Development of Miniaturized Competitive Immunoassays on a Protein Chip as a Screening Tool for Drugs

Hongwu Du,1,5,6 Moutian Wu,2 Weiping Yang,3 Gu Yuan,4 Yimin Sun,5 Yuan Lu,5 Shan Zhao,5 Qingyun Du,3 Jun Wang,4 Sheng Yang,2 Mangen Pan,5 Ying Lu,5 Shan Wang,2 and Jing Cheng1,5,6*

Background: Doping in sports has become a serious problem. Gas chromatography–mass spectrometry (GC-MS) serves as an effective reference method, but it is limited by low throughput and is therefore not suitable for large-scale screening. Use of protein chips for high-throughput screening of all athletes for prohibited substances could become an important complementary tool to GC-MS.

Methods: We developed a protein chip based on an aldehyde-activated glass slide containing 10 physically isolated arrays. The chip was used to screen urine from 1347 athletes for prohibited substances and to screen a negative control group consisting of 200 females and 120 males. Urine samples from 66 individuals known to be abusers, provided by the China Doping Control Center (CDCC), and 129 standard prohibited substances were tested as positive controls.

Results: All 1347 urine samples screened by means of the protein chips were also subjected to reference analysis by GC-MS at the CDCC. There was no qualitative difference between the results obtained with the two methods. The correlation coefficient ($r^2$) for the quantitative results obtained with the protein chip and GC-MS was 0.991.

Conclusions: The protein chip could be used to screen for a series of 16 prohibited drugs in urine samples. This system has the potential to become an effective screening method to test substances prohibited by the International Olympic Committee.

© 2005 American Association for Clinical Chemistry

Cheating in sports through use of prohibited substances has become increasingly common, and drug-abusing athletes are endeavoring to be one step ahead of the testing authorities. To minimize doping in sports and to promote health, fairness, and equality for athletes worldwide, the World Anti-Doping Agency (WADA)7 has initiated a series of efforts to combat this problem. Despite impressive progress made in detecting doping agents, many believe that the current methods alone cannot be effective in preventing doping in sports (1). In fact, no reliable method is available to detect certain substances, such as human growth hormone and other substances with similar chemical structures (2–5).

A change in attitude toward doping, with systematic screening for all athletes in major sporting events, may be an effective way to combat the problem. It should be an indispensable part of the Olympic Games (6, 7), and indeed, the number of athletes screened for doping in recent Olympic Games, such as the Sydney and Salt Lake City games, has increased significantly (8, 9).

There are ~140 substances that have been used for doping (10). They range from small synthetic chemical...
molecules to peptides such as insulin and erythropoietin. The list of prohibited substances, issued by WADA, increases with pharmaceutical developments each year. Because of the vast differences in the physical and chemical characteristics of the prohibited substances, it remains a challenge to find a general method that can detect them all in a single assay.

A combination of gas chromatography (GC) and mass spectrometry (MS) is still the most reliable drug testing method and has been used since the 1972 Olympic Games (11, 12). This method can detect and differentiate minute amounts of analytes. However, its usage as a screening tool is limited because of the cost of the instrument and the lengthy procedures required for sample preparation. The use of this method is further restricted by the limited differential power of the molecular weights of analytes. As a result, the GC-MS method is reserved as a reference tool for low-molecular-weight drugs that may be abused by winners of sporting events (13).

Biochip technology is one of the fastest growing fields in the analytical sciences (14, 15). It is becoming a method of choice for simultaneous and high-throughput measurement of multiple analytes by use of nonporous and porous solid supports combined with fluorescence-based detection (16, 17). Biochip immunoassays promise to revolutionize both clinical diagnosis (18) and drug testing (19) in situations where there is a need to simultaneously detect multiple analytes. We have developed a novel protein chip system based on a competitive immunoassay design for the simultaneous detection of up to 16 prohibited substances in a single drop of urine. In this assay, anti-drug antibody competes for drug immobilized on the protein chip and drug present in the sample. Anti-drug antibody bound to the slide is then quantified by use of a Cy3-labeled second antibody (Fig. 1), and the entire process can be completed in 2 h. This method is highly sensitive and reproducible, and for routine applications one technician can process hundreds of samples per day.

Materials and Methods

Samples from Athletes
Urine samples (from 557 female and 790 male athletes) were collected between June 2002 and March 2003. All urine samples were stored at −20 °C. The negative control group consisted of urine samples provided by 200 female and 120 male athletes and had been confirmed as drug free by GC-MS. Sixty-six confirmed positive urine samples (21 female and 45 male; 30 positive for anabolic steroids, 24 positive for stimulants, and 12 positive for narcotics) were collected by the China Doping Control Center (CDCC) between November 1982 and December 2002.

Antibodies and Reagents
Twenty-three conjugated antigens and their corresponding antibodies were purchased from Fitzgerald Industries International, Inc. Six antibodies were produced by Aviva Antibody Corporation. Five drug–bovine serum albumin (BSA) conjugates were synthesized at the Key Laboratory of Bioorganic Chemistry and Molecular Engineering, Peking University, China. Cy3-labeled goat anti-mouse IgG (H+L; 1.0 mg/vial; product code PA43002; absorbance maximum, 550 nm; emission maximum, 570 nm) was purchased from Amersham Pharmacia Biotech. The CDCC provided 129 standard drugs, including 50 anabolic steroids, 68 stimulants, and 11 narcotics. All test substances were chosen from the 2004 Prohibited List published by the World Anti-Doping Agency (10). The antibodies used for the protein chip assays are summarized in Table 1.

Preparation of Protein Chip Substrates
Glass slides chemically modified with aldehyde groups were used as the substrate to covalently bind BSA-conjugated molecules at the designated locations. The slides were cleaned with 100 g/L chromic acid for 6 h, followed by rinsing with deionized water. Slides were then dipped into a 2 mol/L sodium hydroxide solution and then 4 mol/L hydrochloric acid, each for 30 min, followed by rinsing with deionized water and then drying under a stream of nitrogen. Cleaned slides were silanized for 8 h using 3-glycidoxypropyltrimethoxysilane in etha-
nol (40 mL/L). The glass surface was washed with toluene, acetone, and deionized water, after which the slides were dipped in 4 mol/L hydrochloric acid again for 30 min and then immersed into 50 mmol/L NaIO4 for 1 h to complete the preparation process. The contact angles of the aldehyde-activated slides were measured by use of a contact angle system (Model OCA; DataPhysics Instruments GmbH) for quality-control purposes. Slides were stored in a desiccated box at room temperature for a maximum of 3 months.

Printing of Protein Chips
Ten 9 × 9 arrays of BSA-conjugated drugs were printed on each slide. For a peptide hormone, the peptide was printed directly. On each slide, one sample can be tested on one 9 × 9 array for a variety of analytes, and up to 10 samples can be analyzed in parallel on one protein chip. A contact printing robot (PixSys 5500; Cartesian Technologies) with a stealth microspotting pin (Model SMP3; TeleChem International) was used to print the protein chips on the aldehyde-activated slides. The concentration each the printed protein (drug-BSA) was 500 mg/L in 400 mL/L glycerol or Protein Printing Buffer (TeleChem International). The drug-BSA conjugate was reacted on the protein chip for 6 h in a humidified chamber. The slide was then stored at room temperature for up to 1 month.

Immunoassay Procedure
A competitive immunoassay design was used to test the 16 WADA-prohibited substances on the protein chips. A molded polyester frame was attached to the substrate to partition 10 arrays on the protein chip surface (Fig. 2A). This protein chip consisted of 16 different drug-BSA conjugates and 11 positive or negative controls to form a 9 × 9 array. Each material was printed in triplicate (Fig. 2, B and C). The protein chips were immersed in blocking solution [a 1:10 dilution of sheep serum in phosphate-buffered saline (PBS), pH 7.4] for 30 min at room temperature and then rinsed three times with PBS containing 0.5 mL/L Tween 20, pH 7.4 (PBS-Tween A). A mixture of the anti-drug mouse monoclonal antibodies and urine sample containing the drug was then applied to the gridded reaction chamber formed by the polyester frame covering the surface of the protein chip. The protein chip was then maintained at 37 °C in a humidified chamber for 30 min. The protein chip was then rinsed three times with PBS-Tween A, and the secondary antibody (Cy3-labeled goat anti-mouse IgG) was applied to the protein chip and incubated at 37 °C for 30 min. The protein chip was then washed again and scanned for the presence of bound Cy3-labeled secondary antibody by use of a laser confocal scanner (GenePix 4000B; Axon Instruments) or a charge-coupled device-based scanner (EcoScan-100; CapitalBio Corporation). The analog fluorescent signal was converted to digital signal by data analysis software (GenePix Pro 4.0; Axon Instruments). The results obtained from the protein chip were later compared with those obtained by GC-MS at CDCC.

Results

Prohibited Substances Detected on Protein Chips
An examples of an image of the biochip obtained with a samples negative for amphetamine is shown in Fig. 3. Examples for 14 additional drugs are provided in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue2/. Each drug was arrayed in triplicate on the aldehyde-activated protein chip (boxed areas in Fig. 3). As expected, the three test spots for each drug bound the anti-drug antibody and then the bound Cy3-labeled second antibody to give a fluorescent signal. In each case, all of the mouse IgG control spots (upper and low rows of nine spots and the two central groups of three spots) were positive, as would be expected from reaction of the

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoylcegonine</td>
<td>Aviva</td>
</tr>
<tr>
<td>Boldenone</td>
<td>Aviva</td>
</tr>
<tr>
<td>MDMAa</td>
<td>Aviva</td>
</tr>
<tr>
<td>Methenolone</td>
<td>Aviva</td>
</tr>
<tr>
<td>Isotestosterone</td>
<td>Aviva</td>
</tr>
<tr>
<td>Tetrahydrocannabinol</td>
<td>Aviva</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Barbiturate</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Benzodiazepine</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Caffeine</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Cortisol</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Digoxin</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Estradiol</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>hCG-α</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>hCG-β</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Insulin</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>LH</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Methadone</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Morphine</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Phenylclobidine</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Progesterone</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>TCA</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Testosterone</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Tobramycine</td>
<td>Fitzgerald</td>
</tr>
<tr>
<td>Theophylline</td>
<td>Fitzgerald</td>
</tr>
</tbody>
</table>

a MDMA, 3,4-methylenedioxyamphetamine; hCG, human chorionic gonadotropin; IGF-1, insulin-like growth factor-1; LH, luteinizing hormone; TCA, tricyclic antidepressants.
immobilized mouse IgG control with the goat anti-mouse conjugate used in the assay.

**Influence of Different Matrices on the Fluorescence Signal on Protein Chips**

The potential effect on the fluorescent signal of different samples and solutions, such as urine, water, or PBS, was evaluated. We found that different solutions could dramatically affect the signals for certain tested substances. We have noticed a significant signal decrease for steroids when the solution was changed from PBS to urine. This may be attributable to the binding of some endogenous steroid interferents with the corresponding antibodies. Most of the exogenous drugs have a comparable ratio of PBS to blank urine commonly $<1.50$, but we noticed that certain analytes, such as amphetamine, generated an exceptionally high ratio of $\sim 2.50$ (Table 2). We repeated the assay in a 96-well plate and found that all exogenous
analyses containing amphetamine had a low PBS-to-blank urine ratio of 1.13–1.18. This suggests that some nondoping substances in human urine may have interfered with the interaction of amphetamine and its antibody.

**Detection Limit and Cutoff Value**

In principle, the fluorescent signal at the corresponding location is decreased when a tested substance is present in the sample because the drug in the sample competes with drug immobilized on the protein chip (BSA-drug) for the anti-drug antibody. Within the linear measurement range, the decrease in fluorescent signal was proportional to the amount of drug in the sample. This method can therefore be used for both qualitative and quantitative determination of the presence of substances in a sample. A calibration curve for amphetamine measured with use of the biochip is shown in Fig. 4. Calibration curves for nine additional prohibited substances are provided in the online Data Supplement. The detection limit is defined as the lowest concentration of an analyte that can be detected by the protein chip. This concentration corresponds to a signal that is 3 SD lower than the mean of the negative control and ranged from 0.2 μg/L for morphine to 19 μg/L for methadone. The detection limit and the cutoff values (the 50% inhibitory concentration) for the 10 drugs are summarized in Table 3.

**Assay Precision**

In the precision studies, standard samples, including 200 blank urines confirmed by the doping-control-analysis method at CDCC, were repeatedly analyzed (n = 300) by five technicians using different batches of protein chips. The between-batch CV for all analytes was 16% and the within-batch CV was 13% (Table 4).

**Qualitative Analysis**

In a typical screening procedure, we collected urines from 141 Chinese gymnastic athletes and selected morphine as...
a representative example drug for qualitative analysis. Fig. 5 shows representative protein chip assay results for eight specimens, including a negative specimen (Fig. 5A), five specimens positive for morphine (Fig. 5, B–F), a specimen positive for dihydrocodeine (Fig. 5G), and a specimen positive for pethidine (Fig. 5H). The absence of a fluorescence signal at the boxed location indicated a positive result. We also detected cross-reactivity with the anti-morphine antibodies for samples containing dihydrocodeine and pethidine. The mean (SD) morphine signal was 0.429 (0.12), and the critical value (used in significance testing, which is the value that a test statistic must exceed for the null hypothesis to be rejected) was 0.232 (P < 0.05), which is equivalent to 1.7 μg/L morphine. The sample would be positive for morphine if the measured signal was below the critical value. All positive samples were confirmed by GC-MS at CDCC.

In addition to serving as a means for qualitative screening tool for large numbers of samples, the protein chip can also be used for quantitative analysis. Table 5 shows the results of six replicate tests for samples from four methamphetamine drug abusers detected by both the protein chip and a GC-MS method. The correlation coefficient (r²) for the protein chip and GC-MS results was 0.991, indicating the comparability of the results obtained by these two methods for quantifying methamphetamine in urine.

### Discussion
Protein chips can be applied to functional studies of proteins, can facilitate systematic studies of the function of thousands of proteins, and can provide an integrated understanding of biological systems (20–22). The design of diagnostic protein chips has focused on clinical applications (23, 24). Ekins and Chu (25) suggested that quantitative immunoassays could be developed that use mi-
crospots of antibodies on nonporous solid supports, thereby enabling multianalyte assays, but it was only recently that such testing biochips have become a reality (26–29). A sophisticated clinical profiling system, the Evidence developed by Randox Laboratories Ltd., is the premier example of the commercial multianalyte-analysis protein chip system intended for routine clinical diagnostic applications.

The protein chip technology should be applicable to a wide range of substances, including stimulants, narcotics, anabolic agents, and peptide hormones. In principle, nearly all prohibited drugs can be tested by this technology. The limit at the moment is the availability of the corresponding antibodies and antigen conjugates. In this study, in addition to the available commercial anti-drug antibodies, we also synthesized and produced several drug-BSA conjugates and the corresponding antibodies. More antibodies will need to be produced to detect more prohibited substances.

We have developed a protein chip suitable for use in miniaturized immunoassays for doping control. This protein chip can be used to simultaneously analyze many prohibited drugs on a single array as well as to detect other suspicious drugs. For example, a sample from a female athlete that had been confirmed methamphetamine positive by CDCC tested positive on the protein chip, as expected. However, it also was positive for morphine and human chorionic gonadotropin. Later, this

---

**Table 5. Comparison of methamphetamine concentrations obtained with the protein chip and GC-MS.**

<table>
<thead>
<tr>
<th>Methamphetamine positive</th>
<th>Protein chip, mg/L</th>
<th>GC-MS, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>3.9 (1.1)</td>
<td>4.2</td>
</tr>
<tr>
<td>Sample 2</td>
<td>6.9 (1.3)</td>
<td>6.1</td>
</tr>
<tr>
<td>Sample 3</td>
<td>8.1 (3.6)</td>
<td>7.5</td>
</tr>
<tr>
<td>Sample 4</td>
<td>15.0 (5.1)</td>
<td>14.6</td>
</tr>
</tbody>
</table>

*Mean (SD); n = 6.
sample was retested by CDCC. The positive morphine result was confirmed by GC-MS, and the positive result for human chorionic gonadotropin was confirmed by a pregnancy test.

One of the major factors influencing the performance of protein chips is the development of an appropriate substrate for immobilization of printed proteins (30, 31). In the present work, aldehyde-activated glass slides were found to be the most suitable substrate for protein immobilization. Experimentally, aldehyde-activated slides have some distinct advantages. They were more stable and demonstrated lower CVs for quantitative work. Additionally, proteins immobilized on aldehyde-activated slides were resistant to removal by PBS–100 mL/L Tween 20, acetonitrile, and trifluoroacetic acid during washing steps. This indicated that the aldehyde surface provides better immobilization. In addition, the molecules covalently attached to the aldehyde-activated slide surface retained their ability to interact with target proteins in solution.

Quality control is very important in a biochip immunoassay, and we have set up a series of measures to control the quality of the assay results. We first chose the glass slides from more than 10 available companies and then selected each slide for roughness, flatness, and thickness. We next measured the contact angle after chemical modification of the slides and chose only eligible slides (contact angle in range 60–70 degrees) as substrates for printing protein chips. We then examined the protein chips by Cy5 fluorescence scanning to confirm that the mean CV of the printing was <10%. Finally, if the mean CV of the signal with mouse IgG (control spots) was >20% within a protein chip, the assay was then automatically considered to have failed.

Although specific antibodies against dihydrocodeine and pethidine were not available for this study, samples positive for these two substances could also be detected because of the cross-reactivity of the morphine antibody used in the assay. Such cross-reactivity is well known in immunologic methods for opiates (32) and results from the chemical similarities of dihydrocodeine and pethidine to morphine. The presence of dihydrocodeine and pethidine in the samples was confirmed by ELISA.

Cross-reactivity may be a valuable asset for protein chips because it enables detection of more analytes with limited numbers of antibodies. Although the WADA list of prohibited substances extends to 140 different drugs, their chemical entities can be subgrouped, and some compounds within each group have similar core structures. Cautious screening of antibodies that recognize the core structure, optimization of the antigen immobilization ratio, and suitable data processing may allow the use of ~30–50 antibodies to detect all of the prohibited substances on the list because our protein chip serves the purpose of an initial screen. Under such conditions, all questionable positive samples could be identified and a list of potential substances obtained. The suspicious samples would be confirmed by GC-MS analysis.

When a specific analytical method is not available for a new drug, the cross-reactivity may be a useful means for detection. For example, tetrahydrogestrinone (THG) is a new steroid that caused alarm in the sporting world when the US Anti-Doping Agency first found it in samples collected at the 2003 US Outdoor Track and Field Championships. If an antibody against gestrinone (a steroid structurally related to THG) were available, it might be possible to identify all samples containing gestrinone, THG, or other structurally related substances.

The definition of a screening procedure by WADA is “an analytical test procedure whose purpose is to identify those samples which are suspicious with respect to containing a prohibited substance or metabolite or marker of a prohibited method and which require additional confirmatory testing”. The criteria for accepting a screening result and allowing the testing of the sample to proceed must be scientifically valid and should include negative and positive controls in addition to the samples being tested. Screening procedures for threshold substances are not required to meet quantitative or uncertainty requirements (33). Our preliminary results indicate that protein chips could be used to simultaneously screen for traces of up to 16 popular drugs in urine sample. With further optimization, the current protein chip system may evolve into an acceptable screening method to test for all of the substances prohibited by WADA in the coming Olympic Games in Beijing in 2008.

This work was funded by the Beijing Natural Science Foundation of China (No. H010210640121) and the National Hi-Tech Program of China (No. 2002AA2Z2011). We are grateful to Profs. Larry Kricka and Brian Caddy for critical comments and suggestions on the manuscript.

References


