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Radioimmunoassay of glycyrrhetic acid

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Abstract

A direct radioimmunoassay for glycyrrhetic acid in unextracted serum was developed using 3α-[^{3}H]-3β-hydroxy-glycyrrhetic acid as the labeled antigen. The antiserum was raised in rabbits against glycyrrhetyl-30-glycine-bridged bovine serum albumin, showed highly specific to the glycyrrhetic acid. It can distinguish clearly between glycyrrhetic acid and its C-3 derivatives such as glycyrrhizin, 3-monoglucuronyl glycyrrhetic acid and carbenoxolone. Double antibody method was used to separate antibody-bound and free glycyrrhetic acid. A satisfactory glycyrrhetic acid standard curve for radioimmunoassay was obtained in the range of 0.2 ng—20 ng/tube and measurable range was 2 ng—200 ng/ml using 5 μl of serum as samples. The minimum detectable concentration was 125 pg/tube. The intraassay coefficient of variation was 6.6%, and the interassay coefficient of variation was 7.5%. The present radioimmunoassay eliminates extraction of glycyrrhetic acid from serum and requires only 5 μl of serum for assay.

Key words licorice, glycyrrhizin, glycyrrhetic acid, 3-oxo-glycyrrhetic acid, unextracted serum, radioimmunoassay

Abbreviations BSA, bovine serum albumin ; CV, coefficient of variation ; GA, glycyrrhetic acid ; GL, glycyrrhizin ; HPLC, high performance liquid chromatography ; OH, hydroxy

Introduction

Licorice ("Kanzō") has long been used in traditional oriental medicine as a demulcent, an expectorant and an antispasmodic agent. In the Western world, it has been employed in the treatment of peptic ulcers for many years.[1] Glycyrrhizin (GL) is the active and principal constituent of licorice root. Glycyrrhetic acid (GA), aglycone form of GL, and some of its derivatives have shown the anti-inflammatory,[2] anti-allergic[3] and anti-cholesteremic[4,5] effect. Recently it was demonstrated to have anti-viral effect[6,7] also. However, administration of licorice or GL has resulted in an adverse effect which is reffered to pseudo-aldosteronism[8] inducing hypertension, hypokalemia and suppression of renin-angiotensin-aldosterone system in some recipient. This mineralocorticoid effect of licorice is thought to be caused by GA. Although several methods for the determination of GL or GA has been reported,[9,10] those measures do not give very high sensitivities to elucidate the pharmacokinetics of the GL or GA in human biological fluids in ordinary dosage amounts.

We previously reported an enzyme immunoassay of GA.[11] This method was so sensitive that it could estimate the small amount of GA in the human blood or urine under the usual dosage, however it needs extraction by organic solvent in the case of the assay of biological fluids which

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caused non-specific interference to antigen-antibody reaction of enzyme immunoassay.

Thus we introduced tritieted hydrogen [^{3}H] to C-3 position of GA and developed a radioimmunoassay of GA, which was capable of application to determine GA concentrations in human body fluids without extraction.

Materials and Methods

Reagent and samples : GA purchased from Nakarai Chemicals Ltd., Kyoto, was recrystallized with ethanol, showed one peak on high performance liquid chromatography (HPLC). Bovine serum albumin (BSA) Cohn fraction V and 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide were obtained from Sigma, St. Louis. [^{3}H]-Sodium borohydride ([^{3}H]-$NaBH_4$) was purchased from New England Nuclear (Daiichi Chemical Pharmaceutical Co., Ltd., Tokyo).

N-(18β-glycyrrhetyl) glycine Na salt was kindly supplied from Minophagen Pharmaceutical Co., Ltd. Anti-rabbit IgG (goat serum) from Miles Lab., Inc., was used.

Synthesis of 3-oxo-glycyrrhetic acid performed in our laboratory was detailed elsewhere by one of our colleagues.[12]

Preparation of [^{3}H]-glycyrrhetic acid : The reduction of 3-oxo-GA with sodium borohydride ([^{3}H]-$NaBH_4$) gave 3β-hydroxy-3α-[^{3}H]-GA and 3α-hydroxy-3β-[^{3}H]-GA. 3β-hydroxy-3α-[^{3}H]-GA was separated and purified by thin layer chromatography from 3α-hydroxy-3β-[^{3}H]-GA and unreduced 3-oxo-GA. 3β-hydroxy-3α-[^{3}H]-GA showed one peak on HPLC and its specific activity was 0.72 Ci/mmol.

GA standard : Recrystallized GA with ethanol which showed one peak on HPLC was used as standard. This purified GA was diluted with "GA free serum" obtained from a patient who had taken no meals for several days under intravenous hyperalimentation. A stock solution of 1 mg/ml of GA in ethanol was serially diluted with 10% GA-free human serum and 0.05 ml volumes of the standard solutions obtained (0 to 8 μg/ml) were stored at -20℃.

Antiserum : Glycyrrhetyl-30-glycine BSA[13] was used for immunization. Approximately 600 μg of the conjugate emulsified with complete Freund's adjuvant was made and used for the initial injection in two rabbits. Four boosters of 300 μg were injected biweekly, followed by three boosters at monthly intervals. Blood was obtained one week after the final injection. Antiserum was immobilized at 56 ℃ and stored at -20℃ until use.

Procedure of radioimmunoassay of GA : Serum samples were diluted ten-fold with Buffer B (0.06 M phosphate buffer, pH 7.4 containg 0.01 M EDTA, 0.1% BSA) before assay. Volumes of 50 μl of standards or diluted serum samples were mixed with 100 μl of 1 : 300 dilution of anti-GA serum solution in Buffer B and 50 μl of diluted [^{3}H]-GA solution (5 nCi/tube) were added with thorough mixing. The solution was incubated for 2 hours at room temperature. Then, 50 μl of anti-rabbit IgG (goat serum) (1 : 10) and 20 μl of normal rabbit serum (1 : 100) were added and incubation was continued overnight at 4℃. Then, 1 ml of water was added and the mixtures were centrifuged at 1,800 $\times g$ at 4℃. The supernatant was decanted, and the resulting precipitates were washed with 1 ml of Buffer B. The precipitates were dissolved with 100 μl of 1 N NaOH solution twice. The solution was counted by liquid scintillation counter.

Results

Standard curve

The standard curve for radioimmunoassay of GA is shown in Fig. 1, which exhibited little interference of serum. The minimal amount of GA which could be measured with 95% confidence in the assay was 125 pg/tube and the measurable range was 2 ng$-$200 ng/ml, using 5 μl serum samples. The bile juice as one of the biological fluids other than serum was also examined of its interference effect on GA standard curve. The addition of 5 to 50 μl of bile juice on assay mixture had little influence on GA standard curve as well as serum. Thus the bile juice was also measurable.

Dilution of samples

Four plasma samples containing various con-

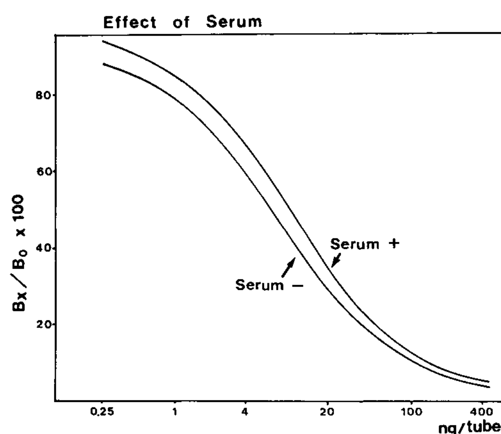

Fig. 1 Standard curve of glycyrrhetic acid radioimmunoassay with or without serum.

centrations of GA were diluted serially with GA free serum and their GA levels were determined by radioimmunoassay. Fig. 2 showed a linear relation between the dilution and the GA concentration for sample.

Specificity

Cross-reactivity of related compounds of GA in this radioimmunoassay system were : 18α-GA, 78.3% ; 3α-OH-GA, 0.19% ; 3α-OH-18α-GA, 0.95% ; 3-oxo-GA, 2.6% ; 3-oxo-18α-GA, 7.2% ; 3-monoglucuronyl-GA, 0.72% ; 30-monoglucuronyl-GA, 39% ; 3, 30-diglucuronyl-GA, 0.19% ; carbenoxolone, 0.95% ; ammonium glycyrrhizin, 0.2% at a level of 50% bound. The cholic acid, dexamethasone and other steroids showed scarcely cross-reacted with this system. This assay system could differentiate GA from GL almost completely (Table I).

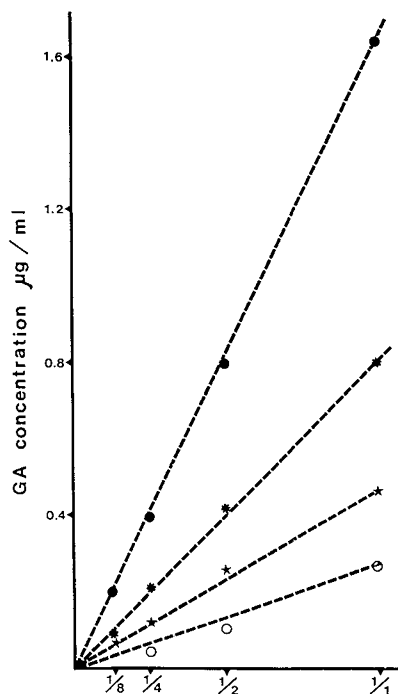

Fig. 2 Effect of dilution of four serum samples.

Accuracy and precision

The recovery of various amounts of GA added to four different pooled serum (mean±S.D.) samples showed 112.9±18.4% at low concentration of GA added (40 ng/ml), 93.2±8.6% at medium concentration (200 ng/ml) of GA added (Table II). The intraassay coefficient of variation (CV) for analyses of 6 samples from pools of high (mean 810 ng/ml) and low (mean 198 ng/ml) GA concen-

Table I Cross-reactivity of the antiserum with various glycyrrhetic acid derivatives and steroids.

Compounds	Cross reaction (%)	Compounds	Cross reaction (%)
GA	100	Ammonium GL	0.2
18α-GA	78.3	Dexamethasone	<0.005
3α-Hydroxy-GA	0.19	Cholic acid	<0.005
3α-Hydroxy-18α-GA	0.95	Aldosterone	<0.005
3-Oxo-GA	2.6	Hydrocortisone	<0.005
3-Oxo-18α-GA	7.2	Estradiol	<0.005
Carbenoxolone	0.95	Estriol	<0.005
3-Glucuronyl-GA	0.72	Testosterone	<0.005
30-Glucuronyl-GA	39	Progesterone	<0.005
3,30-Diglucuronyl-GA	0.19		

Table II Study of accuracy of the method.

GA added	A	B	C	D	Recovery
0 ng	8.1 ng	1.2 ng	2.5 ng	3.8 ng	
2 ng	10.1 9.8 97.0 %	3.2 4.6 144.0 %	4.5 4.9 108.9 %	5.8 5.9 101.7 %	112.9±18.4 %
10 ng	18.1 19.5 107.7 %	11.2 9.6 85.7 %	12.5 11.0 88.0 %	13.8 12.6 91.3 %	93.2± 8.6 %
40 ng	48.1 41.0 85.2 %	41.2 46.0 111.6 %	42.5 45.0 105.6 %	43.8 42.0 95.9 %	99.6±10.0 %
100 ng	108.1 105.0 97.1 %	101.2 115.0 113.6 %	102.5 100.0 97.6 %	103.8 110.0 106.0 %	103.6± 6.8%

Upper value : the expected value for addition. Middle value : the measured value.
Lower value : calculated recovery (%).
Each column A, B, C, and D showed different serum samples.

trations were 8.7% and 6.6%, respectively. The values for interassay CV were 7.5% (mean=426 ng/ml, n=6).

Discussion

Ichikawa *et al.* described the determination of GA and GL in rats blood by HPLC.[14] However, its method could not be applied for the measurement of GA and GL in biological materials of humans, because of its detection limit for GA was 0.125 μg/ml, which was too insensitive to estimate under the usual clinical dosages of GL. In-house HPLC system has also the same detection limit for GA,[15] which is applicable only when high GA concentration is expected in serum. In the recent past, the gas liquid chromatography assay described for carbenoxolone sodium (the hemisuccinate ester of GA) was applied to the assay for GA.[16] But the detection limit of that method was only 1 μg/ml. Blood GA levels were below the limit of detection for the 10 days following licorice (containing 580 mg GL) ingestion.

Nowadays only enzyme immunoassays have been available for the estimation of human serum or urine GA[11] or GL[17] levels under the clinical doses of GL or "Kanzō". In GA enzyme immunoassay, some interferences from ligand-serum constituents interactions makes it difficult to do direct assays. Thus the extraction step is required for the enzyme immunoassay for GA.

The sample amount of our present assay is only 5 μl of serum, and we actually used 50 μl of ten-fold diluted with Buffer B. This amount is very small in comparison with 150 μl of sample in case of the enzyme immunoassay. This small amount of serum caused few interference to antigen-antibody reaction in our present radioimmunoassay. Non-specific interference to antigen-antibody reaction caused by biological fluids seems to be not only in relation to the amount of sample, but also augmented in case of enzyme immunoassay as compared with radioimmunoassay. Under these conditions, the dose-response curves of both assays have almost the same slope expressed "ng/ml" as abscissa, however the curves are different from each other expressed "ng/tube" as abscissa. The lower limit of detection was 125 pg/tube in our present radioimmunoassay system, in comparison with 250 pg/tube in the in-house enzyme immunoassay for GA. These performances of the both assays showed little difference. Furthermore the percentage cross reactions against the most GA related compounds are similar.

In the present assay we use antibody against glycyrrhetyl-glycine conjugated to protein at the C-30 position. The anti C-3 bridged-BSA antiserum for immunoassay of carbenoxolone and GA couldn't separate distinctly between GA and its C-3 derivatives.[13] Thus we chose anti C-30 bridged GA antisera which were obtained

from rabbits immunized with glycyrrhetyl-30-glycine-BSA conjugate. This antiserum clearly distinguished C-3 position, the cross-reaction of 3α-OH-GA, 3α-OH 18α-GA, 3-oxo-GA, 3-oxo-18α-GA, carbenoxolone and 3-monoglucuronyl-GA are 0.19%, 0.95%, 2.6%, 7.2%, 0.95% and 0.72% respectively, in contrast to this specificity, the difference of C-30 or C-18 position showed heavy cross reaction that is 18α-GA and 30-monoglucuronyl-GA cross-reacted 78.3% and 39%, respectively. Low antiserum dilution of the present study (1：300) seems to act with some suppressive role on interference of biological fluid.

Technical ease is one of the requirements for a useful assay of small molecules in biological fluids. Non-extraction radioimmunoassay for serum GA of the present study satisfies this requirement.

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和文抄録

グリチルレチン酸の3位に ^{3}H を導入して抗原とし，ラジオイムノアッセイ法を開発した。抗血清は，グリチルレチン酸の30位にグリシンを導入し牛血清アルブミンを結合させた後，家兎に免疫して得たものであり，グリチルレチン酸にきわめて特異的である。特にグリチルレチン酸の3位の誘導体，すなわち，グリチルリチン，3-モノグルクロニル-グリチルレチン酸，カルベノオキソロンをはっきりと識別する。BF分離は二抗体法で行った。標準曲線は0.2 ng－20 ng/管の間で描け，5 μl の血清で測定可能であり，測定可能性域は 2 ng－200 ng/ml である。最小測定感度は125 pg/管，測定内変動係数は6.6%，測定間変動係数は7.5%であった。この方法は従来の酵素免疫測定法において必要とされた血清からの抽出操作が不用であり，簡便かつ，精度も優れた方法である。

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