Multiplexed immunoassays by flow cytometry for detection of clenbuterol, chloramphenicol and sulfadimidine with high sensitivity and selectivity

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An accurate, rapid and cost-effective detection on the environmental hazardous chemicals is the cornerstone of efficient food safe management. Here we discuss the relevance of an emerging technology, multiplexed competitive immunoassays read by flow cytometry, for the detection of Clenbuterol, Chloramphenicol and sulfadimidine. In these assays, multiple fluorescent microspheres, conjugated to different test antigens, constitute the solid phase for detecting antigens in biological samples based on the competitive ELISA principle. These assays are more sensitive than traditional immunoassays , have a high throughput capacity provide a wide analytical dynamic range and are powerful tools for exposure analysis and assessment offering low-cost screening with minimal sample pretreatment requirements combined and served a better alternative for the instrumental detection on the derivatives of those metabolites that often require expensive instrumentation. The sensitivity for the detection limit of the simultaneous identification of clenbuterol, chloramphenicol and sulfadimidine can reach 0.5ng, 2.0ng and 0.5ng/mL, which shows the promising multiplexing ability. Therefore, we predict a widespread application for a new breed of small, affordable, practical flow cytometrics as field instruments for replacing conventional ELISA and sophisticated GC or HPLC analysis.

Key Words: multiplexed immunoassays, flow cytometrics, clenbuterol, chloramphenicol , sulfadimidine,competitive ELISA

Clenbuterol (2-[*tert*-butylamino]- 1-[4-amino-3,5-dichloro-phenyl]-ethanol hydrochloride) is a major β-agonist drug reported to be used for illegal purposes in man and animals. The use of clenbuterol (CL) to improve athletic performanceis banned by the sport authorities [1]. CL is also fraudulently used to promote growth in meat-producing animals [2-5]. The most serious adverse effect of chloramphenicol is bone marrow depression. Serious and fatal blood dyscrasias (aplastic anemia, hypoplastic anemia, thrombocytopenia and granulocytopenia) are known to occur after the administration of chloramphenicol. An irreversible type of marrow depression leading to aplastic anemia with a high rate of mortality is characterized by the appearance weeks or months after therapy of bone marrow aplastia or hypoplasia. Peripherally, pancytopenia is most often observed, a reversible type of bone marrow depression, which is dose related, may occur. This type of marrow depression is characterized by vacuolization of the erythroid cells, reduction of reticulocytes and leukopenia, and responds promptly to the withdrawal of chloramphenicol. Chloramphenicol is categorised by the IARC (International Agency for Research on Cancer) as probably carcinogenic in humans.

Measurement of those illicit drug levels has in recent years been dominated by instrumental and ELISA methods. While ELISA offers a sensitive approach with detection in the low pg/mL range, the technique is hampered by its heterogenerous format with multiple addition and wash steps and an absorbance readout with substantial variability and interference. Complex pretreatments of derivation did not allow for the popularity of GC and HPLC methods to determine those molecules. Uniquely, this method provides the ability to combine different immunoassay formats in a single detection system: a competitive-inhibition format for the simultaneous measurement of the three representative drugs.

Therefore, an alternative microsphere-based immunoassay by flow cytometrics that offers comparable or higher sensitivity, better reproducibility, greater dynamic range, shorter preparation time, higher throughput capacity and the capability of simultaneous measurement of analytes in biological matrices was initially put forward in this study [6].

MATERIALS

Carboxyl SPHERO^{TM} microspheres (Φ 4.0μm, Spherotech, Libertyville, IL) were applied as solid carriers.. The McAb, hapten of the three analytes

(clenbuterol, chloramphenicol and sulfadimidine) and bovine serum albumin (BSA) were purchased from Wanger Biotechnology Inc. FITC goat anti-mouse IgG antibody conjugate was obtained from USA. N-Hydroxysuccinimide(NHS) and 1-ethy-3-(3- dimethyaminopropyl) carmodiimide hydrochloride (EDC) were purchased from Fluka(switzerland). Activation buffer (0.1 mol L^{-1} NaH_2PO_4, pH 6.2), storage/blocking buffer (PBS, 1%BSA, 0.05% NaN_3 (*Sigma*), pH 7.4), wash buffer (PBS containing 0.05% Tween20; agdia), dilution buffer (PBS, pH 7.4) and coupling buffer (0.05 mol L^{-1} 2-(*N* morpholinoethanesulfonic acid, MES; *Sigma*; pH 5.0). Labeling result is determined by measuring the absorbances at the related wavelengths on a spectrophotometer (Beckman ALTRA HyPerSort System) according to EXPO^{TM} 32 MultiCOMP software.

METHODS

PREPARATION OF MICROSPHERE

All prepared capture antigens diluted in PBS buffer were at a level of 5–10μg/mL. After the sonication for 30s, every 10^4 microsphere was immediately added 200μL PBS, and centrifuged (*Sigma*,3k30 super-speed bench ,UK) at 5,700 rpm for 20 min. The microspheres were washed twice with PBS to secure the pellets were not adhered to the wall of tubes. The supernatant was removed and the rest microspheres were suspended in 80 μL PBS. The level of EDC and sulfo-NHS was adjusted to prepare a final concentration of 5mg/mL. A solution of hapten–BSA(5μg/mL) was added to each aliquot and they were incubated by vortex shaker for 3 h in the dark and centrifuged for 20 min to remove the supernatant, incubated in a blocking buffer for 2h and washed twice and then resuspended in 500μL PNT(PBS+1%BSA), stored at 4！ overnight.

MICROSPHER-BASED COMPETITIVE FLUORENSCENT IMMUNOASSAY

Aliquots of the suspension of functionalized microspheres were recentrifuged, the supernatant was removed. Added 50μL analyte, then added 20μL 4.23μg/ml antibody to the respective microspheres tube, vortexed immediately for 10 s. The mixture was incubated on a rocker at room temperature in the dark for 2 h. The microspheres were centrifuged for 20 min, and then the separated microsphere were washed with wash buffer twice. Next, microspheres were removed supernatants and resuspended in 100μL of 10μg/ml FITC-goat anti-mouse IgG to each tube. The microspheres was shaken in the dark for 30 min and centrifuged for 20 min, washed with wash buffer twice and then diluted in 600μL wash buffer. The tube was shaken vigorously for approximately 1 min to disperse the microspheres.

Flow Cytometric Analysis

A COULTER EPICS ALTRA HYPerSort^{TM} system (Beckman Coulter, Inc. Fullerton,CA) equipped with an air-cooled argon ion laser (488 nm) and HeNe ion laser(633nm) was used for this study. Fluorescence of thousands of beads was reported by EXPO^{TM} 32 MultiCOMP software. To design a protocol for immunophenityping dual-signals, fluorescent light intensities of individual microspheres were measured and four-parameter histograms showed adhesion ratio. To determine the fluorescence intensity of a microsphere, a region of interest (ROI) was drawn around the microsphere and the fluorescence intensity within the ROI measured according four-parameter logistic log fits.

RESULTS

DETERMINATION OF MICROSPHERE-BASED COMPETITIVE FLUORESCENT IMMUNOASSAY PARAMETERS

Flow cytometry displays the intensity of the fluorescent microspheres bound with Antibody-FITC with the most intense signal present in the absence of competing free clenbuterol (see Fig. 2).

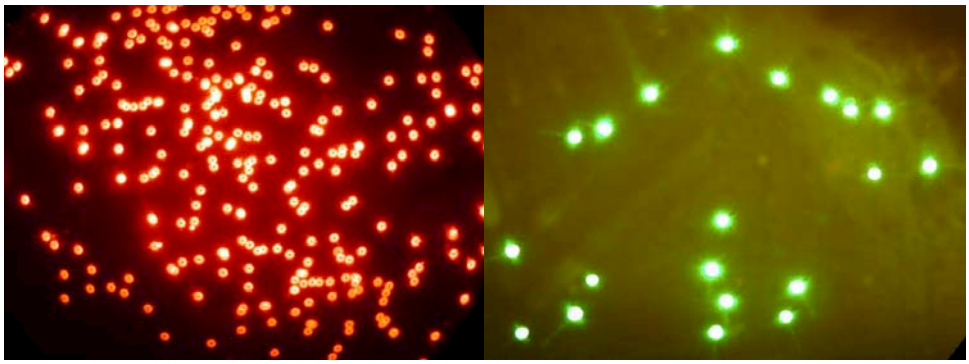
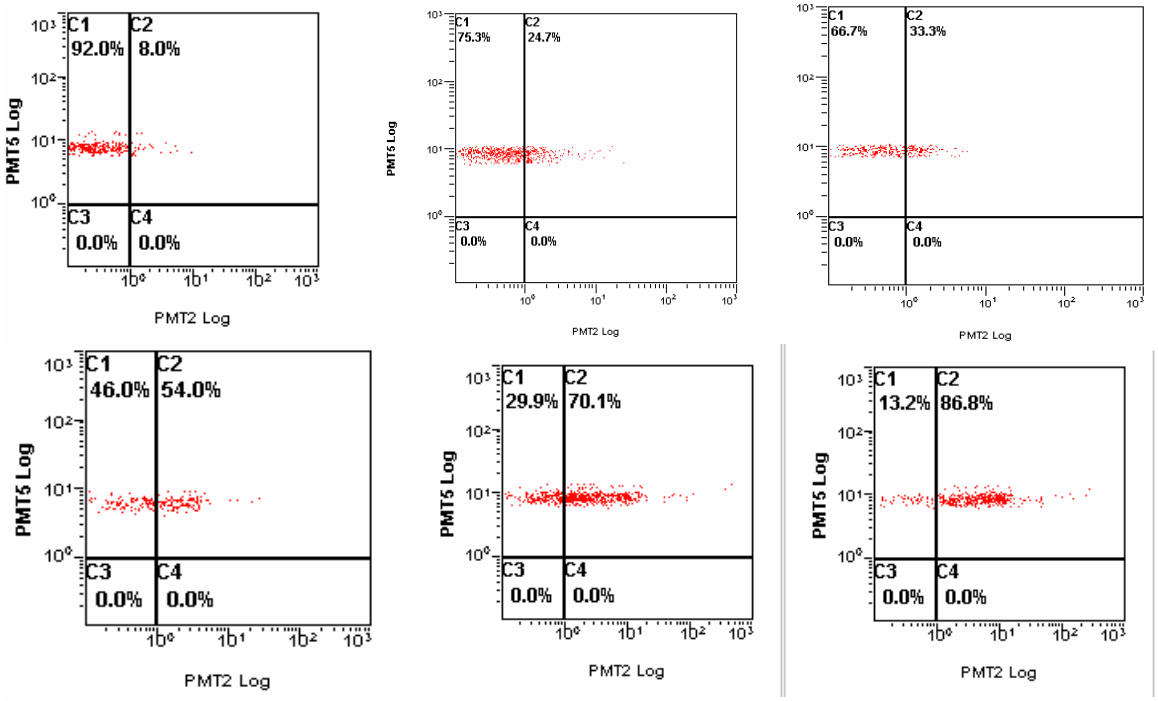
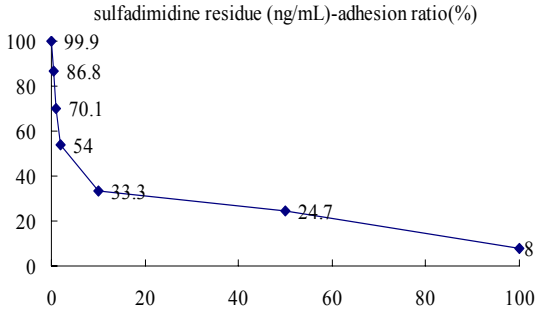


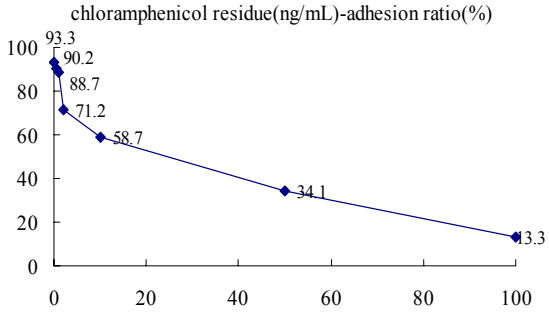
Fig. 2 The fluorescence of microsphere excited by green and red laser

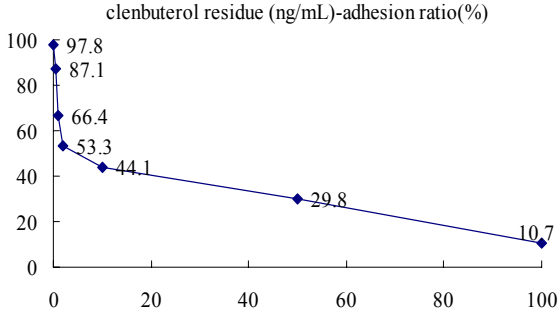
Effect of the Mode of Microsphere-based competitive fluorescent immunoassay

Images showing the analytical and encoding signals were captured by a flow cytometry interfaced with a PMT detector. Highly significant linear relationships were observed.









Comparison of Microsphere-based competitive fluorescent immunoassay with ELISA

(ELISA MDC =0. 5 ng/mL) .This novel method will be exploited to detect immune responses from multiplex veterinary residuals.

Discussion

First, we devised and optimized competitive immunoassays for the individual analyte using labeled BSA immobilized on microspheres. Furthermore, we are contuine studying multianalyte based on this method. To determine

the fluorescence intensity of a microsphere, a region of interest (ROI) was drawn around the microsphere and the fluorescence intensity within the ROI measured according four-parameter logistic log fits. The specificity of this gating is such that two different-sized or different fluorescent particles, each with a different antigen used to bind a different fluorescent ligand, can be used to simultaneously measure multiple analytes.

In the early stage of our investigation, antibodies labeled with a fluorescent dye were used in relatively high concentrations to generate appropriate fluorescent signals. Therefore, the sensitivity of these assays was only modest. To achieve high sensitivity with heterogeneous competitive immunoassays, it is usually necessary to keep the concentrations of the immunoreagents low. Furthermore, low analyte and labeled antigen concentrations are needed to minimize nonspecific effects and interferences between the individual immunoassays of multiplexed assays. Nonspecific binding to the solid surface, cross-reactivity, and aggregation of the individual tracer molecules can decrease the performance of multiplexed immunoanalyses[7]. It appears that it is more difficult to meet these requirements for competitive than for noncompetitive immunoassays because the latter assays are generally more sensitive [8]. Most reported multianalyte immunosensing methods are in the noncompetitive (sandwich) format.

In our experiment, we are previously attributed to decrease microsphere, FITC and monoclonal antibody nonspecific reaction. Coupling BSA to active microspheres, Incubate microspheres for 2 h on a rocker in dark, then centrifugation at 5,700 rpm for 20 min and wash twice with 150 μL wash buffer. Direct coupling monoclonal antibody or FITC goat anti-mouse IgG antibody can perform FITC or monoclonal antibody between microsphere nonspecific connection. For best assay response and sensitivity, we must be optimized to provide maximal labeling efficiency of bound antigen accompanied by minimal nonspecific binding with each other.

The sensitivity, dynamic range, and robustness of the Microsphere-Based Flow Cytometric Immunoassay are comparable to those of ELISA because ELISA is traditional method to determination residues. ELISA, although quantitative, precise, sensitive, and accurate, are designed to measure only one analyte per assay, for example,clenbuterol residual immunoassay kit, yielding 96 data points per plate. The Microsphere-Based Flow Cytometric Immunoassay give accurate, specific and reproducible results while saving time, sample and money, also small simple, multiplexing capabilities, more sensitivity and specificity. As the level of multiplexing is increased, the throughput is increasingly elevated. We will design the simultaneous measurement of several veterinary medicine using microsphere-based competitive fluorescent immunoassay for a total throughput of many analytes per sample.

In future work, the precision of our microsphere-based assays could be increased by using arrays with higher microsphere density, improved immobilization chemistry, and optical imaging fibers and an imaging system better suited for these analyses. Furthermore, it appears that the sensitivity of the analyses could be increased and the interferences between the assays could be minimized by employing even more efficient signal generation material such as quantum dot, a novel and superior fluorescent material.

In conclusion, we devised and optimized Microsphere-based competitive fluorescent immunoassay for the individual veterinary medicine(clenbuterol) residueusing labeled antigen immobilized on fluorescence microspheres. The result proved to be very sensitive for the determination of clenbuterol. Lower limit of detection and quantification was obtained by the new method. Whereas, there are few methods at present for the examination of veterinary residual. This method can be used to monitor clenbuterol residue in edible tissues, pork and dairy products.

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