanine dye-labeled antigen, a competing species for the specific antibody, has been shown in competitive immunoassay (8, 9). For a large molecule such as a protein antigen, a monoclonal antibody to an epitope of the protein antigen is required. The epitope is usually a segment on the surface of the protein consisting of 5 to 10 amino acids. A labeled peptide of the same amino acid sequence can be used as the competing species for immunoassay.

For the direct assay of a protein antigen, modulation of the electrophoretic mobility of an antibody is possible, preferably by using a monoclonal antibody for the protein antigen. Because migration time is a function of the charge-to-mass ratio, the antibody–antigen complex should have a migration time intermediate between those of the antigen and the modified antibody (10).

Thus, if the antibody is fluorophore-labeled and present in excess with respect to the protein antigen, the amount of the complex formed should be directly proportional to the concentration of the protein antigen. Thus, only a single antibody species, rather than the pair of antibodies required for sandwich immunoassays (11) with solid-phase separation, is needed for a quantitative immunoassay using the CE/LIF technique. The use of excess antibody vs antigen in solution should provide more favorable reaction kinetics than that obtained with the traditional solid-phase-based sandwich immunoassay.

In conclusion, the combination of charge modulation of the antibody or antigen and powerful capillary electrophoresis separation, along with laser-induced fluorescence detection, promises to be a powerful tool for quantitative immunoassays.

We thank Josephine Michael for the synthesis of the PCP derivative used for labeling, Julie Kim for providing the anti-PCP antibody, and James C. Sternberg and James C. Osborne for helpful discussions.

References