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The development of a radioimmunoassay for cannabinoids in blood and urine

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Antibodies, for use in radioimmunoassay, have been raised in sheep by immunization with a conjugate of Δ^9-tetrahydrocannabinol hemisuccinate and bovine serum albumin. Antiserum titre and avidity were increased by successive booster doses of conjugate. The high degree of non-specific binding encountered in the radioimmunoassay of cannabinoids was reduced by the use of the solubilizing detergent Triton X-405 and by restricting protein concentration in the assay medium. Plasma samples were deproteinized with ethanol before assay, but urine was directly assayed. High avidity antibodies and high specific activity [^{3}H]-Δ^9-tetrahydrocannabinol permitted the detection of 50 pg of cross-reacting cannabinoids—a sensitivity of 7·5 ng ml^{-1} of plasma and 1·0 ng ml^{-1} of urine. Whilst apparently specific for the three-ringed cannabinoid nucleus, the assay antiserum cross-reacted with several cannabinoids, both natural compounds and metabolites. Partial identification of cross-reacting cannabinoids was achieved by the use of pure compounds and by the assay of plasma and urine samples collected from rabbits given pure cannabinoids intravenously.

The production in sheep of antibodies to tetrahydrocannabinol (THC) suitable for use in a radioimmunoassay (RIA) has been described by Teale, Forman & others (1974b, c). Subsequently, improvements in antiserum titre and avidity and the commercial availability of radio-labelled THC of high specific activity, have enabled the RIA to be improved both in sensitivity and ease of performance, the high sensitivity permitting analysis of microsamples of plasma or urine with an acceptable degree of accuracy and precision.

MATERIALS AND METHODS

Δ^9-*trans*-Tetrahydrocannabinol (Δ^9-THC) was obtained from Makor Chemicals, Jerusalem, Israel and as a gift from the National Institute for Mental Health, Bethesda, Maryland, as were Δ^8-THC, cannabinol (CBN), cannabidiol (CBD) and 11-hydroxy-Δ^8-THC. Synthetic cannabinoids were a gift from Dr. N. K. McCallum then at the Hebrew University of Jerusalem. Pure tetracycline, amphetamine, lignocaine, promazine, diphenhydramine, nicotine, trifluoperazine, ephedrine and amitriptyline were donated by Dr. A. C. Moffat, Home Office Central Research Establishment, Aldermaston, Berks, and [^{3}H]-Δ^9-*trans*-tetrahydrocannabinol (^{3}H-THC) by Dr. E. W. Gill, Department of Pharmacology, Oxford, but subsequently purchased from the Radiochemical Centre, Amersham, Bucks. Bovine serum albumin (Cohn fraction V), charcoal (Norit A) and ethyl-(dimethylaminopropyl)-γ-carbodiimide were purchased from Sigma Chemicals Ltd., bovine γ-globulin (Cohn fraction II) from

Koch-Light Laboratories Ltd., Dextran T-70 from Pharmacia Ltd., Triton X-405, succinic anhydride, other chemicals and all solvents from BDH Chemicals Ltd.

Preparation of THC-conjugate

70 mg Δ^9-THC and 172 mg succinic anhydride were dissolved in 5 ml pyridine. 20 μCi (10 μg) of ^{3}H-THC was added as tracer to indicate the degree of incorporation of THC into the conjugates. The mixture was heated under reflux, in the dark, for 4 h. Pyridine was removed under vacuum at 45° and the residue dissolved in chloroform, washed several times with water and dried over anhydrous sodium sulphate. Chloroform was removed under vacuum at 35° and the residue dissolved in dimethylformamide (5 ml) and added to 100 ml distilled water (buffered with phosphate to pH 5·5) containing 250 mg bovine serum albumin (BSA). Ethyl-(dimethylaminopropyl)-carbodiimide (EDC) (120 mg) was added immediately and the mixture stirred constantly at room temperature. After 30 min a further 40 mg EDC was added and the solution stirred overnight at room temperature. The reaction mixture was extracted three times with 100 ml ether (to remove any unconjugated THC) and the remaining aqueous solution was dialysed against distilled water for 3 days at 4° to remove other small molecules which interfere with immunization.

The dialysed solution containing the THC-BSA conjugate was lyophilized under vacuum at 0°. Measurement of tracer ^{3}H-THC present in the conjugate showed that an average of 25 THC residues were attached to each BSA molecule.

Immunization procedure

THC-BSA conjugate (5 mg) dissolved in sterile water (1·5 ml) and emulsified with Freund's complete adjuvant (3 ml), was injected at six sites into the leg muscles of a sheep. Booster doses containing varying amounts of conjugate (see Fig. 1) emulsified

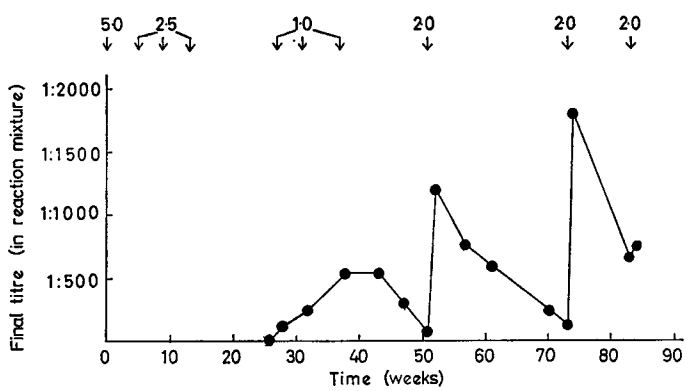

FIG. 1. Titres of antisera harvested from a sheep (S133Y) following immunization with a conjugate of THC-hemisuccinate and BSA. Arrows indicate dose times and values indicate mg of conjugate given.

in incomplete Freund's adjuvant were given intramuscularly at intervals. Blood was collected 8 days after each booster dose, allowed to clot and the serum separated. Thiomersalate was added to a final concentration of 0·025% (w/v) and the antiserum stored at 4° or, after dilution 1:4 in phosphate buffer, lyophilized under vacuum at

0°. Each bleed from each individual animal was coded separately and treated as unique.

Radioimmunoassay

Radioimmunoassay was carried out by conventional techniques. The detailed protocol finally adopted is shown in Table 1. Stock standard THC, prepared by

Table 1. *Assay protocol for RIA of THC cross-reacting cannabinoids in plasma.* Dilutions and additions were made using a Warner Compu-pet (proportioning dispenser).

	Volumes of reagent added (μl)				
Reagent	Total counts tube	Non-specific binding tube	Zero tube	Standard tube	Sample tube
Diluent buffer	380	380	380	280	380
Normal sheep serum (1 : 200)	100	100	—	—	—
Antiserum (1 : 200)	—	—	100	100	100
^{3}H-THC (260 pg)	100	100	100	100	100
THC standard	—	—	—	100	—
Normal plasma extract	20	20	20	20	—
Plasma sample extract	—	—	—	—	20
	Incubate 4 h at 4°				
Diluent buffer	200	—	—	—	—
Dextran-coated charcoal (2·5%)	—	200	200	200	200
	Centrifuge and count 500 μl of supernatant				

dissolving THC in ethanol to a concentration of 1 μg ml^{-1} was stored in the dark at −20°. Working THC solutions were prepared immediately before use by adding the appropriate amount of stock standard to diluent buffer (0·1 M phosphate, pH 7·4, containing 0·2% bovine γ-globulin and 0·1% Triton X-405). Antiserum 133Y/25/4 (collected at week 52, see Fig. 1) was used at a final dilution of 1 : 1200. ^{3}H-THC (Radiochemical Centre TRK.446 Batch 2; specific activity 26 μCi μg^{-1}) was stored in 50 μCi aliquots in ethanol (1 ml) at −20° and diluted 1 : 625 in diluent buffer immediately before use.

^{3}H-THC (260 pg) was placed in each assay tube with 100 μl of diluted antibody and either standard plasma extract or urine added. The total amount of each incubate was 600 μl. Incubations were carried out in glass tubes at 4° for 4 h. Separation of the free from antibody-bound fractions of ^{3}H-THC was effected by the addition of 200 μl of 2·5% dextran-coated charcoal. A 500 μl aliquot of each supernatant was taken for β-counting using a Packard Tricarb (model 2425) liquid scintillation spectrometer.

Dextran-coated charcoal was prepared by mixing a solution of 1 g dextran T 70 in 200 ml of 0·04 M phosphate buffer, pH 7·4, with a suspension of 10 g Norit A charcoal in 200 ml buffer. After stirring the mixture at 4° for 18 h the charcoal was removed by centrifugation. The supernatant was discarded and the charcoal resuspended in 400 ml of fresh buffer. Each batch was stored at 4°.

Plasma (or serum) was separated from red cells as soon as practicable after collection and stored at −20° until extracted with ethanol before analysis. One volume of

plasma was mixed thoroughly with twice its volume of absolute ethanol using a vortex mixer and centrifuged. The supernatant was removed and either assayed directly or stored at −20° until required. The ethanolic supernatant was added directly to the assay tube without further processing (see Results). Standard curves were constructed by adding an ethanolic extract of normal plasma to the assay tubes containing the THC standards.

Urine samples were assayed by direct addition to the assay tube (see Results). Standard curves were constructed by adding normal urine to the assay tubes containing THC standards.

Acute administration of cannabinoids to rabbits

Pure natural cannabinoids were given by rapid injection into the lateral ear veins of Dutch rabbits. The dose (80 μg kg^{-1}) was prepared by mixing 0·2 ml of a solution of cannabinoid in ethanol (5 mg ml^{-1}) with 1·3 ml of sterile physiological saline. Blood was collected at frequent intervals for up to 3 h and treated as described above. Urine from each animal was collected for 24 h after each intravenous dose.

Three groups of 3 rabbits were used: animals in group 1 were given THC intravenously, those in group 2 were given cannabidiol (CBD) and those in group 3 were given cannabinol (CBN). Plasma and urine was analysed for THC cross-reacting material using the RIA.

RESULTS

Radioimmunoassay

Titre. Fig. 1 shows the variation in titre (final dilution in reaction mixture) during the immunization schedule as well as the time and amount of each booster. The working dilution of antiserum was taken as that which bound 45% of the label.

Specificity. Antiserum 133Y/25/4, used for the assay of samples, was assessed in detail for its cross-reactivity with THC, other cannabinoids and a number of different drugs. With cross-reacting naturally occurring or synthetic cannabinoids standard curves were constructed using ^{3}H-THC as label. From these curves Scatchard plots were constructed which permitted calculation of the avidity constant for each compound. Table 2 lists the values obtained for cannabinoids which cross-reacted with the antiserum. Table 3 lists the non-cross-reacting cannabinoids together with compounds related structurally to cannabinoids and other drugs that were tested for cross-reactivity with antiserum. One μg of each compound showed no cross-reaction.

One hundred plasma samples, collected from hospital patients and volunteers taking a wide range of drugs, excluding THC, were extracted and assayed for THC cross-reactivity. No cross-reaction was encountered with any of the samples.

Assay conditions

Effect of Triton X-405. 0·1% Triton X-405 in the final reaction mixture was found to be the optimum concentration. This had a minimal effect on antibody binding of the radio label but retained the capacity to solubilize at least 2 ng of THC. Therefore assay conditions were arranged to ensure that each tube contained this concentration of detergent and not more than 2 ng of THC (or other cannabinoids).

Table 2. *Naturally-occurring and synthetic cannabinoids exhibiting reaction with antiserum under assay conditions.* The avidity constant of each reaction and the amount of each compound required to reduce the binding of ^{3}H-THC to antiserum by 50%.

Cannabinoid	Avidity constant (litres mole^{-1})	Amount reducing label binding by 50% (ng)
Δ^9-THC	$6{\cdot}0 \times 10^9$	0·4
Δ^8-THC	$6{\cdot}0 \times 10^9$	0·4
11-Hydroxy-Δ^8-THC	$6{\cdot}0 \times 10^9$	0·4
Cannabinol	$6{\cdot}0 \times 10^8$	0·4
11-Hydroxy-cannabinol	$2{\cdot}2 \times 10^7$	35·0
*8α-Hydroxy-HHC	$6{\cdot}0 \times 10^9$	0·4
*Cannabinol-11-al acetate	$6{\cdot}0 \times 10^9$	0·4
*Δ^8-THC-11-oic acid methyl ester	$6{\cdot}0 \times 10^6$	>100
*Δ^8-THC-11-oic acid methyl ether	$9{\cdot}8 \times 10^6$	>100
*8-Acetoxy-9-hydroxy-HHC	$8{\cdot}8 \times 10^6$	>100

HHC = hexahydrocannabinol.
* Synthetic

Table 3. *Cannabinoids and other compounds exhibiting no reaction with antiserum when present in the assay in amounts up to 1 μg.*

Cannabinoids	
Cannabidiol	*4-Carbomethoxy-Δ^8-THC
Cannabichromene	*Δ^8-THC-11-oic acid methyl ester
Cannabicyclol	methyl ether
Other compounds	
Morphine	Diphenhydramine
Codeine	Nicotine
Heroin	Trifluoperazine
Methadone	Ephedrine
Aminoglutethimide	Amitriptyline
Sulthiame	Lysergic acid
Phenobarbitone	Salicylate
Phenytoin	2-hydroxybiphenyl
Tetracycline	Carvone (terpenes)
Amphetamine	α-Pinene (terpenes)
Lignocaine	Piperitone (terpenes)
Promazine	Pulegone (terpenes)

* Synthetic.

Adequate solubilization was achieved only in glass tubes (approximately 35% of the label was adsorbed by plastic tubes); thus, all assays were performed in glass tubes.

Recovery of THC from plasma during ethanol extraction. As THC could not be assayed in plasma added directly to the assay reaction mixture, the proteins were precipitated by ethanol, in which THC is soluble, and the alcoholic extract was added to the assay mixture. A ratio of 2 parts ethanol to 1 part of plasma was necessary for maximum recovery of ^{3}H-THC. Using this ethanol:plasma ratio, a further series of plasma samples to which THC had been added were extracted and the

supernatants analysed for THC content by RIA. Recoveries were 97, 101, 92 and 132% for THC amounts of 50, 25, 10, and 5 ng ml^{-1} plasma; the respective coefficients of variation were 14·6, 15·9, 34·2 and 40·9%.

Effect of ethanolic plasma extract on assay characteristics. Up to 20 μl of ethanolic extract could be added to the system before the decrease in label-binding fell below 50%. Up to 50 μl of urine had no effect on label-binding under the assay conditions adopted.

Precision and reproducibility of the assay. The assay was sensitive to the addition of 50 pg THC to the assay tube. This was sufficient to produce a 3–5% reduction in bound ^{3}H-THC compared to a plasma blank and was equivalent to a sensitivity of less than 7·5 ng ml^{-1} THC in plasma or 1 ng ml^{-1} in urine. Between batch precision (c.v.) of the assay was 15% at 25 ng THC ml^{-1} rising to 40% at 5 ng ml^{-1}.

Animal experiments

The amount of THC cross-reacting cannabinoids recovered from the plasma of the 3 rabbits given THC fell from a peak of 230–245 ng ml^{-1} at 15 min to approximately 50 ng ml^{-1} at 1 h and then to 20–10 ng ml^{-1} after 3 h. For the 3 animals given CBN the peak was 55–57 ng ml^{-1} at 5 min falling to 15–10 ng ml^{-1} at 3 h. The responses were similar amongst animals of each group. No THC cross-reacting cannabinoids were detected in the plasma of rabbits given CBD.

The amount of THC cross-reacting cannabinoids recovered in the 24 h urine specimens collected from the rabbits given THC varied between 18·3 and 38·0 μg and of rabbits given CBN 16·0 and 38·6 μg. No cross-reacting material was detected in the urine of rabbits given CBD.

DISCUSSION

The commercial availability of high specific activity ^{3}H-THC in combination with the production of a high avidity antiserum to THC have largely overcome problems in the lack of sensitivity of the RIA in measuring THC in biological fluids. Difficulties due to non-specific binding of THC to proteins and its extreme lipophilicity remain.

Reduction of non-specific binding to a minimum and solubilization of cannabinoids in an aqueous assay medium was achieved by using bovine γ-globulin. Triton X-405 (0·1%) proved suitable for solubilizing THC without affecting antigen-antibody interaction (Triton X-100, even at very much lower concentrations, completely inhibited antibody binding).

The addition of plasma albumin and lipoproteins to the assay system even in the presence of a solubilizer, reduced the binding of radio-label to antibody to unacceptably low levels, thereby precluding direct assay of unextracted plasma. Presumably once dissolved in the lipid moiety of lipoprotein THC cannot be removed even by the most avid antibody. Consequently, whilst urine samples can be assayed directly, plasma samples must first be extracted. Several techniques are available but it is possible to use ethanol as combined protein precipitant and solvent. While this permits almost complete recovery of cannabinoids, the volume of extract that can be

added to the assay tube is restricted to 20 μl or less if antibody binding is not to be reduced to unacceptably low levels. This limits the sensitivity of the assay in its present form. Paradoxically successive increases in antiserum titre and avidity has made the system more sensitive to additions of ethanol.

The use of sheep for antibody production has yielded antihapten antibodies not only of higher avidity than those produced by rabbits but also at higher titre and in larger amounts. The assay characteristics of each antiserum vary, not only from animal to animal, but also from bleed to bleed.

Preliminary studies with rabbits (Teale, Forman & others, 1974a) provided information on the optimum number of THC derivatives per carrier molecule and the optimum length of the spacer group between them during conjugate preparation. Maximum immunogenicity was achieved with about 20 hapten residues linked through a 4-carbon chain to a carrier molecule. These values are in agreement with studies on other drugs (Robinson, Morris & others, 1975). Antisera raised against a 1-carbon link conjugate provided the basis of our first THC assay system (Teale & others, 1974b) but subsequent booster immunizations with the conjugate failed to improve avidity or antiserum titre which remained impracticably low. The 4-carbon conjugate used in the same way led to production of antibodies which increased in avidity and titre after boosting at suitable intervals (Fig. 1). Even with a monthly immunization schedule, antiserum of sufficient titre for use in an RIA took 9 months to produce. The antiserum used in the current assay was that harvested one year following primary immunization.

Antibodies raised in rabbits, whilst apparently capable of binding an iodinated THC-copolymer showed no reaction with ^{3}H-THC itself. Similar observations have been reported for THC by Van Vunakis & Levine (1974).

Whilst antisera raised against THC conjugates apparently react exclusively with cannabinoids, they seemingly cannot discriminate between several compounds within that general group (Table 2). Conjugation to carrier protein through the hydroxyl group of THC apparently masks the area around that functional group, including the 11-position. The cross-reactive behaviour of antiserum 133Y/25/4 is similar to that exhibited by an antiserum raised against a carboxymethyl-THC-carrier conjugate (Van Vunakis & Levine, 1974). In a recent report Gross, Soares & others (1974) reported that the use of azo derivatives of THC, presumably linked at position 2 or 4 thereby exposing the area near to the C_{11}, apparently resulted in the production of antibodies which showed only partial cross-reactivity with 11-hydroxy-THC. Like 133Y/25/4 the antiserum raised by Gross & others (1974) reacted only poorly with THC-11-oic acid (or its methyl ether) but unlike 133Y/25/4 it also cross-reacted with cannabidiol though only weakly.

In the animal experiments, since no THC cross-reacting material was detected in either the plasma or the urine from rabbits given CBD, it can be concluded that not only CBD but none of its metabolites in rabbits are measured in the assay. 11-hydroxy-CBN does not react markedly with antiserum 133Y/25/4 and, if it is a major metabolite of CBN, the cross-reacting cannabinoids observed in plasma following the intravenous injection of CBN can be attributed largely or solely to CBN itself. The fall in concentration with time can be considered as being due to disappearance of CBN. If THC behaves similarly the rise in plasma cross-reacting cannabinoids observed 10 to 15 min after intravenous injection of THC could represent mainly

THC metabolites which have entered the vascular compartment from the tissues—the most likely metabolites are 11-hydroxy-THC (Lemberger, Silberstein & others, 1970) but almost certainly not THC-11-oic acid since this does not cross-react with antiserum 133Y/25/4.

The high group specificity of the present assay system for three-ringed cannabinoids without absolute specificity for THC has advantages as well as disadvantages in practice. Because it cross-reacts with 11-hydroxy-THC the present assay can be employed to detect cannabis use in epidemiological studies, case finding and for clinical purposes by examination of the urine. An assay capable only of detecting THC on the other hand would be useless for these purposes since unchanged THC is not excreted in the urine (Hollister, Kanter & others, 1974).

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