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Development of test strips for rapid buprenorphine detection *in vitro*

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ABSTRACT

Objectives: Buprenorphine (BUP) is the primary treatment for narcotic addiction, but it is often abused by opioid-dependent patients in many countries. For timely and effective detection and controlling the amount of BUP used in therapy, a rapid and sensitive test is needed. In the present study, we describe the development of test strips using monoclonal antibodies (MAbs) for the detection of BUP.

Design and methods: The MAbs were generated from hybridomas, and purified MAbs were used to create colloidal gold–antibody conjugates that were placed in the test strips.

Results: The BUP test strips had a limit of detection (LOD) of 12.5 ng/mL and did not cross-react with other drugs tested at physiological levels.

Conclusions: Therefore, this assay has sufficient sensitivity and specificity for BUP detection in urine specimens so that the dosage of BUP given to individuals being treated for opioid dependence can be monitored.

Introduction

Buprenorphine (BUP) is a derivative of opium and has been marketed in the United States for many years for the treatment of pain. It has been used for the prevention or treatment of moderate to severe chronic pain with therapeutic doses of 0.3–0.6 mg/day and also used in treatment of heroin addiction as an alternative to methadone with doses of 1–32 mg/day. In October 2002, the US Federal Drug Administration (FDA) approved BUP for the treatment of opioid dependency, and it is now available as a prescription medication [1]. However, BUP has the potential to become a drug of abuse when taken above the recommended dosage or without a doctor's approval [2]. BUP is more effective than morphine in relieving pain and can be administered intravenously, sublingually, or transdermally. In addition, BUP is used to treat patients dependent on other opioid drugs, such as heroin. While other opioid drugs have harmful withdrawal effects leading to psychological and physical pain, the withdrawal effects of BUP are minor and BUP dependency rarely occurs [3]. Therefore, switching patients to BUP is a useful way to treat patients with opioid dependency. After this switch, the addiction will hopefully subside, and opioid drugs will no longer be needed. When the use of BUP became legal, non-medical consumption also increased due to its increased availability and relatively low price [4]. This increased use has amplified the need for BUP testing. A rapid and sensitive assay for screening individuals for abuse and drug monitoring would be valuable, although confirmation methods, including gas or liquid chromatography (GC, LC) combined with mass spectrometry (MS), are necessary for the specific detection of BUP and its metabolites [5]. Test strips using BUP antibodies are a potential method for BUP detection. BUP detection in the urine should be effective as this drug has an elimination half-life of 24 to 72 h [6–8]. Additionally, test strips are an ideal method because they allow for quick, non-invasive, inexpensive testing that can be performed anywhere.

In this study, mice were inoculated with BUP conjugated to either bovine serum albumin (BSA) or bovine thyroglobulin (BTG, T1001 thyroglobulin from bovine thyroid) [9], and monoclonal antibodies (MAbs) recognizing BUP were developed. These antibodies were then purified, and colloidal gold–antibody conjugates were created for test strip production. Finally, the cross-reactivity of these test strips with different drugs was tested, and the antibody test strips were optimized.

Material and methods

Preparation of anti-BUP MAbs

MAb production and selection were performed by standard protocols. Specifically, BALB/c mice (8 weeks old) were subcutaneously immunized three times with 100 μg of BUP conjugated to either BSA or BTG [10]. Splenocytes from immunized mice with the highest antibody titer after a boost immunization with 25 μg of BUP-BSA or BUP-BTG were harvested

Abbreviations: BUP, Buprenorphine; MAbs, Monoclonal antibodies; GC, Gas chromatography; LC, Liquid chromatography; MS, Mass spectrometry; BSA, Bovine serum albumin; BTG, Bovine thyroglobulin; ELISA, Enzyme-linked immunosorbent assay; PBS, Phosphate-buffered saline; TMB, Tetramethylbenzidine; OD, Optical densities; SD, Standard deviations; MDEA, 3,4-Methylenedioxyethylamphetamine; BCOCB, Enzoylecgonine; THC, Δ-9 Tetrahydrocannabinol; NBUP, Norbuprenorphine; BUP-Gluc, Buprenorphine-3-β-D-glucuronide; NBUP-Gluc, Norbuprenorphine-3-β-D-glucuronide.

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and fused with SP2/0 myeloma cells. Cell culture supernatants from the wells containing hybridoma colonies were screened by enzyme-linked immunosorbent assay (ELISA), and strongly positive hybridoma cell clones were subcloned by the limiting dilution method and used for scale-up production of MAbs by injecting the cells into the abdomens of BALB/c mice.

Selection of MAbs by ELISA

The conjugated drugs BUP-BSA, BUP-BTG, ketamine-BSA, and ketamine-BTG were diluted to 5 μg/mL in coating buffer (sodium bicarbonate, pH 9.5), respectively. Then, 100 μL of each solution was added to a 96-well ELISA plate, and the plate was incubated at 4 °C overnight. The coated plates were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) four times, and the residual liquid was removed. Blocking buffer (150 μL; PBST containing 0.5% casein) was added to each well, and the plate was incubated for 2 h at room temperature. The plates were washed four times with PBST, 100 μL of cell culture supernatants were added to each well (except for the blank well), and the plates were incubated at 37 °C for 30 min. The plates were washed four times with PBST, a 1:5000 dilution of goat anti-mouse IgG-horseradish peroxidase conjugate (Biosource, Camarillo, CA, USA) was added, and the mixture was incubated at 37 °C for 1 h before the addition of tetramethylbenzidine (TMB) reagent. Optical densities (OD) were measured by a microplate reader (MK3, Thermo Labsystems, Grand Rapids, OH, USA) at 450 nm. The cut-off value was determined based on the average absorbance value of 10 negative mouse serum samples diluted at 1:100 plus five standard deviations (SD) [8]. The reaction results were classified into five groups according to the net values (OD of the test sample subtracted from the blank control) as follows: OD ≥ 2.0 (++++), 2.0 > OD ≥ 1.5 (+++), 1.5 > OD ≥ 1.0 (++), 1.0 > OD ≥ 0.5 (+), OD < 0.5 (±), and OD < cut-off value (−).

Purification of MAbs against BUP

MAbs were purified by ammonium sulfate precipitation [11]. In brief, ascitic fluids containing MAbs were centrifuged at 6000 rpm for 10 min, and the supernatants were collected. The supernatant (4 mL) was added to 4 mL of 30% (w/v) ammonium sulfate. The antibody mixtures were shaken vigorously on a flat shaker for 1 h and then allowed to sit at room temperature overnight. The mixtures were centrifuged at 8500 rpm for 30 min, and the supernatant and precipitate were collected separately. The supernatant was diluted 2-fold with 35% (w/v) ammonium sulfate, shaken vigorously on a flat shaker for 1 h, and then allowed to sit at room temperature overnight. The mixtures were centrifuged at 8500 rpm for 30 min, and the supernatant and precipitate were collected separately. The precipitate was resuspended in 2 mL of Tris–HCl (10 mM, pH 8.0) and desalted with a 10 mL Econo-Pac 10DG Column (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The desalted fractions were collected, and the OD at 280 nm was measured. The purified antibodies were analyzed by 12% SDS-PAGE gels.

Preparation of colloidal gold-antibody conjugates (S4)

To produce colloidal gold–antibody conjugates, MAbs were added to a 1× colloidal gold solution to a final concentration of 75 μg/mL. The optimal conditions for antibody binding to colloidal gold were determined by adding 0.1 M K_2CO_3 to adjust the pH so that the color of the conjugated solution matched that of the 1× colloidal gold solution. The mixtures were allowed to sit for 10 min at 37 °C, and 1× Protector (Artron BioReseach Inc., Burnaby, BC, Canada) was added to the colloidal gold–antibody-conjugated solutions to a final concentration of 5% (v/v). The solutions were then centrifuged at 4000 rpm for 45 min and allowed to sit for another 30 min before the precipitated conjugates were collected. The precipitated conjugate concentrations were measured at 532 nm, and 25% of 1× Protector was added to adjust the concentration to an OD_{532} of 25. The conjugated solutions were spread onto nonwoven fabrics and dried for 72 h at 37 °C.

Preparation of coated membrane (S1)

BUP-BSA and BUP-BTG were diluted to 200 ng/mL, 250 ng/mL, 300 ng/mL, and 400 ng/mL in 10 mM Tris–HCl (pH 8.0) and coated, respectively, on a test region (T line) of nitrocellulose membrane using a coating machine (Bio-Dot). Goat anti-mouse IgG (1 mg/mL) was applied as an internal process control and coated on the control area (C line) of the membrane. After drying at 37 °C overnight, the coated membrane was blocked with blocking buffer (PBST + 3% BSA) for 2 h. The membrane was washed three times with PBST with shaking and allowed to dry at 37 °C overnight.

Test strip assembly

Colloidal gold–antibody-conjugated fibers (S4) were assembled with the coated membrane (S1), absorbing pad, glass fiber, and thick and thin fibers (sample pad) on a plastic backing board (Fig. 1). All layers were assembled with slight overlaps for a rapid lateral flow. The test strips were made with different combinations of S4 widths (1.2 cm, 1.0 cm, 0.7 cm, and 0.6 cm) and S1 BUP-BTG and BUP-BSA concentrations (200 ng/mL, 250 ng/mL, 300 ng/mL, and 400 ng/mL). Finally, the test strips were cut into 3 mm widths by a cutter.

Sensitivity

Sensitivity was assessed by establishing the limit of detection (LOD). Drug-free urine samples collected from patients in the Laboratory Medicine Department, the Second Affiliated Hospital of Chongqing Medical University (China), were spiked with BUP at the following concentrations: 5 ng/mL, 7.5 ng/mL, 10 ng/mL, 12.5 ng/mL, and 15 ng/mL, respectively. Then, the LOD of BUP was determined using the colloidal gold–antibody-conjugated test strips.

Specificity

To assess the specificity, high concentrations (100 μg/mL) of potentially interfering licit and illicit drugs were added to drug-free urine samples. The following drugs and metabolites (in bracket with its parent drug) were evaluated: acetaminophenol, alprazolam, amobarbital, amitrityline, amphetamine, butabarbital, benzoylecgonine, codeine, cocaine, clomipramine, clonazepam, clobazam, diphenhydramine, diazepam (nordiazepam, oxazepam, temazepam), dextromethorphan, estazolam, ecgonine, flunitrazepam, flurazepam, fentanyl, methadone, heroin (6-acetyl-morphine), hydrocodone, hexobarbital, imipramine (desipramine), ketamine, lorazepam, maprotiline, methaqualone, morphine (morphine-β-glucuronide), methamphetamine, 3,4-methylenedioxyethylmethamphetamine (MDMA), nitrazepam, nortriptyline, phenobarbital, prazepam, promethazine, phencyclidine, protriptyline, (*S*,*S*)-(+)-pseudoephedrine, (*R*,*R*)-(−)-pseudoephedrine, secobarbital, trizaolam, trimipramine, Δ-9 tetrahydrocannabinol (THC), and (±)THC-COOH. The spiked urine samples were analyzed with the colloidal gold–antibody-conjugated test strips.

Precision

To assess the precision and random error of visual interpretation of the colloidal gold–antibody-conjugated test strips, three individuals performed the assay and observed the test results. An identical panel (20 samples of every concentration) of coded specimens containing 5 ng/mL, 7.5 ng/mL, 10 ng/mL, 12.5 ng/mL, 15 ng/mL, and 20 ng/mL BUP was provided to each operator for testing.