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DETERMINATION OF QUASSIN IN PICOGRAM QUANTITIES BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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Key Word Index—*Quassia amara*; Simaroubaceae; enzyme-linked immunosorbent assay; quassin; 18-hydroxyquassin.

Abstract—A double-antibody, enzyme-linked immunosorbent assay has been developed for the detection of quassin, neoquassin and 18-hydroxyquassin, the bitter natural products of *Quassia amara* and related species. Antiserum was raised in rabbits using 18-hydroxyquassin–bovine serum albumin as immunogen. The assay described is able to detect these closely related seco-triterpenes at concentrations as low as 5 pg per 0.1 ml sample, and the antiserum shows little cross-reactivity with other quassinoids. The distribution of quassin within small plants of *Q. amara*, *Q. indica* and *Picrasma quassioides* is described.

INTRODUCTION

The intensely bitter natural product quassin (**1**) is the best known of an important group of seco-triterpenes, the quassinoids [1], synthesized by members of the tropical family Simaroubaceae. Quassin has uses as a food additive, a pharmaceutical and an insecticide [2]. Other quassinoids have been identified as showing powerful anti-cancer [3] and anti-amoebic [4] properties, as well as insecticidal action [5].

Currently we are studying the biosynthesis of quassin by plant cells in culture. As the expression of genes for secondary product formation by cultured cells is usually poor, it is desirable to be able to select a few high-yielding strains from numerous microcultures. Since there is also evidence that metabolite productivity in cell culture is related to the type of tissue from which the culture is derived [6], an analysis of plant material may assist in establishing cell lines of higher yield. Therefore, our requirement is for an efficient screening method with which crude extracts of a very large number of samples may be assayed simply, accurately and at high sensitivity. Immunoassays are clearly the method of choice in this situation. Of the many forms of immunoassay available, a microtitration plate enzyme-linked immunosorbent assay (ELISA) offers a number of advantages, such as ease of automation, the need for comparatively low-cost equipment, and simplicity. We have previously described a form of double-antibody ELISA applicable to low MW, non-immunogenic compounds [7–9] and have developed a broad-specificity assay for a range of quassin-like structures by raising antiserum against an isoquassinic acid–bovine serum albumin conjugate [10]. This assay has a sensitivity of 10 ng quassin per 0.1 ml sample, which is comparable to that reported for a number of radioimmunoassays for low MW plant metabolites [11–14], but not in the same order as a number of ELISAs recently reported [7–9]. The insensitivity is due to the way in which the immunogen is synthesized, involving the isomerization of quassin to form isoquassinic acid.

In order to develop an ELISA of greater sensitivity and selectivity towards quassin, we have isolated 18-hydroxyquassin [15] and used this to form a conjugate in which the precise stereochemistry of quassin is maintained [R. J. Robins and D. T. Coxon, unpublished results]. When conjugated to bovine serum albumin (BSA) and injected into rabbits, a new anti-quassin antiserum is produced. This serum shows the desired properties, and in this communication we report the characterization of an ELISA and its application to the determination of the distribution of quassin in tissues of *Quassia amara* L., *Q. indica* (Gaertn.) Nootebloom and *Picrasma quassioides* (Buch-Ham.) Bennett.

RESULTS AND DISCUSSION

ELISA standard curve for quassin

Preliminary experiments, carried out as in ref. [10], established that satisfactory conditions for the standard curve sensitivity were achieved using a coating conjugate concentration of 1 μg/ml and primary and secondary antibody titres of $1:5 \times 10^4$ and 1:1500, respectively (see Experimental). Figure 1 shows a standard curve in these conditions for quassin. It also shows that there was no significant desensitizing effect when a final concentration of 10% methanol was incorporated in the competition stage of the ELISA. The ability to include methanol in the competition reaction simplified the assay of tissue extracts, which could thus be made in methanol, diluted in PBST to 10%, and assayed directly. The assay was extremely sensitive, giving a dose–response curve over the range 5–100 pg quassin (Fig. 1). This compared favourably with the dose–response curve shown by an anti-isoquassinic acid antiserum [10] which had a sensitivity of 5 ng quassin. Clearly the effect on the stereochemistry of quassin caused by the need to provide a stable acid function for conjugation to protein in immunogen synthesis was considerable.

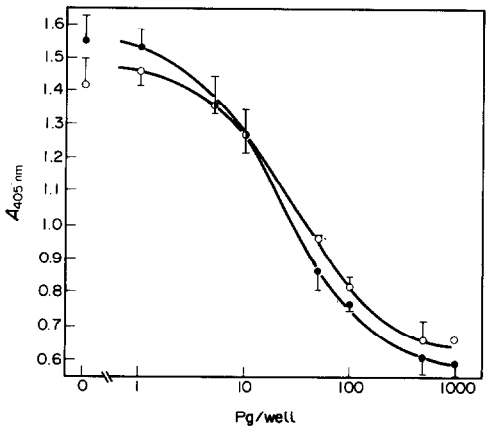


Fig. 1. Standard curves of the ELISA for quassin in PBST (○) and PBST containing a final concentration of 10% (v/v) methanol (●). Means ± s.e. for triplicate sample are shown. Assay conditions are as described in the Experimental.

Cross-reactions in the ELISA

Table 1 shows the extent to which various quassinoids cross-reacted with the antiserum raised against 18-hydroxyquassin. While extremely slight cross-reactions occurred with a number of compounds, the antiserum showed considerable selectivity for the closely related compounds of Class 1. It is well established that those substituents on the side of a hapten distal to the point of conjugation, and in this case particularly the structure of the quassinoid A-ring [10], are crucial factors determining antiserum recognition. The choice of 18-hydroxyquassin for derivatization and immunogen synthesis provided a ring structure of almost identical stereochemistry to that of quassin [R. J. Robins and D. T. Coxon, unpublished results]. This was in contrast to the use of isoquassinic acid in which limited isomerization had occurred and where consequently the antiserum showed much less selectivity [10].

As expected, quassin and 18-hydroxyquassin showed comparable reactivity. So did 12-hydroxyquassin (**3**), in which the only alteration was at a position adjacent to the point of conjugation and therefore probably masked by the BSA carrier. The precise structures of isomers 'a' and 'b' of neoquassin are yet to be fully determined, though preliminary ^{1}H NMR analysis [R. J. Robins and D. T. Coxon, unpublished results] indicates that they are the 16α- and 16β-isomers, respectively. The antiserum showed rather better recognition of the β-isomer than of quassin, whereas the stereochemistry of the α-isomer, with a hydroxyl group protruding down below the plane of the molecule, appeared to inhibit recognition significantly. Changes in the D-ring structure, as in 14,15-dehydroquassin (**5**) and isoquassinic acid, caused marked loss in recognition.

Other than neoquassin and 12-hydroxyquassin, the only compounds showing any significant cross-reaction were the nigakilactones A and B. In both these compounds the side of the molecule distal to the point of conjugation showed a change only from a ketone to a hydroxyl in the C-11 position, and they are thus stereochemically similar to quassin. Further changes, such as the acetylation of the C-11 hydroxyl group, led to a virtually total loss in binding, as with nigakilactone E.

Table 1. Cross-reactivity of anti-18-hydroxyquassin antiserum

Class	Compound	% Cross-reaction*	Range (pg)†
1	Quassin (**1**)	89	5–100
	Neoquassin a (**2**)	189	5–100
	Neoquassin b (**2**)	11.5	10–100
	12-Hydroxyquassin (**3**)	128	5–100
	18-Hydroxyquassin (**4**)	100	5–100
	14,15-Dehydroquassin (**5**)	10.7	5–500
	Isoquassinic acid	0.4	10–5000
2	Nigakilactone A (**6**)	34	10–2000
	Nigakilactone B (**7**)	45	5–500
	Nigakilactone E (**8**)	0.09	10^4–10^5
	Nigakilactone F (**9**)	2.3	50–5000
	Nigakihemiacetal A (**10**)	1.0	500–5×10^4
3	Picrasin B (**11**)	0.1	10^4–10^5
	6-Hydroxypicrasin B (**12**)	0.009	10^4–5×10^5
	Klaineanone (**13**)	0	—
4	Glaucarubine (**14**)	0.08	10^4–5×10^5
	Glaucarubinone (**15**)	0	—
5	Chaparrinone (**16**)	0	—
6	Bruceine A (**17**)	0	—
	Bruceine B (**18**)	0.06	10^4–10^5
	Isobruceine B (**19**)	0.01	10^4–10^6
8	Picrasin A (**20**)	0.4	10–5000
	Simarolide (**21**)	0	—
9	Sergeolide (**22**)	0	—

*At four different levels of quassin (10, 30, 50 and 100 pg), the amount of cross-reactant giving the same degree of response in the assay (absorbance at 405 nm) was expressed as a % of the reaction given by the relevant amount of quassin. The % cross-reactivity is the mean of these four percentage figures.This is necessary when the reaction curves of the two compounds are non-parallel. A standard curve of 'mixed' quassins was conducted on each plate.

†The range of reaction of cross-reactant is shown to indicate whether or not the reaction curves are parallel.

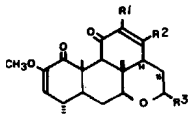
The antiserum is very much more selective than the broad specificity serum previously described [10], and will only detect quassin and a few closely related metabolites.

Tissue distribution of quassin

Although *Q. amara* is the conventional source of quassin, we chose to examine other species, *Q. indica* and the Japanese *Picrasma quassioides*, as possible alternatives for quassin bio-production by plant cell cultures. *P. quassioides* is used pharmaceutically in Japan and has been shown to contain quassin and a number of nigakilactones [16, 17].

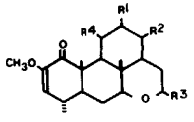
The extreme sensitivity of the ELISA described here makes it possible, if required, to examine the quassin content of a few μg of tissue. Using samples each of a few mg, we looked at the distribution of quassin throughout plants of these three species. Although it was not possible with this assay to distinguish between quassin and the few

Class 1



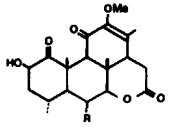
		R^1	R^2	R^3
1	Quassin	OMe	Me	=O
2	Neoquassin	OMe	Me	HOH
3	12-hydroxyquassin	OH	Me	=O
4	18-hydroxyquassin	OMe	CH_2OH	=O
5	14,15-dehydroquassin	OMe	Me	=O

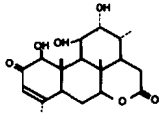
Class 2



		R^1	R^2	R^3	R^4
6	Nigakilactone A	OH	Me,H	=O	OH
7	Nigakilactone B	OMe	Me,H	=O	OH
8	Nigakilactone E	OMe	Me,OH	=O	OAc
9	Nigakilactone F	OMe	Me,OH	=O	OH
10	Nigakihemacetal A	OMe	Me,OH	H,OH	OH

Class 3

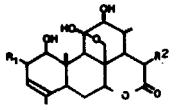




		R
11	Picrasin B	H,H
12	6-hydroxypicrasin B	H,OH

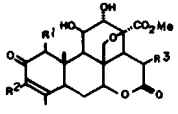
13 Klaineanone

Class 4 & 5



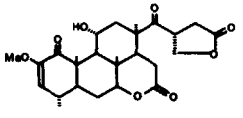
		R^1	R^2
14	Glaucarubine	=OH	
15	Glaucarubinone	=O	
16	Chaparrinone	=O	H,H

Class 6

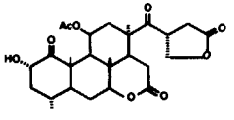


		R^1	R^2	R^3
17	Brucein A	H,H	OH	$OCOCH_2CHMe_2$
18	Brucein B	H,H	OH	OAc
19	Isobrucein B	H,OH	H	OAc

Class 8

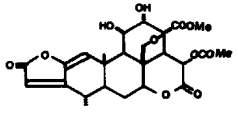


20 Picrasin A



21 Simaroiide

Class 9



22 Sergeolide

biosynthetically closely-related metabolites also recognized by the antiserum, the biosynthetic capacity was still measured. Furthermore, quassin constituted at least 60% of the quassinoid in the wood of *Q. amara* and 18% of that extracted from *P. quassioides* wood, nigakilactones A and B being 18 and 5%, respectively [16]. *Q. indica* has not previously been examined.

All the tissues analysed showed some quassin present (Fig. 2) but there was over a 10^4-fold variation in concentration. In green tissue from all three species, young expanding leaves contained more quassin than older leaf material, and the midrib consistently had a higher concentration than the lamella. Mature petioles contained a concentration approximating to that of the mature midrib, although in *Q. indica* it was rather higher. Young petioles, in contrast, contained very much less quassin than young midribs, reflecting a possible switch from synthesis to transport as the leaf matured.

Of the non-green tissues, seed was very low in quassin. As expected, the wood of mature *Q. amara* had a very high level as did the woody part of twigs from this species. In contrast, the wood from twigs of *P. quassioides* contained substantially less quassin than the green tissue, while in *Q. indica* this difference was even more marked.

It is very probable that as some of the enzymes of terpenoid biosynthesis are chloroplastic [18, 19], triterpenes are synthesized in leaves and transported to other parts of the plant. Quassimarin, a Class 6 quassinoid, has been isolated from the sap of *Q. amara* [20] and could represent a form of quassin modified for transport. This survey has shown that species other than *Q. amara* are capable of synthesizing quassin to a comparable extent, although they do not accumulate it in the wood to such a high level. The presence of this synthetic capacity, however, makes them important as potential sources for the production of quassin.

EXPERIMENTAL

Materials. Quassin was obtained from Koch-Light Laboratories Ltd. (Colnbrook SL3 OB7, U.K.). This preparation, referred to as 'mixed' quassins, is a mixture of the quassinoids from the wood of *Q. amara*, and was used to purify a number of these [15]. The term 'quassin' is reserved for the pure product. Other quassinoids were the kind gifts of Dr. J. Polonsky (CNRS Institut de Chemie des Substances Naturelles, Gif-sur-Yvette, France), Dr. T. Murae (Department of Chemistry, University of Tokyo, Japan) and Dr. J. D. Phillipson (School of Pharmacy, London, U.K.).

BSA (fraction V) and 4-nitrophenyl phosphate tablets were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.); keyhole limpet hemocyanin(KHLH) from C.P. Labs. Ltd. (Bishops Stortford, U.K.); and alkaline phosphatase (EC 3.1.3.1) labelled goat anti-rabbit Igg immune serum from Miles Labs. Ltd. (Slough, U.K.). The microtitration plates used were NUNC Immunoplate I (with certificate) and were washed using a Dynawasher II. The reaction density was determined at 405 nm on a Kontron SLT 210 auto-plate reader.

Plant material. Rooted cuttings of *Quassia amara* L. were generously supplied by the Royal Botanic Gardens (Kew, Richmond, U.K.) and mature leaf and stem tissues by the City of Liverpool Botanic Gardens (Calderstones Park, Liverpool, U.K.). The latter were stored in liquid N_2 until required. Seedlings of *Quassia indica* (Gaertn.) Nootebloom were germinated from seed sent by the Botanic Gardens of Indonesia (Kebun Raya, P.O. Box 110, Bogor, Indonesia) from whom seed of *Q. amara*

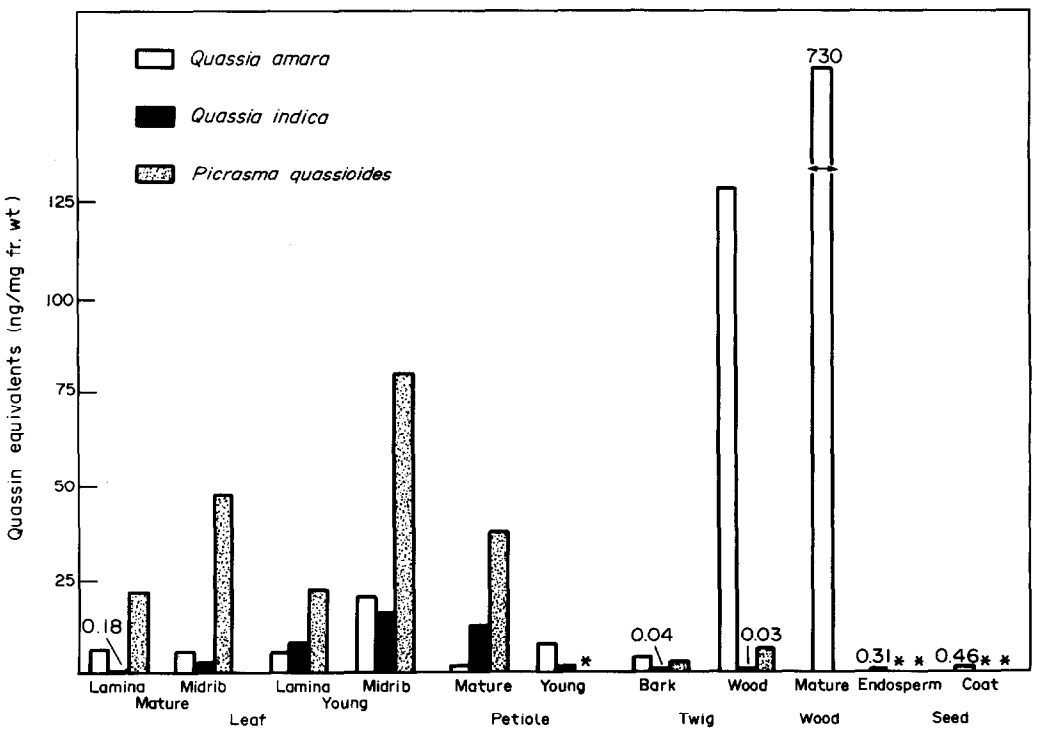


Fig. 2. Distribution of quassin in various tissues of three species of Simaroubaceae. Samples were extracted in methanol and analysed at suitable dilution as described in the text. * = Material unavailable.

was also obtained. *Picrasma quassioides* (Buch-Ham.) Bennett was kindly supplied by the Royal Botanic Garden (Edinburgh, Scotland, U.K.). Plants were maintained under glass with supplementary heating and illumination, and at high humidity.

Purification of component quassinoids from the 'mixed' quassins. 18-Hydroxyquassin for conjugation was purified by prep. HPLC [15]. The other components used to test for cross-reactions were purified immediately prior to use by analytical HPLC [10, 15].

Preparation of 18-hydroxyquassin–protein conjugates. (i) *Synthesis of 18-hydroxyquassin hemisuccinate*: 18-Hydroxyquassin (40 mg) and succinic anhydride (200 mg) were dissolved in C_5H_5N (1 ml) and left in a stoppered vessel for 4 days at room temp. The C_5H_5N was removed by rotary evapn and the residue dissolved in $CHCl_3$ (10 ml). After washing with H_2O (2 × 2 ml), the $CHCl_3$ was removed by rotary evapn to give a crude 18-hydroxyquassin hemisuccinate preparation.

(ii) *Synthesis of 18-hydroxyquassin–BSA*: The 18-hydroxyquassin–BSA conjugate was synthesized by the mixed anhydride technique [21]. The crude hemisuccinate was dissolved in 1,4-dioxan (2 ml) and tri-*n*-butylamine (0.05 ml) and cooled to 12°. After 10 min *iso*-butylchlorocarbonate (0.025 ml) was added, and the mixture was left at 12° for a further 15 min. The mixture was then added to a soln of BSA (80 mg) in H_2O (4.3 ml) which had been adjusted to pH 9.5 with 0.1 M NaOH. The pH was quickly raised to 7.3 with alkali, and the mixture was left at 4° for 4 hr. After dialysis against tap H_2O (15 hr) and distilled H_2O (3 × 5 l; 48 hr), and lyophilization, 74 mg of conjugate was obtained.

(iii) *Synthesis of 18-hydroxyquassin–KHLH*: This conjugate was synthesized as in (i) and (ii) above, substituting KHLH (80 mg) for BSA.

Immunization schedule. Rabbits were immunized, and serum was collected and stored as described previously [7].

Preparation of microtitration plates for ELISA. Microtitration plates were coated with 18-hydroxyquassin–KHLH. A soln of the conjugate (1.0 mg/l. in 0.05 M carbonate/bicarbonate buffer, pH 9.6) was filtered and 300 μl placed in each well. Plates were covered and left for 15 hr at 2°, washed 5 × with H_2O, and stored under desiccation until required.

ELISA procedure. Before use, coated plates were washed 5 × with phosphate-buffered saline, pH 7.4, containing Tween 20 (PBST [22]). Standards or samples to be assayed (100 μl in PBST or PBST containing 10% MeOH) and anti-18-hydroxyquassin antiserum (100 μl diluted $1:5 \times 10^4$ in PBST) were added to appropriate wells. After incubation for 15 hr at 2°, the plates were washed 5 × with PBST and 200 μl alkaline phosphatase-labelled anti-rabbit Igg antiserum (diluted 1:1500 in PBST) was added to each well. After incubation at 35° for 3 hr, the plates were again washed 5 × with PBST prior to adding 200 μl of substrate soln (4-nitrophenyl phosphate at 1 mg/ml in 0.05 M carbonate/bicarbonate buffer containing 0.5 mM $MgCl_2$) per well. After incubation for 1 hr at 35°, the absorbance of the soln in each well was recorded.

Extraction of plant tissues. Small pieces of tissue (1–25 mg) were excised from various parts of the plant, weighed and extracted with MeOH (1.0 ml) at 50° for 30–45 min. The extract was diluted with PBST to give a final soln containing 10% MeOH. Serial dilutions were then made in PBST containing 10% MeOH to provide several samples on the range of the standard curve for the ELISA. Triplicate aliquots were assayed for quassin content, quantitation being by reference to the standard curve conducted on each plate.

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