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Poly-N-Isopropylacrylamide Hydrogel Based Fluoroimmunoassay of Methyltestosterone

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Abstract: A novel immunoassay method for methyltestosterone (MT) in serum was developed. The antigen of MT (Ag_{MT})was synthesized by covalently bonding MT to BSA and raising its antibody (Ab_{MT}). Ab_{MT} was conjugated to poly-N-isopropylacrylamide (p-NIPAAm) to form Ab_{MT} bound thermally reversible hydrogel p-NIPAAm-Ab_{MT}. A competitive immunoassay method based on the competition of fluorescein isothiocyanate (FITC) labeled MT antigen (FITC-Ag_{MT}) and free MT with limited amount of p-NIPAAm-Ab_{MT} was established. The separation in the assay process was achieved by precipitation of the immuno-complex above its critical solution temperature. The detection limit for MT is 50 ng/ml. The recoveries of MT from human serum are satisfactory.

Keywords: Methyltestosterone, immunoassay, poly-N-isopropylacrylamide, thermally reversible hydrogel.

Introduction

Methyltestosterone (MT）is a synthesized steroid. Athletes use synthetic steroids to increase their sport grade. This, from the view of ethics, violated the original rules of sport. Therefore, the monitoring of the dope abuse has become more and more important worldwide. So far, the monitoring is commonly carried out by chromatographic separation followed by mass spectrometric analysis. The target samples are normally urine[1~2]. The monitoring based on blood sample is mainly used as a complementary method to overcome the shortcoming from the analysis using urine sample.

Poly-N-isopropyl acrylamide (p-NIPAAm) is one of the most frequently used thermally reversible hydrogels. It has a lower critical solution temperature (LCST) of 31~33°C. When the solution temperature is above LCST p-NIPAAm precipitates out from the solution and when it is below LCST the precipitates redissolves into the solution and thus showing excellent phase separation property[3]. This property is very useful in immunoassay because it can combine the advantages of homogeneous immunoassay in speed and simplicity, and heterogeneous immunoassay in sensitivity [4~5].

In this study, MT antigen (Ag_{MT}) was synthesized by conjugating it to bovine serum albumin (BSA). The polyclonal antibody (Ab_{MT}) of the antigen was raised. The antibody prepared was immobilized on a thermally reversible hydrogel p-NIPAAm to produce a hydrogel and antibody conjugate p-NIPAAm-Ab_{MT}. A competitive

immunoassay method based on the competition of fluorescein isothiocyanate (FITC) labeled Ag_{MT} (Ag_{MT} -FITC) and free MT with limited amount of p-NIPAAm-Ab_{MT} was established. The recoveries of MT from serum were satisfactory.

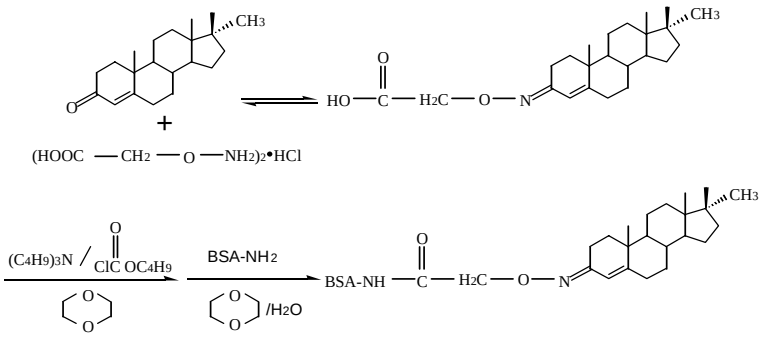
Materials and Instruments

Methyltestosterone (MT) is a product of Sigma Co. Hydrochloric acid-carboxymethyl hydroxylamine is from Bailingwei China Chemical Corporation Limited. Bovine serum albumin (BSA) is from B. M. Company. Fluorescein isothiocyanate (FITC) is from Zhaohui Pharmaceutical Factory of the Second Military Medical College, Shanghai. N,N,N′,N′-tetramethylethylenediamine (TMEDA) is a product of BIB Company. Ammonium persulfate is from Beijing Chemical Factory. N-isopropyl acrylamide (NIPAAm) was synthesized according to a previous report[6]. N-acrylsuccinimide (NAS) was also synthesized by a reported method[7]. Beckman DU-600 UV spectrophotometer, Beckman GS-15R high speed centrifuge, Hitachi M-850 fluorospectrometer and Bio-Rad 3550 microplate reader were used in this study.

Experimental

Preparation of MT antigen (Ag_{MT}). It was prepared according to a previous report[8]. The procedure for the preparation of MT antigen is shown in **Figure 1**.

Figure 1 The procedure for the synthesis of MT antigen



Raising of polyclonal antibody of Ag_{MT}. The polyclonal antibody of Ag_{MT} was raised from a female chinchilla rabbit in our own lab. Its titers measured by agar double-diffusion and enzyme-linked immunoassay (ELISA) methods were 1:32 and $1:10^4$ respectively.

Preparation of Ab_{MT} bound thermally reversible hydrogel (p-NIPAAm-Ab_{MT}) [9]. Ab_{MT} reacts with NAS to give a polymerizable monomer. The monomers copolymeraize with NIPAAm in the presence of ammonium persulfate and TMEDA to give *Ab_{MT}* bound thermally reversible hydrogel (p-NIPAAm-Ab_{MT}). It was purified by repeated washing and centrifuging separation at 38 ^oC.

Thermally reversible hydrogel based immunoassay of MT. In a centrifuge tube 100 μl of p-NIPAAm-Ab_{MT} in PBS solution (pH 7.4), 100 μl of different dilutions of MT and

5 μl of Ag_{MT}-FITC were mixed, and 295 μl of PBS solution was added to make a total volume of 500 μl. The solution was mixed well on a vortex mixer. The reaction was allowed to proceed at room temperature for 30 min and 35 ^{o}C for 10 min. Then, it was centrifuged at 35 ^{o}C and 4000 rpm for 5 min. The supernatant was removed by decanting. The precipitate was washed by adding 500 μl of ice-cooled PBS (pH 7.4) to re-dissolve the precipitate, and then separated again at 35 ^{o}C by centrifuging. This washing step was repeated three times. Finally, the precipitate was dissolved in ice-cooled PBS solution and the relative fluorescence intensity was measured on a Hitachi M-850 fluorospectrometer with excitation at 490 nm and emission at 520 nm. Both excitation and emission slits were maintained at 5 nm. For sample measurement the sensitivity of instrument was set at "high" and for other measurements it was set at "normal".

Results and Discussion

The recovery of p-NIPAAm-Ab_{MT}-FITC upon repeated precipitation. High recovery of p-NIPAAm-Ab_{MT} in the process of repeated precipitation is essential for its successful application in immunoassay. For convenience, p-NIPAAm-Ab_{MT} with labeled FITC (p-NIPAAm-Ab_{MT}-FITC) was used for recovery evaluation. After each thermal precipitation, a short centrifuging separation was followed. The precipitation media used include ice-cooled PBS, PBST and PBS-5%BSA.The precipitate after each precipitation separation was dissolved in corresponding precipitating medium and the fluorescence intensity was measured. The results showed that after 4 times of thermal precipitation, the recoveries for p-NIPAAm-Ab_{MT}-FITC were all above 85％(**Table 1**), indicating that

Table 1 . The recovery of p-NIPAAm-Ab_{MT}-FITC upon repeated precipitation

Precipitation medium / Times	PBS	PBST	PBS＋5％BSA
	Recovery (%)	Recovery (%)	Recovery (%)
1	96.1	99.6	93.4
2	95.7	99.4	92.7
3	89.2	98.1	88.3
4	89.1	95.9	85.9

the separation based on the thermal precipitation was satisfactory. Although the recovery from PBST is high this buffer showed high background fluorescence. For subsequent study, PBS was used.

Calibration curve for MT and its recoveries in serum. Under pre-optimized conditions ([p-NIPAAm-Ab_{MT}]=5 mg/ml, [Ag_{MT}-FITC]=5 μl), the calibration curve for the measurement of MT in synthetic samples was made. The linear range is 0.05~7.5 μg/ml (r=0.98). The sensitivity for MT is 50 ng/ml. For recovery measurement, 100 μl of 0.5 mg/ml MT in dioxane-water (1:2, v/v) was added to 1ml of serum from healthy person. It was diluted 10 fold and used as a mimetic serum sample. Then, 17 μl and 222 μl of the mimetic samples were used for recovery experiment. The results are summarized in **Table 2**.

Table 2. The recoveries of MT from human sera

MT added (ng/ml)	Replicates	MT measured (ng/ml)	Recovery (%)	RSD (%)
150	5	140	93.4	7.1
2000	5	1940	97.0	3.6

The data showed that the recoveries of MT from human serum were satisfactory. This method may be used in practical analysis of MT for doping control in sports.

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