ELSEVIER

Journal of Chromatography A, 680 (1994) 587–591

JOURNAL OF CHROMATOGRAPHY A

Analysis of structural specificity in antibody–antigen reactions by capillary electrophoresis with laser-induced fluorescence detection

Ramon A. Evangelista*, Fu-Tai A. Chen

Advanced Technology Center, Beckman Instruments Inc., 2500 Harbor Boulevard, Fullerton, CA 92634, USA

Abstract

A rapid and simple procedure for screening antibodies for binding to an antigen is proposed. A fluorescent hapten–dye conjugate was prepared by labeling the amino moiety of the hapten with a commercially available reactive cyanine dye, Cy5 (excitation maximum: 650 nm, emission maximum: 670 nm). A fixed amount of the Cy5–hapten was titrated with serial dilution of the antibody. Each of the titration mixture was analyzed by capillary electrophoresis (25 cm × 20 μm column) monitored by laser-induced fluorescence (laser: 10 mW helium–neon, 632.8 nm). Free and antibody-bound Cy5-hapten were analyzed simultaneously on the electropherogram. Competitive immunoassay of hapten was demonstrated with low-end sensitivity of $5 \cdot 10^{-8}$ M, about 10× more sensitive than the present drug screening methods. Using morphine as an example, the screening of various antibodies (from different vendors) and cross-reactivity of morphine analogues using the present procedure will be discussed.

1. Introduction

Screening of urine samples for presence of drugs of abuse and their metabolites is routinely performed in toxicology laboratories in cases pertaining to crime, accidents, rehabilitation and increasingly as a prerequisite to employment. The National Institute on Drug Abuse has established guidelines for detecting and reporting five drug classes: amphetamine/methamphetamine, cannabinoids, cocaine, morphine/codeine and phencyclidine [1].

The most common analytical techniques currently used are TLC, GC–MS and immunoassays [2,3]. TLC uses only low-cost equipment but is labor-intensive, requires high skill and experience and is subject to misinterpretation by the person viewing the color of the sprayed spots. Current commercial immunoassays offer rapid quantitation, high specificity and sensitivity but reagent costs are high because enzyme–antibody or enzyme–drug conjugates are employed. The results of GC–MS analysis are considered confirmatory but the equipment is expensive and requires great expertise.

The analyte we selected as an example, morphine, is the major metabolite of heroin and codeine and is excreted in the urine either in the free form or conjugated as the glucuronide. The sensitivity of the assay for morphine can be increased by hydrolysis of the glucuronide either by acid treatment or by the action of β-glucuronidase [3].

We report here the use of capillary electro-

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00677-6

phoresis (CE) with laser-induced fluorescence (LIF) detection as a tool for screening of antibodies for binding to a particular antigen and for the study of cross-reactivity of an antibody towards compounds of similar structures and we describe the immunochemical analysis of morphine by CE–LIF. This method exploits the high affinity and specificity of antibody–antigen binding, the great resolving capability of CE and the sensitivity of LIF. Previous reports have dealt with the use of CE for study of immunochemical reactions [4–6].

Our selected fluorophore, Cy5, a cyanine dye [7–9] has a high extinction coefficient (215 000 M^{-1} cm^{-1} at 650 nm,), good quantum yield (0.28 for protein conjugates), absorption and emission in the long wavelength visible region which do not overlap with those of other fluorescent compounds, good water solubility and photostability. These properties allow detection of 10^{-10} M of this label in spite of the small window of our narrow (20 μm) fused silica capillary with excitation by a low-cost, stable, long-lived 10 mW 632.8 nm helium–neon laser.

2. Experimental

The fluorescent morphine–Cy5 conjugate was prepared by the reaction between normorphine and Cy5 reactive dye (Biological Detection Systems, Pittsburgh, PA, USA) in hydrogencarbonate buffer pH 8. The principal product was purified by reversed-phase HPLC through a Poros II R/H 4.6/100 P002H026 246 column (Perseptive Biosystems, Cambridge MA, USA) using a linear gradient of 0.01 M phosphate buffer pH 7.0 and methanol as eluting solvent. The fluorescent drug conjugate was chromatographed three times with concentration of the HPLC eluent using the Speed Vac Concentrator between each purification step. The methanol gradient was from 30 to 80% in 8 min in the first two purifications and from 40 to 65% in 7 min in the third. The concentration of the morphine–Cy5 conjugate was determined from its extinction coefficient (215 000 M^{-1} cm^{-1}) at 650 nm. The conjugate was stored at −20°C in the HPLC aqueous methanol eluent.

CE–LIF analyses were performed using the Beckman P/ACE System 2100 (Fullerton, CA, USA) with a fused-silica capillary (25 cm × 20 μm) at 20 kv, 20-s injection, using a separation buffer composed of 90% 100 mM borate pH 10.2 and 10% ethylene glycol. Detection was achieved with the Beckman LIF detector module with a 10 mW 632.8-nm helium–neon laser (Melles Griot, Irvine, CA, USA) as excitation source and a 665-nm emission filter (Barr Associates, Westford, MA, USA).

Stock solutions of the morphine–Cy5, the antibodies and the morphine calibrators were dissolved in the Beckman ICS diluent containing 500 μg/ml human IgG. The CE samples for the antibody screening were prepared by mixing 10 μl of $1 \cdot 10^{-7}$ M morphine–Cy5, 10 μl of 1:50 dilution of the commercially available morphine antibody and 50 μl human IgG diluent. The CE solutions for testing cross-reactivity of opiates were composed of 10 μl $1 \cdot 10^{-7}$ M morphine–Cy5, 50 μl $1 \cdot 10^{-5}$ M opiate and 10 μl 1:50 diluted sheep morphine N polyclonal antibody (Biodesign International, Kennebunkport, ME, USA). The CE samples for the morphine competitive assay were prepared by adding 10 μl of 1:50 antibody solution to a mixture of 50 μl morphine calibrator in the human IgG diluent and 10 μl $1 \cdot 10^{-7}$ M morphine–Cy5.

3. Results and discussion

The addition of an amount of morphine antibody (10 μl of 1:50 dilution) sufficient to capture 1 pmol of the morphine–Cy5 conjugate results in almost complete conversion of the sharp fluorescent drug peak to that of the antibody–antigen complex with a shorter migration time due to the large molecular mass of the antibody (Fig. 1). The relatively broad peak for the complex is due to the fact that a polyclonal antibody was used.

The screening of four commercially available morphine antibodies by CE–LIF showed that the Biodesign morphine N antibody was best suited for the immunochemical binding assays (Fig. 2).

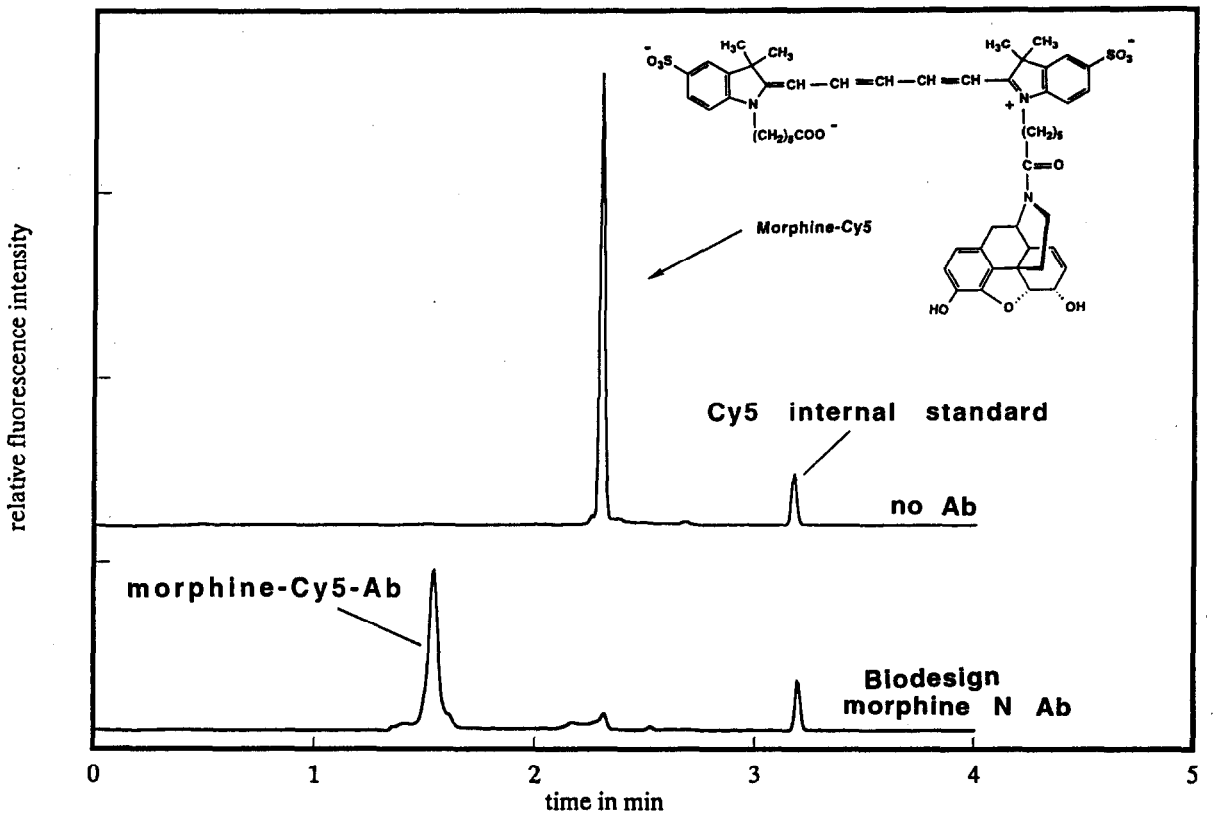

Fig. 1. Binding of sheep polyclonal antibody to morphine–Cy5. A 1-pmol amount of Cy5 was captured by 10 μl 1:50 antibody solution. Analysis by CE–LIF in 25 cm × 20 μm fused-silica capillary at 20 kV/17 μA.

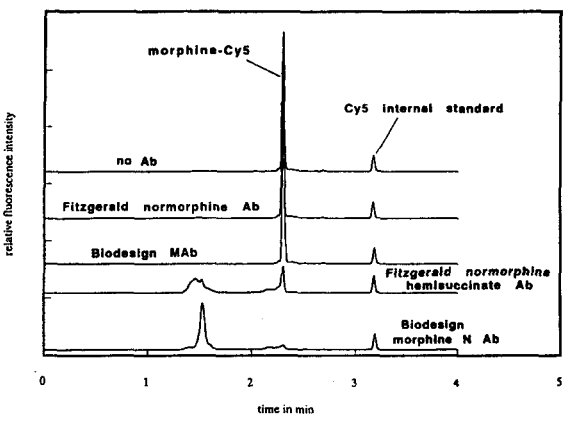

Fig. 2. Screening of four commercial antibodies for binding to morphine–Cy5. Mixtures contained 50 μl human IgG diluent, 10 μl $1 \cdot 10^{-7} M$ morphine–Cy5 and 10 μl 1:50 dilution of morphine antibody.

The electropherograms obtained with the other antibodies displayed evidence of poor affinity and/or antibody heterogeneity. Two of the antibodies did not display any significant binding to the labeled antigen, most likely due to incompatibility between the structure of our labeled drug and the immunogen used in the production of those antibodies. For immunochemical reaction with a labeled small molecule, the site of attachment of the label must be the same as the site of attachment of the carrier protein in the immunogen.

The cross-reactivity of nine opiate compounds towards the morphine antibody was tested by competition with the labeled morphine for the antibody binding site by CE–LIF (Fig. 3). The strength of binding of morphine derivatives to

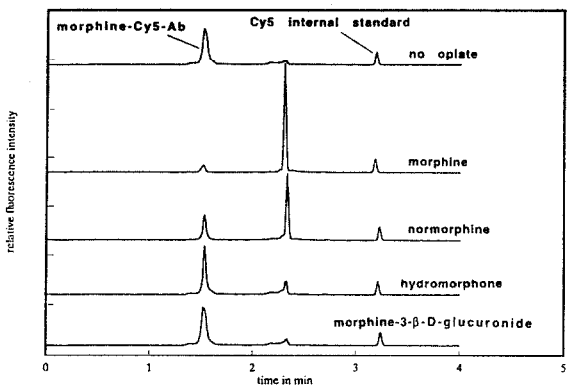

Fig. 3. Cross-reactivity of opiate compounds towards morphine N antibody. Mixtures contained 50 μl $1 \cdot 10^{-5}$ M opiate or blank diluent, 10 μl $1 \cdot 10^{-7}$ M morphine–Cy5 and 10 μl 1:50 dilution of morphine N antibody.

the morphine antibody is as follows: morphine> normorphine> hydromorphone> morphine-β-D-glucuronide. The codeine derivatives and levorphanol did not show any significant cross-reactivity with the antibody. These results indicate that the antibody has poor affinity to those compounds which are substituted at the 3-phenolic oxygen. This is expected for an antibody which was produced from an immunogen wherein the carrier protein was attached to the nitrogen of morphine.

The electropherograms for the competitive

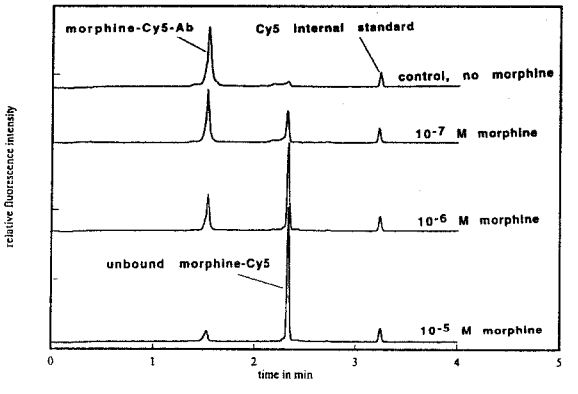

Fig. 4. Competitive binding assay of morphine by CE–LIF. Assay mixtures contained 50 μl morphine calibrator solution, 10 μl $1 \cdot 10^{-7}$ M morphine–Cy5 and 10 μl of 1:50 dilution of morphine N antibody.

immunochemical binding assay for morphine show increasing signal for the unbound labeled morphine and decreasing signal for the labeled antigen–antibody complex with increasing morphine calibrator concentration (Fig. 4). The limit of detection is $5 \cdot 10^{-8}$ M (14 ng/ml) morphine, well below the established cut-off concentration of $1 \cdot 10^{-6}$ M (300 ng/ml) for positive result in forensic urine drug testing is [3]. Thus, the present method is applicable for practical drug screening. The detection limit for competitive immunoassay using labeled antigen is highly dependent on the concentration of the labeled drug and the affinity between antigen and antibody. Thus, for analytes which are present at lower concentrations, the amount of labeled antigen and antibody can be adjusted to achieve lower detection limits.

4. Conclusions

The use of CE–LIF for the rapid screening of antibodies for binding to an antigen was demonstrated. Our results indicate that CE–LIF can be used to study an antibody population in a heterogeneous mixture and can also be used to determine immunochemical dissociation constants. The same CE–LIF method was utilized to study cross-reactivity of compounds of closely related structures towards an antibody by competition with the labeled antigen. A rapid and highly sensitive method based on competitive immunochemical binding by CE–LIF has been developed for quantitation of a biologically active analyte.

References

[1] B. Levine, *Anal. Chem.*, 65 (1993) 272A–276A.
[2] A.C. Moffat, J.V. Jackson, M.S. Moss and B. Widdop, (Editors), *Clarke's Isolation and Identification of Drugs*, Pharmaceutical Press, London, 2nd ed., 1986, pp. 524–525, 790–791.
[3] C.D. Klaassen, M.O. Amdur and J. Doull (Editors), *Casarett and Doull's Toxicology, The Basic Science of Poisons*, Macmillan, (New York), 3rd ed., 1986, pp. 863–866.

[4] P.D. Grossman, J.C. Colburn, H.H. Lauer, R.G. Nielsen, R.M. Riggin, G.S. Sittampalam and E.C. Rickard, *Anal. Chem.*, 61 (1989) 1186–1194.
[5] N. Schultz and R.T. Kennedy, *Anal. Chem.*, 65 (1993) 3161–3163.
[6] K. Shimura and B. Karger, *Anal. Chem.*, 66 (1994) 13–19.
[7] L.A. Ernst, R.K. Gupta, R.B. Mujumdar and A.S. Waggoner, *Cytometry*, 10 (1989) 3–10.
[8] R.B. Mujumdar, L.A. Ernst, S.R. Mujumdar, C.J. Lewis and A.S. Waggoner, *Bioconjugate Chem.*, 4 (1993) 105–111.
[9] F.-T.A. Chen, A. Tusak, S. Pentoney, Jr., K. Konrad, C. Lew, E. Koh and J. Sternberg, *J. Chromatogr. A*, 652 (1993) 355–360.