STUDIES OF CANNABINOID METABOLITES – A FREE RADICAL IMMUNOASSAY

Michael CAIS*, S. DANI, Y. JOSEPHY and A. MODIANO
Department of Chemistry, Technion-Israel Institute of Technology, Haifa, Israel

H. GERSHON
School of Medicine, Technion-Israel Institute of Technology, Haifa, Israel

and

R. MECHOULAM
School of Pharmacy, Hebrew University, Jerusalem, Israel

Received 22 May 1975

1. Introduction

The societal implications of the growing use of cannabis drugs finds expression also in the noticeable recent expansion of cannabinoid research in biological, chemical, pharmacological and medical sciences [1]. Rather surprisingly, however, there have been relatively few reports of attempts to develop immunological techniques for the study and identification of cannabinoid metabolites. One reason for this may be due to the serious difficulties which have been encountered in the production of anti-cannabinoid antibodies [2]. A radioimmune assay for Δ^1-tetrahydrocannabinol (Δ^1-THC $\equiv \Delta^9$-THC) recently described [3] utilized goat antiserum obtained [4] against a mixture of isomers of azo-Δ^1-THC. The authors claim [3] potential use of their method for the detection of THC in the plasma of chronic marijuana users, if the assay is carried out within 15 min after the smoking act. A similar time-limit for the detection of THC in plasma applies to a recently described [5] assay employing a combination of gas liquid chromatography and mass spectrometer analysis. Another radioimmune assay, using a sheep antisera and [^{3}H] THC has been reported [6] to detect THC in the blood or urine of a person following the smoking of a single cigarette impregnated with 5 mg of pure THC.

In this communication we wish to report the synthesis of protein conjugates with Δ^6-tetrahydrocannabinol (Δ^6-THC) derivatives II-V, the production of specific antibodies to the II to V-haptenic groups, the preparation of spin-labelled haptens, VII-IX, (fig.1) and the use of these materials for the detection of cannabinoid metabolites by a free-radical immunoassay, (fig.2). These results have been obtained as a part of a systematic study undertaken to determine the capability of the cannabinoid structural skeleton to trigger specific antibody formation. In addition, in view of previously reported difficulties [2] we have been aiming at the production of optimal antisera preparations for suitable general use in immunoassays.

2. Materials and methods

The starting material, Δ^6-THC, I, was obtained by previously described methods involving solvent extraction of hashish, chromatographic purification and appropriate chemical transformations [1]. The cannabinoid acids II to VI were prepared by standard organic synthesis procedures and all had satisfactory elementary and spectroscopic analyses.

The haptens (fig.1) were coupled to two immuno-

* To whom inquiries should be addressed.

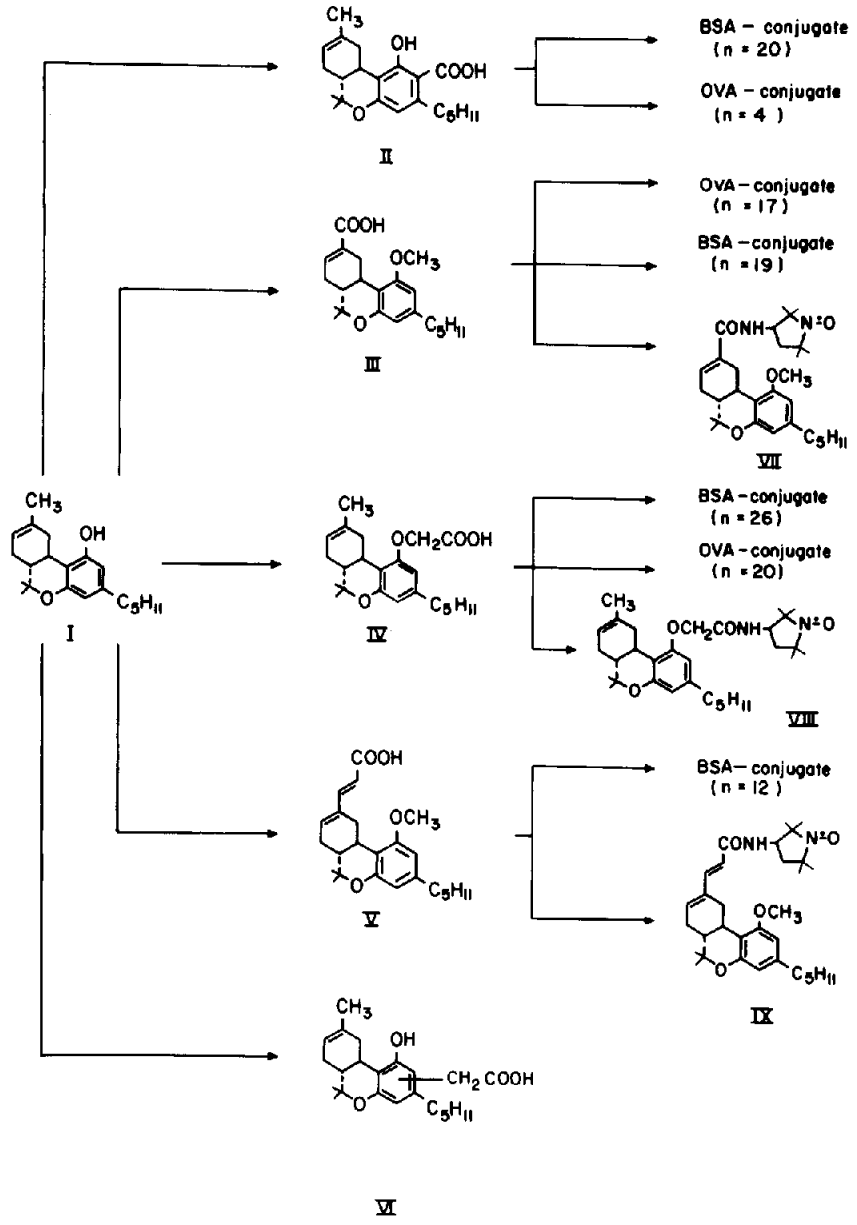

Fig.1. Derivatives of Δ^6-THC coupled to protein carriers (BSA and OVA, n = number of hapten molecules per protein molecule) and to a stable free radical (3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl).

logically non-crossreacting carriers, bovine serum albumin (BSA) and ovalbumin (OVA), by the mixed anhydride procedure using isobutylchlorotormate in dimethyl formamide. The degree of conjugation, n, (fig.1) was determined by differential u.v. analysis. Rabbits were immunized by multiple subcutaneous injections along the flank, nape and rump with an emulsion of complete Freund's adjuvant and the appropriate cannabinoid-BSA conjugate. Bleeding was carried out prior to immunization (for normal serum) and at weekly intervals subsequent to immunization. Booster injections were given at regular six-weekly intervals. Sera from bleedings between boosters were pooled. Immunoglobins were preparated from pooled antisera and normal sera by precipitation in ammonium sulfate followed by dialysis against 0.01 M phosphate buffered saline, pH 7.3 (PBS). Immunological assays were carried out using both classical precipitin reactions with hapten inhibition of precipitation and free radical immunoassay techniques. The precipitin curves obtained in the quantitative assays showed that the antisera produced contained antibodies to the hapten as well as to the carrier (BSA). This was demonstrated by the precipitation of BSA with or without hapten conjugation, by the precipitation of hapten-OVA conjugate by antisera which did not recognize the OVA carrier alone and by inhibition of precipitation of hapten-OVA by free hapten. The normal sera prior to immunization were not found to react with any of the antigens tested. The amount of specific anti-cannabinoids antibodies in various sera was found to vary from 0.3 mg/ml to 1.9 mg/ml.

The spin labelled haptens VII-IX, prepared by coupling the respective carboxylic acid derivatives with 3-amino-2,2,5,5-tetramethylpyrrolidine-1-oxyl (using dicyclohexyldiimide as coupling reagent), were employed to characterize the anti-cannabinoid antibodies by a competitive binding free-radical technique analogous to that already described for anti-morphine antibodies [7]. A solution containing appropriate known amounts of specific γ-globulin and spin-labelled hapten was titrated with increasing amounts of the unlabelled hapten. The amount of competitively displaced spin-labelled hapten was monitored by a Varian E-4 electron spin resonance (ESR) instrument. A typical curve obtained in the determination of antiserum specificity is shown for anti-hapten III (fig.2) together with cross-reactivity curves measured for closely related cannabinoids.

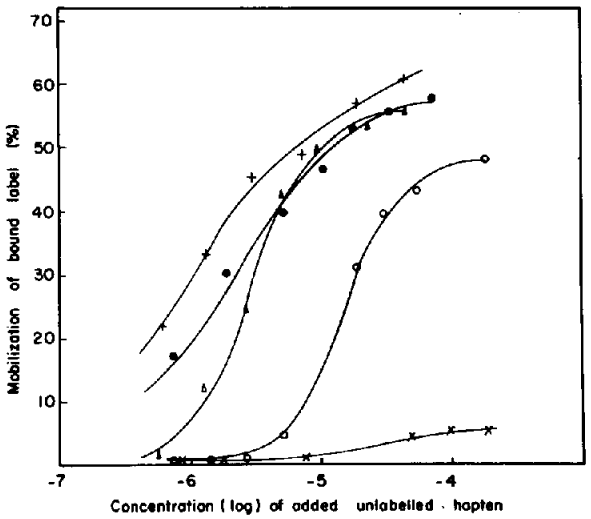

Fig.2. Mobilization of antibody bound-spin-labelled 7-nor-1-carboxy-3′-methoxy-Δ^6-THC (III) by increasing amounts of unlabelled THC derivatives. (•) 7-nor-1-carboxy-3′-methoxy-Δ^6-THC (III); (+) 7-nor-1-carboxyvinyl-3′-methoxy-Δ^6-THC (V); (△) 7-hydroxy-3′-methoxy-Δ^6-THC; (○) 3′-*O*-carboxymethyl-Δ^6-THC (IV); (x) Codeine.

3. Results and discussion

Recent reviews [1,8] on metabolic studies with cannabinoids show that, whilst a number of important facts have become known, the general picture is still rather confused and controversial. This has imposed restrictions on our choice of haptens for the production of anti-cannabinoid antibodies. Our selection was governed in the first stage by structural analogies to identified THC metabolites. Subsequently we aimed at collecting information on the ability of the various cannabinoid features to trigger specific antibody production.

Data obtained so far indicate that antibodies elicited with hapten-III antigens possess greater affinity than those produced with antigens carrying haptens IV or V. Acid III bears a close structural relationship to 7-hydroxy-Δ^1-THC which has been found in the urine of marijuana smokers [9]. Metabolic oxidation of the 7-methyl function to a carboxyl group, leading to analogs of III, is known [10].

This relationship also finds expression in the degree of cross-reactivity by 7-hydroxy-3′-methoxy-Δ^6-THC in the mobilization of the antibody bound spin-labelled-acid III (fig.2).

Anti-hapten II antibodies also appear to be of satisfactory affinity and specificity. However, some uncertainty must be attached to the interpretation of results obtained from work with II due to the reported instability of the latter (II is known to undergo facile decarboxylation). We are currently investigating hapten VI which retains all the functional groups of Δ^6-THC (including the phenolic group) and hopefully will not exhibit the instability reported for II.

The applicability of these results to urine analysis for cannabinoid metabolites was checked by spiking normal urines with the Δ^6-THC derivative, III, and measuring the degree of detection with our reagents. Variations in the ESR signal of 100 randomly collected normal urines are given on the left side of the histogram in fig.3. On the right side of the histogram are shown the variations in the ESR signal obtained directly with the same urines spiked with 0.7 μg/ml of the acid derivative III. The mirror-image like symmetry, of the two parts of the histogram, is a gratifying pictorial outcome of the fact that every spiked urine sample gave a higher ESR signal than its non-spiked counterpart.

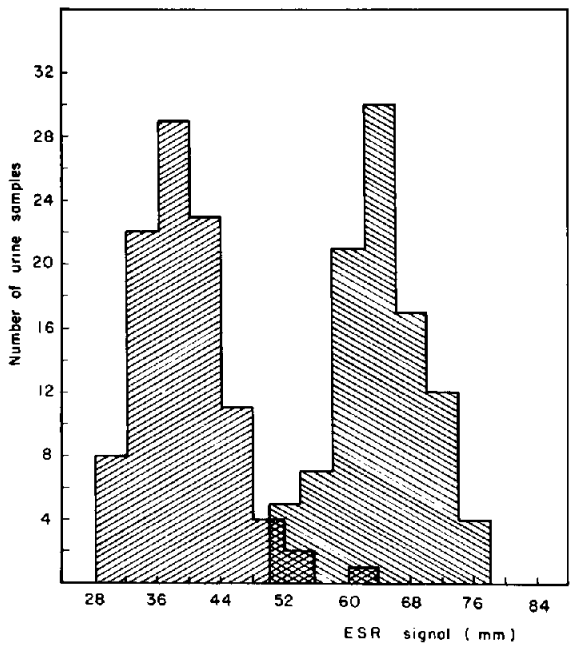

Fig.3. ESR signal response distribution from 100 normal (negative) urine samples and the same urines spiked to a level of 0.7 μg/ml 7-nor-1-carboxy-3′-methoxy-Δ^6-THC (III). (▫) Negative urines. ▪ Spiked urines

Preliminary free radical immunoassay experiments with extracts from ten human urines collected from both casual (less than once a month) and habitual (at least once week) hashish smokers indicate that a positive, significant increase in the ESR signal should occur with the samples given any time, from 5 to 72 hr after the smoking of hashish. Urine samples collected between 0.5 – 2.5 hr subsequent to hashish smoking did not give positive tests. A larger number of urine samples from hashish users must be tested before the scope of our assay can be fully assessed. We have been having some difficulties in obtaining such samples.

Acknowledgement

We wish to thank Syva Research Institute, Palo Alto, Calif., for partial financial support and Miss S. Adamovich for technical assistance.

References

[1] Mechoulam, R., (ed.), (1973): Marijuana, Chemistry, Pharmacology, Metabolism and Clinical Effects, Academic Press, New York, N.Y.
[2] Tsui, P. T., Kelly, K. A., Ponpipom, M. M., Strahilevitz, M., and Sehon, A. H. (1974) Can. J. Biochem. 52, 252, also personal communication from Professor A. H. Sehon.
[3] Gross, S. J., Soares, J. R., Wong, S-L. R. and Schuster, R. E. (1974) Nature 252, 581.
[4] Grant, J. D., Gross, S. J., Lomax, P. and Wong, R. (1972) Nature, New Biol. 236, 216.
[5] Agurell, S., Gustafsson, B., Holmstedt, B., Leander, K., Lindgreen, J. E., Nilsson, I., Sandberg, F., and Asberg, M. (1973) J. Pharma. Pharmac. 25, 554.
[6] Teale, J. D., Forman, E. J., King, L. J. and Marks, V. (1974) The Lancet 553.
[7] Leute, R. K., Ullman, E. F., Goldstein, A. and Herzenberg, L. A. (1972) Nature, New Biol. 236, 93
[8] Mechoulam, R., McCallum, N. D. and Burstein, S. (1975) Chem. Rev., in press.
[9] Woodhouse, E. J. (1972) Amer. J. Publ. Health 62, 1394.
[10] Burstein, S., Rosenfield, I. P. and Wittstruck, T. (1972) Science 176, 422.