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The effects of pH and temperature on the *in vitro* bindings of delta-9-tetrahydrocannabinol and other cannabinoids to bovine serum albumin

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Abstract: Albumin is a major carrier of drugs and fatty acids in biological fluids. These protein–drug complexes serve to solubilize, transport these compounds to sites of action, and have been associated with increased half-life for these compounds. The authors are interested in the pH and temperature effects of the binding of delta-9-tetrahydrocannabinol to albumin. Ultrafiltration techniques were used in the separation of free to bound compounds. Cannabinoids bind to bovine serum albumin rapidly. The cannabinoid binding sites are more sensitive to temperature changes (37–47°C) than changes in pH with 37°C and pH 7.4 resulting in optimal binding. These conditions would result in the greatest viability in the cells, while allowing for the use of a variety of compounds in *in vitro* studies for the administration of compounds to isolated cells and cell lines.

Keywords: *Cannabinoid; marijuana; high-pressure liquid chromatography.*

Introduction

Mecholaum in the late 1960s identified the pschoactive component of marijuana as delta-9-tetrahydrocannabinol (D9THC) [1, 2]. Since then, 63 compounds have been identified and synthesized. The health effects of these compounds are being actively investigated because of their high use and abuse worldwide. Cannabis compounds are very lipophilic and are quite readily absorbed into the cell. One of the most important factors that determines the absorbability of a compound into the cell is its physical state.

Previous investigations have shown that cannabinoids can exist bound to naturally occurring proteins in biological fluids. Albumin, alpha-1-lipoprotein, and beta-1-lipoprotein are some of the major proteins that bind to cannabinoids. These protein–drug complexes serve to solubilize, transport these compounds to sites of action, and have been associated with increased half-life for these compounds [3–6]. The authors are interested in the pH and temperature effects of the binding of D9THC to albumin. Albumin is a major carrier of drugs and fatty acids in biological fluids and therefore the determination of pH and temperature effects on D9THC binding to albumin will be useful in determining a suitable carrier for the delivery of D9THC and other cannabinoids to lymphoid cells in tissue culture studies.

The authors have employed ultrafiltration techniques in the separation of free to bound compound because: (1) this technique does not require dilutions as in other techniques; and (2) it is a method that has been widely used in a variety of basic sciences and clinical studies (7–9).

Materials and Methods

Materials and equipment

Phosphate buffered saline (PBS) at pH 7.4 was diluent for bovine serum albumin (BSA) in the studies below. BSA when stored at 2–8°C was stable for 3 months. Lipids were removed from the BSA by extraction with diethyl ether in a 1:4 (BSA–diethyl ether) volume ratio for 10 min. The extracts were then evaporated and reconstituted in a 100 μl of methanol–ethyl acetate (2:1, v/v), and 6 μl injected into a Hewlett–Packard 5020A gas chromatograph equipped with a flame-ionization detector. The column employed was a 5 ft × 2 mm i.d. (3 mm o.d.) 5% DEGS-PS column (Supelco, Supelco Park, PA) with an injection tempera-

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ture of 275°C, and a temperature program from 120–290°C at 10°C min^{-1}. The detector temperature was 300°C and the resulting chromatogram was compared with known fatty acid (FA) standards.

Delta-9-tetrahydrocannabinol (D9THC), cannabinol (CBN), cannabidiol (CBD), and delta-8-tetrahydrocannabinol (D8THC) were obtained as powders from Applied Science Corporation (State College, PA). The purity of the cannabinoids was checked by gas chromatography–mass spectroscopy (GCMS). A few grains of a solid standard or 0.5 μl of a liquid standard was placed in an aluminium crucible (Finnigan Mat, San Jose, CA) with the liquid sample being evaporated on a hot plate (50°C). The sample was then injected via a solid probe into a Finnigan 112 MAT GCMS system with electron voltage set at 70 eV, filament current at 0.2 μamps, and scanning at 1 s scan in the range *m/e* 35–800 [10]. The spectra generated were directly compared with standard spectra in order to rule out the presence of interfering fragment ions. Stock solutions of 200 ng ml^{-1} of each cannabinoid were added to a set of siliconized glass tubes, the methanol being evaporated by placing these tubes under a gentle stream of nitrogen. BSA solutions were placed in standard test tubes (12 × 100) and vortexed with the appropriate cannabinoid standard to achieve the desired concentration.

HPLC conditions and adsorption study

It was wished to eliminate the possibility that the D9THC was not binding to the glass surface of the test tubes. Therefore, six test tubes were silanized with hexadichlorodisilazane (5% in $CHCl_3$) by passing the tubes through the following sets of solutions: (1) soapy water with subsequent oven drying; (2) rinse in methanol (MEOH); (3) dichlorodisilazane solution in test tubes for 20 min; and (4) acetone and methanol rinse. To each tube 200 ng of D9THC was added and these tubes were incubated at room temperature for 0, 15, 30, 45, 60 and 75 min, respectively. After incubation, 20 μl of the solution was injected into a Perkin–Elmer Series 6 liquid chromatograph using a C_{18} 5-μm particle size column. The mobile phase was methanol–water (70:30, v/v) with ultraviolet detection at 254 nm and flow rate at 1 ml min^{-1} [11–13]. Quantitation was achieved by direct comparison with standards.

Sample treatment

"Syva-free" filters (Syva, Palo Alto, CA) were employed in BSA–THC binding studies with the filtration unit attached to the bottom of a 1 ml syringe according to the standard Syva protocol [7]. Twenty microlitres were injected into the Perkin–Elmer Series 6 chromatograph using the above described analytical parameters.

A preliminary experiment was performed to determine the greatest amount of filtrate produced from a variety of BSA concentrations (Table 1). A series of solutions containing 10, 20, 35, 60 and 80% BSA were drawn through filters. Thirty-five percent BSA yielded the greatest amount of filtrate in the least amount of time. Concentration and time effects were investigated with BSA in a second set of experiments, using 50, 100 and 200 ng ml^{-1} of D9THC and other cannabinoids at a series of temperatures 27, 37 and 47°C and at pH values of 6, 7.4 and 10. Two millitres of 35% PBS–BSA were mixed with 200 ng of D9THC in a test tube (12 × 100) and vortexed for 10 min. Three different PBS–BSA solutions were studied at pH 6.0, 7.4 and 10, respectively, the pH being adjusted by a dropwise addition of 2 M NaOH and 2 M HCl, respectively (cf. Table 2). The test tubes were then incubated for 15–70 min in a water bath at 27, 37 and 47°C, respectively. The samples were then passed through the Syva membrane to separate free from bound drugs and the filtrate analysed by the HPLC technique described above.

Results and Discussion

The physical state of D9THC and other cannabinoids when binding to protein mol-

Table 1
Amount of filtrate obtained when varying concentrations of BSA were filtered through "Syva-Free" membranes

%BSA	Filtrate, μl (±SD)
10	25 ± 2
15	42 ± 6
20	56 ± 8
25	79 ± 3
30	140 ± 12
35	200 ± 10
40	195 ± 9
45	170 ± 8
50	150 ± 6
55	145 ± 4
60	132 ± 7
70	65 ± 5
80	45 ± 3

Table 2
Percent BSA binding as a function of concentration, temperature and time of incubation of cannabinoids on BSA

	50 ng ml^{-1}			100 ng ml^{-1}			200 ng ml^{-1}		
	27°C	37°C	47°C	27°C	37°C	47°C	27°C	37°C	47°C
15 min	2	9	4	8	14	11	14	23	18
30 min	4	12	15	11	19	16	14	30	22
45 min	4	10	8	10	19	14	13	28	22
60 min	3	9	5	7	19	14	14	28	20
75 min	3	19	9	10	19	13	15	29	20

ecules at various pH and temperature conditions is of critical importance in the expression of its function when these compounds were incubated with cells in culture. The authors are interested in determining if D9THC would interact with a suitable protein carrier facilitating its delivery to cells in tissue culture and the pH and temperature parameters governing this binding.

A variety of BSA solutions (10–80%) were drawn through the membrane filters as shown in Table 1. Thirty-five percent BSA yielded 200 plus or minus 10 μl of filtrate. In the second study, 50, 100 and 200 ng ml^{-1} of D9THC, D8THC, CBN and CBD were added to 2 μl of PBS–BSA at 27, 37 and 47°C (Table 2). These studies were performed in quadruplicate to insure statistical validity. D9THC and CBN bind to a greater extent to BSA than D8THC and CBD. For each compound tested, a graphical representation of percent BSA binding versus temperature (Fig. 1) shows that maximum binding for each compound occurs at 37°C. If the temperature of incubation is 27 or 47°C the percent D9THC binding decreases from 28 to 12% or 18%. It is very probable that changes of temperature from 37 to 47°C or to 27°C limits the number of binding sites on BSA for cannabinoids.

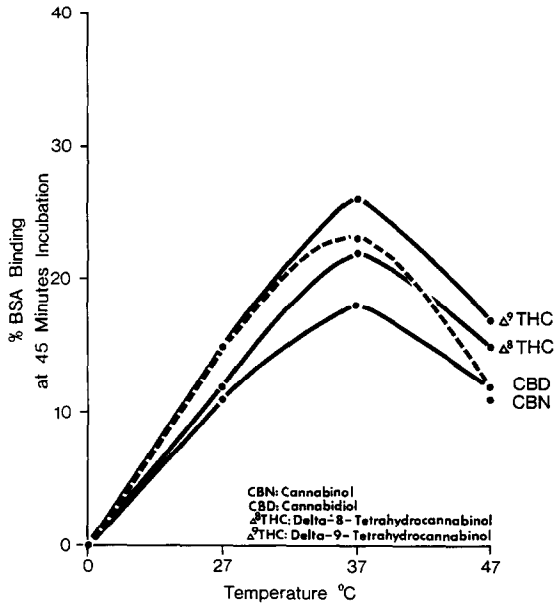

Figure 1
Effect of temperature on cannabinoid BSA binding after 45 min incubation.

In the third set of experiments, the pH was altered from pH 7.4 to 5, 6, 10 and 12 by the addition of 2 M HCl or to 200 ng ml^{-1} D9THC, CBN, CBD, D8THC and 2 μl of PBS–BSA. Figure 2 shows the effect of time on the binding of cannabinoids to BSA at pH 6.0, 7.4 and 10. Generally, binding increases to a maximum at 15 min. The figure also shows that at pH 7.4 optimal binding occurs. A decrease in pH to 6 and an increase to 10 results in dramatic decreases in binding (25 to 8% for pH 6 and 25 to 4% for pH 10). The presence of excess hydroxide ions decreases the number of binding sites to a greater extent than excess hydrogen.

In summary, cannabinoids bind to BSA rapidly. The cannabinoid binding sites are more sensitive to temperature changes (38 to 47°C) than changes in pH with 37°C and pH 7.4 resulting in optimal binding. These are excellent conditions for the administration of compounds to isolated cells and cell lines because tissue culture systems generally exhibit a narrow temperature tolerance before viability is decreased dramatically. Since D9THC contains an unsaturated acyl group similar to that found in fatty acids, the nature of D9THC binding to BSA would be similar to binding of BSA to fatty acids. Hydrogen ion concentration (pH) has been shown in the literature to affect a number of drug binding sites and their *in vitro* dissociation constants [6]. Other studies have shown that temperature affects the free concentration of drugs in the serum. A higher concentration of free phenytoin was detected at 37°C than at room temperature in a series of clinical trials. In contrast, in the present study a minimal

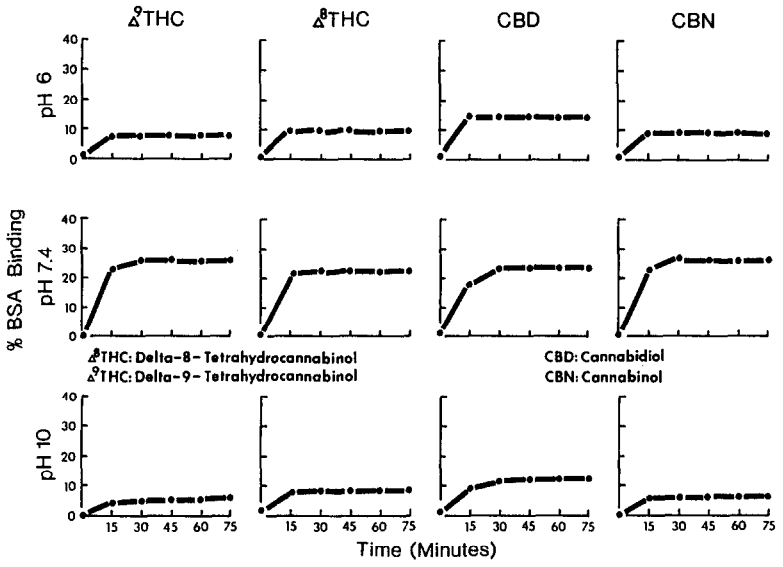

Figure 2
Binding of D9THC, D8THC, CBN, CBD to BSA.

amount of free cannabinoid existed at 37°C. This could be due to an unknown critical difference in the binding site structure and/or conformation.

In conclusion, BSA can function as a carrier molecule for cannabinoids under a variety of *in vivo* and *in vitro* conditions (pH 7.4, 37°C) which allows for optimum binding. These conditions would result in the greatest viability in the cells while allowing for the use of a variety of compounds in the present *in vitro* studies.

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