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Development of an Indirect Competitive ELISA for Glycocholic Acid Based on Chicken Single-Chain Variable Fragment (scFv) Antibodies

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Abstract

Glycocholic acid (GCA) is an important metabolite of bile acids, whose urine levels are expected to be a specific diagnostic biomarker for hepatocellular carcinoma (HCC). A high-throughput immunoassay for determination of GCA would be of significant advantage and useful for primary diagnosis, surveillance and early detection of HCC. Single-chain variable fragment (scFv) antibodies have several desirable characteristics and are an attractive alternative to traditional antibodies for the immunoassay. Because chicken antibodies possess single heavy and light variable functional domains, it is an ideal framework for simplified generation of recombinant antibodies for GCA detection. However, the chicken scFvs have rarely been used to detect GCA. In this study, a scFv library was generated from chickens immunized with a GCA hapten coupled to bovine serum albumin (BSA), and anti-GCA scFvs were isolated by a phage-displayed method. Compared to the homologous coating antigen, using a heterologous coating antigen resulted in about an 85-fold improvement in sensitivity in the immunoassay. This assay, under optimized conditions, had a linear range of 0.02 ~ 0.18 μg/mL, with an IC_{50} of 0.06 μg/mL. The assay showed negligible cross-reactivity with various related bile acids, except for taurocholic acid. The detection of GCA from spiked human urine samples ranged from 86.7 to 123.3 %. These results, combined with the advantage of the scFv antibodies, indicated that a chicken scFv-based ELISA is a suitable method for high-throughput screening of GCA in human urine.

For TOC Only

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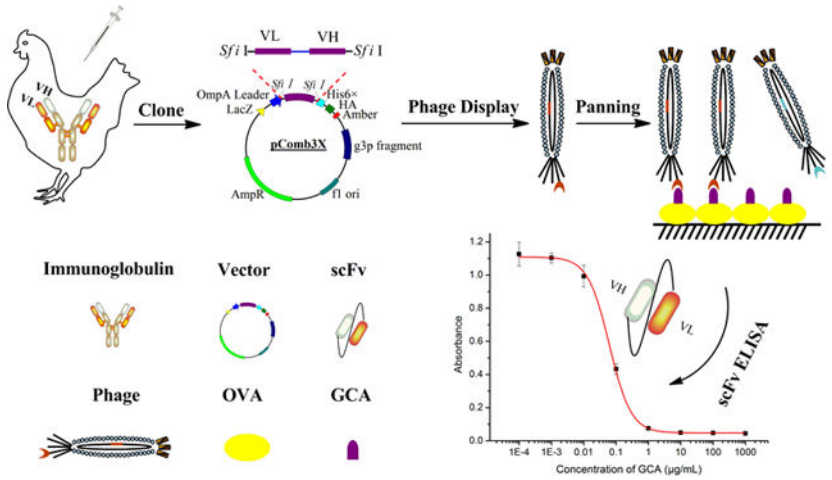

Introduction

Hepatocellular carcinoma (HCC), the most predominant type of primary liver cancer, is the fifth most common cancer in men worldwide and the ninth in women.[1] HCC has a high mortality rate being the second leading cause of cancer-related deaths worldwide among men and the sixth in women.[1] However, the burden of HCC is not distributed evenly throughout the world, since almost 85 % of the cases occur in developing countries.[2] China alone accounts for more than 50 % of cases worldwide.[3] In some areas, including the United States, the incidence of HCC is increasing, primarily because of chronic hepatitis C infection and chronic alcohol use.[4] At present, alpha-fetoprotein (AFP) is the most widely used biomarker for HCC; unfortunately, it has been criticized for low sensitivity and specificity.[5,6] The emerging fields of metabolomics, which is the comprehensive profiling of metabolic changes occurring in the living systems, could provide a powerful platform to discovery novel biomarkers and biochemical pathways for distinguish diseased and non-diseased patient.[7] After analysis of urinary metabolic profiling, the glycocholic acid (GCA) metabolism pathway was found to be the most altered functional pathway associated with HCC.[8] The average concentration of GCA of HCC patients in urine (11.5 μg/mL) was found to be about three times greater than that of healthy volunteers (3.9 μg/mL).[9,10] GCA, shown in figure 1, is being considered as a new biomarker for HCC.[8,11]

GCA, a secondary bile acid, is a derivative of steroid acids (http://www.hmdb.ca/). Many instrumental methods have been established for the qualitative and quantitative determination of GCA in various samples, such as high-performance liquid chromatography (HPLC) (the limit of detection (LOD) reported as 5.6 μg/mL),[12] ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS-MS) (LOD of 0.25 ng/mL),[13] liquid chromatography-electrospray tandem mass spectrometry (LC-MS-MS) (LOD of 1 ng/mL),[14] matrix-assisted laser desorption/ionization time-of-flight-mass spectrometry (MALDI-TOF-MS) (LOD of 4.2 μg/mL).[15] Even though these conventional instrumental methods are sensitive and accurate for the metabolites, they require a lot of time for sample preparation, a high capital expenditure, and a trained operator. Therefore, for large sample loads, where a single analyte is of interest, an economical, simple, rapid, and high-throughput method is

needed, especially in less developed countries that do not have the infrastructure to support complex instrumentation. Over the years, immunoassays based on the highly specific antigen-antibody reactions have emerged as an alternative to the traditional methods that can meet such demands. These techniques have been extensively used for the determination of environmental contaminants,[16,17] food additives,[18,19] and biological metabolites.[20,21] In the previously work, a polyclonal antibody (PAb) based radioimmunoassay has been reported to monitor GCA, with a half-maximum signal inhibition concentration (IC_{50}) of 2.2 μg/mL.[22]

Generally, monoclonal antibodies (MAb) mostly derived from murine hybridoma cell lines, along with PAbs from sera of rabbits, sheep, and other mammals, are the traditional antibody sources used in immunoassay techniques. PAbs can be easily produced in relatively large quantities; but because of the variable immune response among animals, they do not offer consistency from batch to batch. This can be a limiting factor for the PAbs in large-scale application or commercial production. Compared to PAbs, MAbs are much more consistent and more easily standardized.[23] Once the desired hybridoma has been generated, MAbs can also be produced in unlimited quantities. However, MAbs still face difficulties, as the generation of hybridomas and subsequent production of MAbs is expensive, laborious and time-consuming. Valuable MAb clones can be lost. What is more, despite optimized immunization and screening methods, it is not always easy to generate the high-affinity MAbs against small molecules.[24]

These limitations of the conventional PAbs and MAbs have led to recombinant antibodies (RA) as credible alternatives. RAs can be isolated together with their coding sequences from antibody gene libraries from any species by a phage-displayed method. Single-chain variable fragments (scFv) are the most commonly used RA format. scFvs consist of a variable region of heavy (VH) and light (VL) chains, which are joined together by a flexible peptide linker that typically can be well expressed in functional form in *Escherichia coli* (*E. coli*), yielding high affinity and stability.[25] Several studies successfully generating scFvs using mice,[26,27] rabbit,[28,29] and sheep[30,31] have been reported. Compared with other animals, generation of RAs from chickens is preferred, due to the peculiar mechanism of immunoglobulin gene diversification. Since chicken antibodies possess only one functional V segment and one J segment in the immunoglobulin heavy and light chain loci, rearranged immunoglobulin genes rely on gene conversion and somatic mutation in order to create a diverse repertoire.[32,33] So, it is possible to amplify the whole B-cell repertoire using only one set of primers designed around conserved regions flanking these variable segments. During polymerase chain reaction (PCR) amplification, the single primer set not only saves time, but reduces rare transcript loss due to the differences in primer efficiencies.

In the present study, a high-affinity chicken scFv antibody against GCA was isolated by phage display, and was applied to develop an indirect competitive enzyme-linked immunosorbent assay (icELISA) for detecting GCA.

Experimental section

Safety.

All items coming in contact with phage were autoclaved before being discarded. The tubes containing urine samples were discarded as biological waste. GCA and its analogues were discarded as hazardous waste according to campus policies of the University of California, Davis.

Details of hapten synthesis, immunization, scFv phage library construction, expression and purification of the scFv and immunoassays are given in Supporting Information.

Two four-month-old female chickens were immunized intramuscularly with 500 μg of a conjugate of GCA and bovine serum albumin (GCA-BSA) mixed with Freund's complete adjuvant. Total RNA extracted from spleen cells of the immunized chickens was transcribed into first strand cDNA. The phage-displayed scFv library was constructed by cloning PCR amplified scFv genes into the pComb3X phagemid vector. Phage-displayed scFvs against GCA were selected by panning with increasing selection stringency mediated by gradually decreasing concentration of coating antigen and free GCA. After sequencing, phagemids extracted from bacteria clones with unique sequences were transformed into TOP 10F′ cells to express scFv fragments. ELISA with scFv antibody was used to determine assay sensitivity. The assay with the highest sensitivity was chosen for sample analysis.

Result and discussion

Construction of Phage-Displayed scFv Library

Two adult female chickens were immunized intramuscularly with a conjugate of GCA-BSA five times every two weeks. Spleens were harvested and total RNA was then prepared from each spleen sample using RNAiso Plus, and cDNA was generated from each RNA sample using oligo(dT) primer and the PrimeScript™ 1st Strand cDNA Synthesis Kit, according to the manufacturer's instructions. A set of primers were used for amplification of scFv genes (Table S1, Supporting Information). The scFv genes were obtained by colony PCR – a primary PCR that amplified VH and VL gene segments separately and a secondary overlap PCR which randomly combined VH and VL via a common nucleotide sequence of the flexible linker.[32,34] The amplified scFv genes were cloned into the pComb3X phagemid vector, which was then electroporated into electrocompetent ER2738 cells to construct the phage-displayed scFv library. Colony PCR confirmed that all individual clones tested contained full-length scFv inserts, with sequence diversity revealed by DNA sequencing (data not shown). The initial size of the phage-displayed scFv library was estimated at approximately 3.3×10^7 colony forming units (cfu)/mL.

Isolation of anti-GCA Phage-Displayed scFvs

The constructed phage-displayed scFv library was screened against GCA-OVA through four rounds of panning. The stringency of panning was increased over the four rounds of panning (Table S2, Supporting Information). In attempt to capture high-affinity binders, the concentration of coating antigen was gradually decreased from 1 μg/well to 0.01 μg/well,

while the concentration of GCA was decreased by 10-fold from the initial concentration of 100 μg/mL in the each subsequent round of panning. To remove nonspecific and weakly binding phage-displayed scFvs, the number of washes over four rounds of panning were gradually increased from 10 to 20 times. Also, nonspecific phage-displayed scFvs were minimized by post absorption of phage output to the carrier, OVA.

Under such stringent conditions, the ratios of the output and input phages increased steadily, with an approximately 200-fold increase of phage recovery after the fourth round of panning compared with the first round, which suggested that there was an efficient enrichment of antigen-specific phage-scFvs. To confirm enrichment had occurred after the fourth round of panning, phage-scFv ELISA was performed against both the GCA-OVA conjugate and the carrier protein, OVA. There was a strong conjugate-specific response after the fourth round of panning (> 10 times the background signal), while minimal cross-reactivity against the carrier protein, OVA was observed (Figure S1, Supporting Information). This indicated that the panning strategy was effective in enrichment of antigen-specific phage-scFvs.

To isolate a scFv antibody with high affinity, 48 individual phage clones from the fourth round of panning were selected at random, cultured individually, and analyzed by phage ELISA. All 48 clones gave a positive signal against coating antigen, GCA-OVA, with varying intensities (Figure S2, Supporting Information). Among them, four clones that exhibited inhibition binding by GCA were selected for subsequent analysis (Figure S3, Supporting Information).

Sequence Analysis of Isolated scFvs

Sequencing analyses of the four positive clones were confirmed by BLAST search to be consistent with functionally rearranged chicken VH and VL regions. Deduced amino acid sequences of heavy and light chains are shown in Figure 2. Compared with the chicken germline, all four phage-displayed scFv antibodies contained conserved sequences in the framework regions (FRs) and variable sequences in the complementarity determining regions (CDRs), demonstrating that the isolated scFv antibodies were indeed generated from the antigen-driven response rather than the naive antibody repertoire.

The four positive scFvs differed greatly in their respective CDR-encoding amino acid sequences in VL the chains, while the lengths of their CDRs also varied. However, the VH of the four positive scFvs were only slightly different from each other. These clones that expressed the same heavy chain with two different light chains, for example, G11and G29, indicated that the light chain may be less important for binding to GCA. The ability of these scFvs to bind to GCA was controlled primarily by the VH. Since the residues in CDRs directly interact with antigen determinants, the CDRs with variation in both the sequence and length, especially in VH, may play a dominant role in the antibody-antigen interaction, which was in agreement with the previous reports.[29,32]

scFv Based ELISA

Plasmids from scFv G11, that was the most sensitive among the four positive clones, were transformed into nonsuppressor TOP 10F′ cells to produce soluble proteins containing His-

tags. The scFv-His fusion proteins were affinity-purified using a Ni-NTA metal-affinity column and analyzed by SDS-PAGE. The molecular weight of the scFv was about 34 kD.

The purified scFv G11 antibody was subsequently used to evaluate their performance by an indirect competitive ELISA. Prior to ELISA, the optimal concentration of coating antigen and dilutions of scFv were determined by a checkerboard assay.[35,36] To evaluate the potential interferences that may be encountered, for example, in human urine samples, it was necessary to study the effects of pH and ionic strength on the ELISA. As shown in Figure 3A, the ELISA was more sensitive under neutral and slightly alkaline conditions than under slightly acidic ones. This indicated that the interaction between the scFv antibody and the target analyte was most favored at pH 7.4 ~ 8.0. On the other hand, the assay was very sensitive to higher concentration of salt solution (Figure 3B). As the ionic strength of the assay buffer increased, the binding between antibody and antigen was progressively suppressed. These results are consistent with the previous report.[37] We chose a compromise between the maximal signal (A_{max}) value and assay sensitivity by using the ionic strength of 1 × PBS for the assay buffer.

Since GCA is highly lipophilic, it is only slightly soluble in aqueous solution (about 3.3 mg/L, http://www.hmdb.ca). Organic cosolvents are often added in the assay buffer to improve the solubility of hydrophobic analytes. However, high concentrations of the organic cosolvent often significantly influence assay sensitivity and maximum absorbance. Therefore, the effect of organic cosolvent was investigated in the optimization of the immunoassay. Methanol (MeOH) is the most commonly used organic solvent in immunoassay, because of its often smaller effect on antigen-antibody binding.[35] As shown in Figure 3C, when the final concentration of MeOH increased from 5 % to 10 %, the IC_{50} values varied between 4.52 and 7.96 μg/mL and the A_{max} declined from 1.24 to 1.04. However, when the final concentration of MeOH reached 20 %, both the sensitivity and the A_{max} were greatly decreased. These data indicated that a high final concentration of MeOH may interfere with the binding of scFv to both the coating antigen and the target. Thus, in this work, based on the A_{max}, IC_{50} values, and the ratios of A_{max} and IC_{50}, a MeOH concentration of 5 % was selected for the optimized condition for assay development.

A heterologous format in ELISA refers to the haptens used for the immunogen being different from that used for the coating antigen. Not only the carrier protein but also the length and structure of the spacer between hapten and carrier protein should be different, and preferably, the conjugation position should be different. The heterologous approach is commonly used to eliminate the strong affinity which is elicited by the spacer arm and the carrier protein. Thus, in the heterologous format, antibody usually has a higher affinity towards the target compound compared to the coating antigen, which should improve the assay sensitivity substantially.[38,39] What is more, previous reports show that the most sensitive competitive immunoassays are those developed in the heterologous format.[40,41] Several GCA derivatives (Figure 1) were synthesized and used in our study. Haptens GCA, G1 and G2 were conjugated by a mixed anhydride method,[42] click chemistry method,[43] and an active ester method,[44] respectively (Table S3, Supporting Information). Click chemistry, which was first proposed in 2001,[45] has the advantages including mild reaction conditions, high efficiency and good selectivity. However, only few articles reported the click chemistry

being used in immunoassay.[41,46] This study demonstrated how to construct effective conjugates based on a unique click chemistry for improvement of sensitivity in immunoassay, which would widen the application of click chemistry in immunoassay. As shown in Table 1, the IC_{50} values obtained from the two heterologous coating antigens, G1-CON (0.71 μg/mL) and G2-OVA (0.06 μg/mL), were much lower than those from the homologous coating antigen, GCA-OVA (5.08 μg/mL). Similar improvement in LOD was also found. After conjugation, G2-OVA was partially hydrolyzed under basic conditions. However, the replacement of the methyl ester by free acid does not improve the assay sensitivity (data not shown). Comparing with the two heterologous coating antigens, G1-CON and G2-OVA, heterology in spacer group structure of coating antigen also brought a remarkable improvement in assay sensitivity, which is in agreement with some data described previously.[38] Thus, G2-OVA was identified as the best coating antigen evaluated.

The optimized ELISA used a coating antigen of G2-OVA at a concentration of 4 μg/mL and scFv antibody at a dilution of 1/200 in PBS before the competition. This hapten gives heterology of the coupling position. The plate coated with the coating antigen was blocked with 5 % skim milk. The analyte was prepared in assay buffer containing 10 % MeOH in 1 × PBS, pH 7.4. Under these conditions, the heterologous assay had a linear range (IC_{20}~ IC_{80}) of 0.02 ~ 0.18 μg/mL of the target and an IC_{50} value of 0.06 μg/mL. The limit of detection (LOD) in the buffer was 0.01 μg/mL (Figure 4). In contrast with the previous radioimmunoassay (RIA),[22] the sensitivity of the scFv-based ELISA was increased approximately 36-fold.

Cross-Reactivity Studies

To determine antibody selectivity, various bile acids with structural similarity to GCA, including taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurolithocholic acid (TLCA), glycochenodeoxycholic acid (GCDCA), and glycolithocholic acid (GLCA), were investigated for cross-reactivity (CR). Table 2 shows the CRs that were found by the scFv-based ELISA, expressed as a percentage of the IC_{50} of GCA.

The CRs of various bile acids evaluated provide insights into the binding preferences of the scFv antibody. The GCA, GCDCA and GLCA, which shared the same glycine moiety, cross-reacted < 0.58 %, possibly because the antibody binding pocket generated against the glycine moiety was too small. On the other hand, lack of hydroxyl on the C ring of GCDCA and TCDCA and both hydroxyls on the B ring and C ring of GLCA and TLCA resulted in low CR, suggesting that the steroid nuclei were important for binding efficiency. In addition, the reduced CR toward TCA (40 %) probably indicated that the scFv antibody generated against the immunizing hapten likely has more affinity to the presence of both steroid nuclei and glycine moiety rather than only steroid nuclei or glycine moiety.

Among the various bile acids, the scFv antibody showed relatively higher CR to TCA. This is expected with the taurine conjugate acting as an isosteric for mimic of the glycine conjugate. Because glycine-conjugated bile acids represent a major fraction of the total metabolites of a particular bile acid, the scFv antibody may be utilized for preliminary screening of human urine samples, probably with minimal interference from TCA. In addition, the group separation of the glycine and taurine conjugated bile acids can be

achieved prior to analysis.[47,48] Alternatively, the TCA/GCA mixture may prove to be useful biomarker.

Assay Validation

Matrix interference is one of the common challenges for accurate and reliable determination in many analytical methods. In ELISAs, the matrix interference is mainly caused by inhibition of enzyme activity, binding between analyte and antibody, or both. Sample cleanup procedures using solid-phage extraction could result in sample concentration and a relatively clean extract to remove the interferences, however, additional steps increase time, cost, and variation in the assay. Alternatively, the calibration curve could be prepared in the buffer containing blank matrix to account for the matrix effect. Also, dilution of the sample is an effective way to eliminate matrix interference.[49] In this study, an artificial urine matrix was used in order to have an easy to access and easy to handle solution mimicking human urine samples.[50,51] The influence of the dilution of artificial urine with buffer was determined. When the assay was performed in the buffer containing 2 ~ 8 % of the artificial urine matrix, the sensitivity did not change dramatically compared to that of the assay conducted in the buffer (Figure S4, Supporting Information). However, a decrease in A_{max} was observed when the content of artificial urine matrix increased to 8 %. In order to minimize the matrix effect, the urine content in the assay buffer should be less than 4 %.

To validate the assay, we carried out a spike-and-recovery analysis with the newly developed scFv-based ELISA. In the previous reports, the concentration of GCA in the urine of HCC patients was reported to be up to 17.67 μg/mL.[9,10] Taking into account these data, the urine samples from healthy subjects were spiked with GCA at four different concentrations (1, 5 10, and 25 μg/mL). Urine samples spiked with high concentrations of GCA had to be largely diluted to obtain a signal in the linear range. What is more, the higher dilution of the urine sample would decrease the amount of interfering matrix and facilitating quantitatively analysis. Thus, the urine samples spiked with low concentration of GCA (1, 5, 10 μg/mL) were diluted 40-fold, while the high concentration (25 μg/mL) 100-fold. As shown in Figure 5, the linear regression analysis of ELISA results showed a good correlation (R^2 = 0.99) between spiked and detected levels. In addition, good recoveries in a range of 86 ~ 123 % were obtained. Recoveries in a range of 75 ~ 125 % are usually acceptable for spiked samples.[17,52] In addition, four samples spiked with GCA ranging from 1 to 25 μg/mL were then analyzed by both scFv-based ELISA and LC-MS/MS in a blind fashion (by different operator). Good correlation (R^2 = 0.99) was obtained between the two methods (Figure S5, Supporting Information). Thus, the results demonstrated that the scFv-based ELISA was suitable for the detection of GCA in human urine samples.

Conclusion

In this study, we isolated a high-affinity GCA-specific scFv antibody from immunized chickens by phage display. Because chickens possess single heavy and light variable functional domains, it is a good source to generate scFv against small molecular weight compound, such as GCA. In this case, the sensitivity of the ELISA with heterologous coating antigen was approximately 85-fold better than the homologous ELISA. Under the

optimized conditions, the scFv based indirect competitive ELISA showed a working range from 0.02 to 0.18 μg/mL, with LOD of 0.06 μg/mL Validation results from scFv based ELISA and LC-MS/MS were in good agreement with each other. Thus, this study showed that chicken scFv could be successfully used to develop a sensitive immunoassay for GCA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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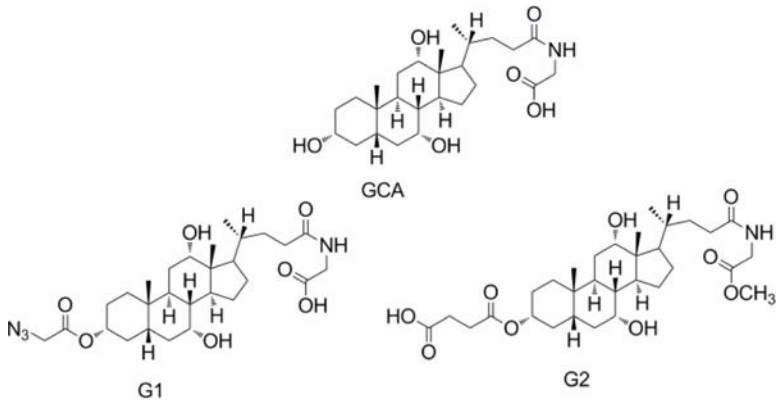


Figure 1.
Structures of GCA and its haptens. Conjugate of GCA with BSA was used as immunogen.

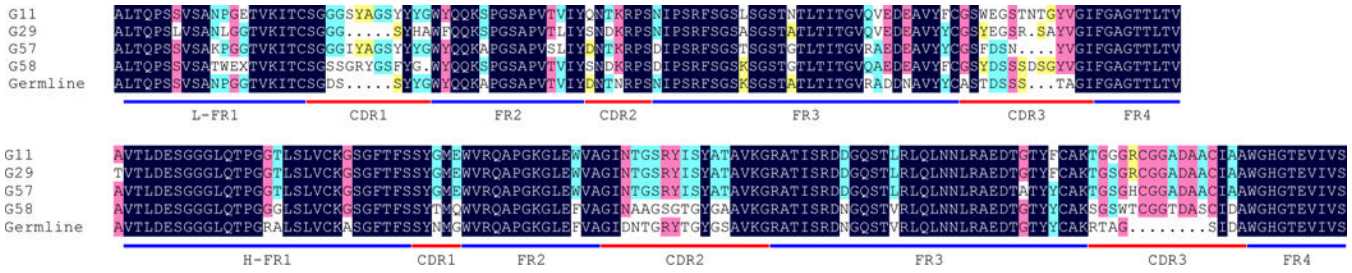


Figure 2.

Schematic alignment of deduced amino acid sequences from four isolated scFv antibodies and chicken germline. FR and CDR regions are indicated below the germline sequence. Absence of corresponding residues is denoted with dot (·). Lettering color indicates degree of homology; dark blue (100 %), pink (≥ 75 %), blue (≥ 50 %), yellow (≥ 33 %).

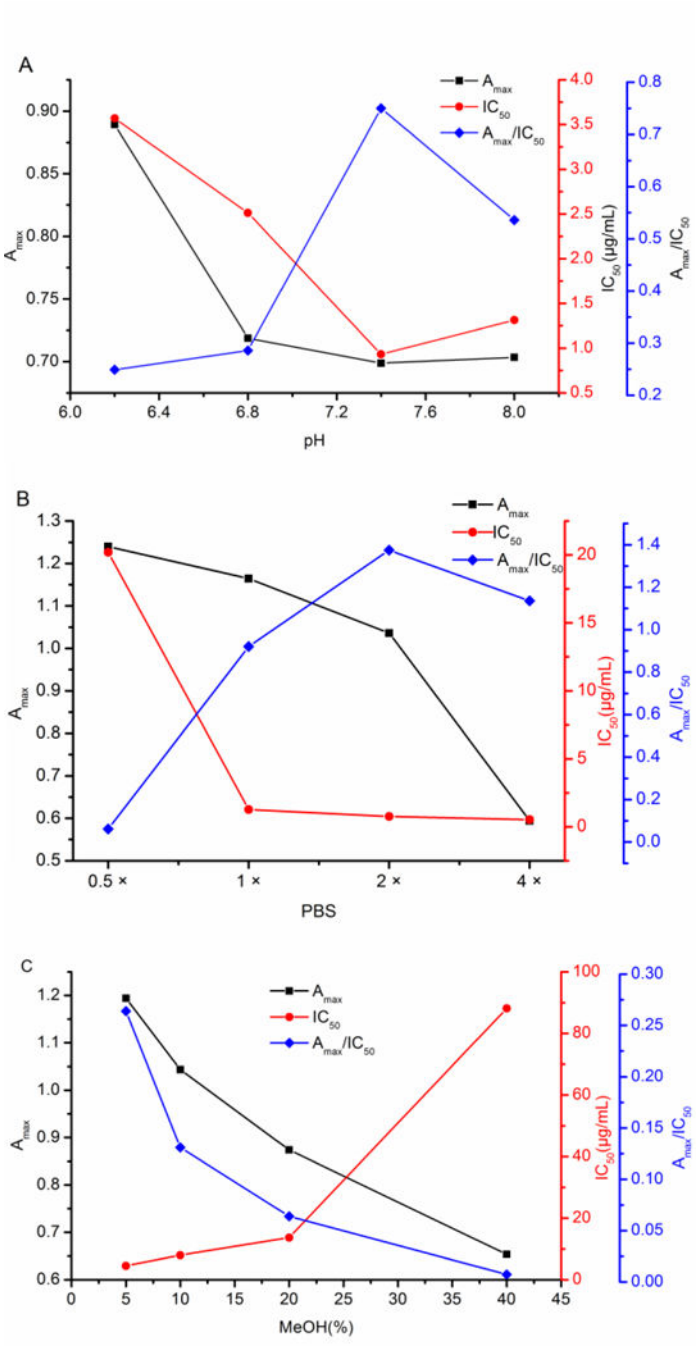


Figure 3.

Influence of pH (A), ionic strength (B), and organic cosolvent (C) on the assay sensitivity. Assay conditions: coating antigen (GCA-OVA) (2 μg/mL); scFv antibody (1/1600); and goat anti-HA tag IgG-HRP (1/5000).

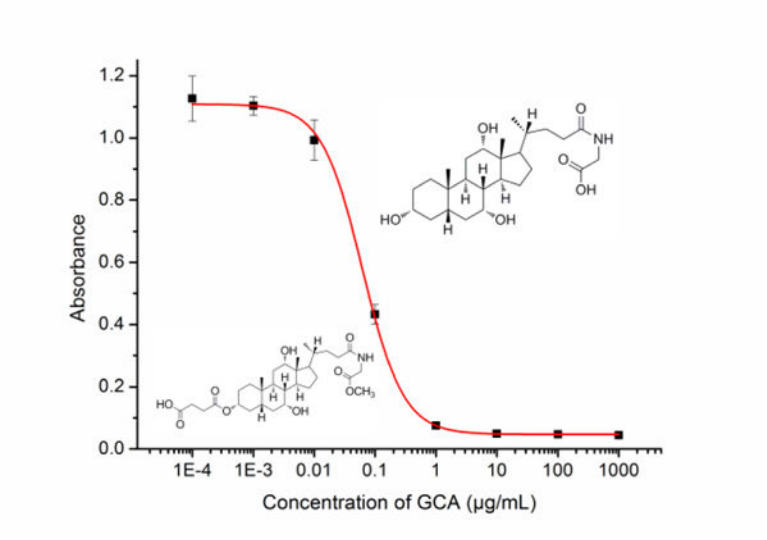


Figure 4.

GCA competitive curve with scFv antibody. The microplate was coated with G2-OVA conjugate (on the left) at 4 μg/mL. Serial dilutions of GCA in PBS containing 10 % MeOH were mixed with an equal amount of a 1/200 dilution of the scFv in PBS. A volume of 100 μL of the mixture was added into the wells. The bound scFv was detected with a 1/5000 dilution of goat anti-HA tag IgG-HRP.

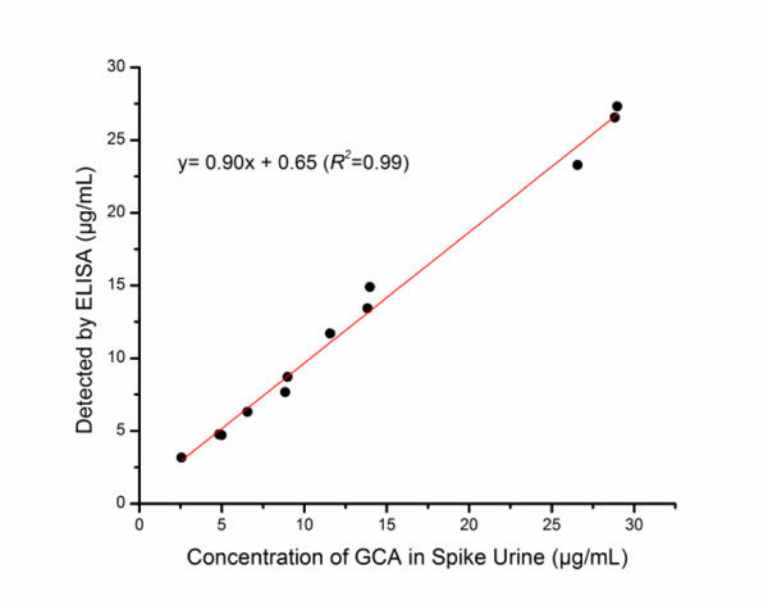


Figure 5.

Relationship between spiked GCA in urine and measured by ELISA. The dilution of GCA spiked urine was mixed with an equal volume of scFv antibody in PBS before analysis.

Table 1.

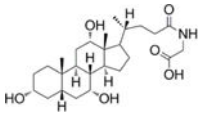
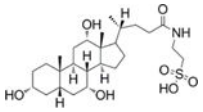
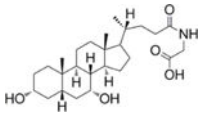
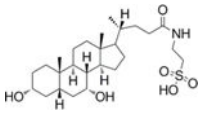
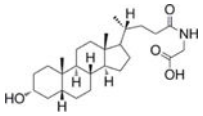
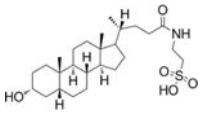
Standard Curve Characteristic of scFv-based ELISA Using Homologous and Heterologous Coating Antigens[a]

Immunogen	Coating antigen	Antibody Dilution	A_{max}	IC_{50} (μg/mL)	LOD (μg/mL)	linear range (μg/mL)
GCA-BSA	GCA-OVA (2 μg/mL)	1/1600	0.98	5.08	0.94	1.75 ~ 14.79
	G1-CON (1 μg/mL)	1/1600	0.89	0.71	0.15	0.26 ~ 1.88
	G2-OVA (4 μg/mL)	1/400	1.11	0.06	0.01	0.02 ~ 0.18

[a]The parameters of the assays were obtained from four-parameter sigmoidal fitting. Several rounds of optimization of assay conditions were used with each coating antigen before generating the above data.

Table 2.

Cross-reactivity (CR, %) of Various Bile Acids Structurally Related to GCA[a]

Analogues	Chemical structure	% CR
GCA		100
TCA		40
GCDCA		0.58
TCDCA		0.32
GLCA		<0.1
TLCA		<0.1

[a]The microplate was coated with G2-OVA conjugate at 4 μg/mL. Serial dilutions of GCA or tested compounds in PBS containing 10 % MeOH were mixed with an equal amount of a 1/200 dilution of the scFv in PBS. A volume of 100 μL of the mixture was added into the wells. The bound scFv was detected with a 1/5000 dilution of goat anti-HA tag IgG-HRP.