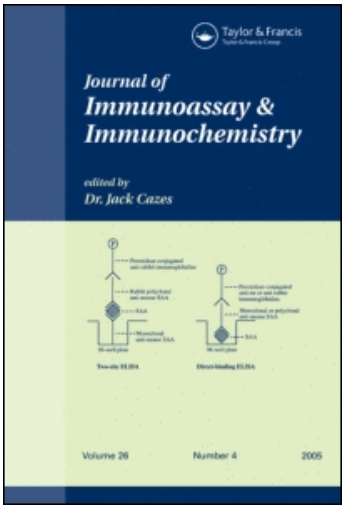

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RADIOIMMUNOASSAY FOR COLCHICINE : SYNTHESIS AND PROPERTIES OF THREE HAPTENS

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ABSTRACT

For the development of radioimmunoassay procedures for colchicine, three haptens, N-ethylamino-colchiceinamide, 4-formylcolchicine - (O-carboxymethyl) oxime and 4-hydroxymethylcolchicine O-hemisuccinic acid were synthetized and characterized by mass and proton magnetic resonance spectrometries. The conjugates obtained by coupling the haptens to bovine serum albumin were employed to immunize rabbits and goats.

INTRODUCTION

Colchicine (I) is one of the oldest drugs used in the treatment of acute gouty arthritis. Because of its great toxicity and its high therapeutical activity the treatment doses are low (1 or 2 mg per day). As a result, for thorough study of the metabolism, pharmacokinetics and clinical monitoring of this drug, sensitive and specific procedures are required for the determination of colchicine levels in biological fluids.

Previous studies of colchicine metabolism utilized analytical techniques such as indicator dye methods (1), isotopic dilution technique coupled with purification by thin-layer chromatography (2), and fluorescent labelling (3). These methods are tedious, require extraction of the drug from biological fluids and are not convenient for routine clinical monitoring.

On the other hand, radioimmunoassay (RIA) allows rapid analysis of numerous samples without purification. Ertel et al (4) reported a sensitive RIA (detection of 50 pg per tube) based on antibodies produced with a conjugate for which the hapten was coupled to the bovine serum albumin through the ketone group of the tropolone ring ; this technique was applied to the determination of colchicine in human plasma and urine following administration of a therapeutic dose. Previously Boudène et al (5) reported a procedure in which the hapten was the N-desacetylthiocolchicine (II) but it was not sufficiently sensitive (5 ng). We have recently developed an RIA, using an antibody obtained in goats after injection of the same hapten (II), which allows detection of colchicine with sensitivity down to 70 pg per sample (6).

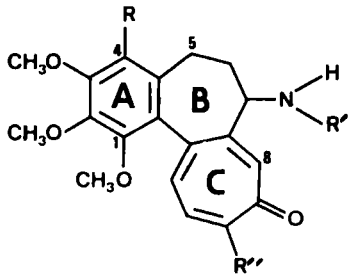
The specificity of antibodies previously described is not well established owing to the fact that not many metabolites of colchicine are actually known (7). For the purpose of establishing the optimal mode and site of conjugation which would lead to elicitation of antibodies of highest specificity, the present approach toward a sensitive, specific and rapid RIA for colchicine, was to prepare new conjugates for which the points of attachement of the drug to the carrier molecule are different. Our early work (6) being devoted to an antigen for which the attachement to the protein was on the ring B, in this study we prepared three colchicine deri-

vatives with substituents at positions 4 (III - IV) in the ring A and 10 (V) in the ring C.

This paper describes the synthesis and characterization of three new conjugates, the production of antibodies to the conjugates in rabbits and goats and the titers of the antisera.

MATERIAL

Colchicine was obtained from Fluka, bovine serum albumin (BSA) from Sigma, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) from Merck, O-carboxymethyl hydroxylamine hemihydrochloride from Ega-Chemie, Freund's adjuvants from Difco.



COMPOUNDS	R	R'	R"
I	-H	$-COCH_3$	$-OCH_3$
II	-H	-H	$-SCH_3$
III	$-CH=NOCH_2CO_2H$	$-COCH_3$	$-OCH_3$
IV	$-CH_2OCOCH_2CH_2CO_2H$	$-COCH_3$	$-OCH_3$
V	-H	$-COCH_3$	$-NHCH_2CH_2NH_2$
VI	-CHO	$-COCH_3$	$-OCH_3$
VII	$-CH_2OH$	$-COCH_3$	$-OCH_3$

Fig.1 : Colchicine and related derivatives.

(2,3-^{14}C) succinic anhydride was synthetized from the corresponding acid which was a gift from Service des Molécules Marquées, C.E.N. Saclay, France.

The homogeneities of all synthetized compounds were determined by thin-layer chromatography on silica gel pre-coated sheets (60 F_{254}-Merck) which were developed with benzene-methanol-diethylamine (35:10:5) or methanol-diethylamine (50:1).

Mass spectra (MS) were recorded on a Varian CH7 (samples applied by direct inlet), IR spectra on a Beckman IR 4250 (KBr disk), UV spectra on a Beckman UV 5230 spectrometers.

Proton magnetic resonance ^{1}H-NMR spectra were obtained on spectrometers operating at 90 mHz (Perkin Elmer R-32) or 100 mHz (Bruker WP 100) in deuterated chloroform (C.E.N. Saclay-Service des Molécules Marquées) at 0.2 N concentration. The chemical shifts are expressed in ppm from TMS and the coupling constants in Hz ; abbreviations : s = singlet, d = doublet, m = multiplet, br = broad.

METHODS

Synthesis of N-ethylamino-colchiceinamide (V)

Compound V was prepared according to the modified technique of Ewins et al (8).

Colchicine (0.53 mmole) dissolved in ethanol (3 ml) was treated with 0.3 ml of a 28% alcoholic solution of ethylenediamine in the dark, at room temperature for 20h. The reaction mixture was evaporated to dryness and chromatographed on a silica gel column using methanol-diethylamine (99 : 1) as the eluent. The amino derivative (0.45 mmole, 85%) was obtained as a yellow product. P M R : 1.5 (s (br), 2H, NH_2) ; 1.98 (s, 3H, $COCH_3$) ; 1.85 - 2.75 (m,

2H - C (5) and 2H - C (6)) ; 3.15 (m, 2H, CH_2-CH_2 - NH_2) ; 3.37 (m, 2H, NH - CH_2- CH_2) ; 3.63, 3.90 and 3.95 (3s, 3 x 3H, OCH_3) ; 4.70 (m, H - C (7) ; 6.54 (s, H - C (4)) ; 6.67 (d,J = 11, H - C (11) ; 7.47 (d,J = 11, H - C (12)) ; 7.50 (m, NH - CH_2) ; 7.57 (s,H - C (8)) ; 8.33 (d,J = 6, HN - C (7)). M S : 427 (M^+), 410 ($M^+ - NH_3$), 397 ($M^+ - CH_2 = N^+H_2$), 384, 355, 296, 28 (100%).

Coupling of V to BSA

The amino group of the hapten was linking to the amino groups of the carrier macromolecule using glutaraldehyde (9). Compound V (45 mg, 0.11 mmole) and BSA (225 mg) were dissolved in 6.0 ml of 0.1 M phosphate buffer pH 7.0 ; then 2.0 ml of 0.1 M glutaraldehyde was added dropwise with continuous stirring. The mixture which turned deep yellow was incubated for 2h at room temperature in the dark, then dialysed against distilled water during 2 days.

Analysis of the conjugate V -BSA

Gel filtration with Sephadex G-25 of an aliquot of the dialysed mixture separated the remaining unreacted hapten from the conjugate (first peak). Determination of the protein content (10) and spectral measurements by the technique of Erlanger et al (11) at 251 mμ indicated that the extent of conjugaison was 20 molecules of haptens per molecule of BSA. 7% of the amino derivative of colchicine present in the conjugate were not coupling to the protein.

Synthesis of 4-formylcolchicine-(O-carboxymethyl) oxime (III)

A solution of 427 mg (1 mmole) of 4-formylcolchicine VI (12) and 110 mg (1 mmole) of O-carboxymethyl hy-

droxylamine hemihydrochloride in 15 ml of ethanol containing 1 ml of 0.5 N KOH, was allowed to stand for 2 h at room temperature. After extraction with methylene chloride the oxime was recrystallized twice from ethyl acetate (yield 95%) ; mp 245° C ; I R : 1755 cm^{-1} ; P M R : 1.50 - 2.50 (m, 2H - C (5) and 2H - C (6)), 2.00 (s, $COCH_3$), 3.61, 3.91, 3.94 and 3.98 (4s, 4 x 3H, OCH_3), 4.60 (m, H - C (7)), 4.67 (s, 2H, CH_2- COOH), 6.90 (d, J = 11, H - C (11)), 7.30 (d, J = 11, H - C (12)), 7.67 (s, H - C (8)), 8.13 (d, J = 6, HN - C (7)), 8.40 (s, HC - C (4)), 8.72 (s, (br), COOH). M S : 500 (M^+ not seen), 424 (M-76), 337 (M-76-28-59).

Coupling of III to BSA

Compound III was conjugated to BSA by use of carbodiimide condensation (13). A solution of protein (137 mg) in 8 ml of 0.15 M NaCl was added to a solution of III (50 mg, 0.1 mmole) in 5 ml of 0.15 M NaCl and 1.7 ml of dioxane. With constant stirring, we added, dropwise, 45 mg of EDC dissolved in 3 ml of 0.15 M NaCl. The reaction was allowed to proceed in the dark, at room temperature overnight. The resulting conjugate was dialysed exhaustively for 3 days against distilled water, with 3 changes per day. The molar radio hapten-macromolecular carrier calculated in the same manner that for BSA-V was found to be only 3/1.

Synthesis of hemisuccinate of 4-hydroxymethylcolchicine (IV)

1 mmole of 4-hydroxymethylcolchicine VII (12) in 5 ml of pyridine was added to a solution of 1.5 mmole of (2,3 - ^{14}C)-succinic anhydride (210 µCi) in 5 ml of pyridine. The mixture was stirred at 100° C for 3h. Pyridine was

removed under vacuum and the residue dissolved in chloroform, washed several times with water, dried ; evaporation afforded IV (480 mg) ; IR : 1730 cm^{-1} ; P M R : 2.02 (s, $COCH_3$), 2.67 (s, CH_2 CH_2 COOH), 3.66, 4.05 and 3.98 (3s, 2 x 3H and 6H, OCH_3), 4.58 (m, H - C (7)), 5.19 and 5.35 (AB quarted, Jgem = 12, CH_2 - C (4)), 7.01 (d, J = 11, H - C (11)), 7.44 (d, J = 11, H - C (12)), 7.74 (s, H - C (8)), 7.85 (s (br), COOH), 8.83 (d (br), HN - C (7)). M S : 529 (M^+), 429 (M^+ - 100), 352, 324, 309, 101.

Coupling of IV to BSA

The hemisuccinate derivative IV (0.1 mmole, 140 µCi) was coupled to BSA (210 mg) in 0.15 M NaCl (14 ml) plus 1 ml of dioxane, in the presence of EDC (1 mmole), stirred 2 h at room temperature in the dark and dialysed against distilled water with 3 changes per day (1.5 days at room temperature, 2 days at 4° C). At the end of the procedure the amount of radioactivity in the product indicated that 17 moles of hapten were joined to each mole of protein.

Immunisation

The three dialysed solutions containing the conjugates were fractionated then lyophilized and stored at -20°C. Each hapten-protein conjugate (3 mg) was dissolved in normal saline (1.5 ml) and emulsified with complete Freund's adjuvant (3 ml). Each conjugate was injected subcutaneously into eight to twelve different sites on the back of New Zealand albino rabbits and intramuscularly in four points on the back of goats (0.5 mg per rabbit and 2.5 mg per goat).

At 4-week intervals thereafter the injections were repeated with an emulsion prepared as already described, except that complete Freund's adjuvant was replaced by incomplete adjuvant ; for rabbits booster doses contained half the amount of antigen. Blood was collected 8 days after each booster injections, allowed to clot ; the antiserum was stored at -20°C.

The antibody titre of the antisera was determined according to a procedure previously described (6), which required an incubation of 1 h at 0°C and a separation phase with dextran-coated charcoal.

RESULTS

The chemical structures of the three haptens synthetized were confirmed by NMR specially and MS. The ^{1}H-NMR characteristics of the new compounds are similar to those already reported for colchicine and derivatives (14).

Because of configurational isomerism about the C = N double bond the oxime derivative (III) can exist in two populations of rotamers, *syn* and *anti*, relative to whether the proton is *cis* or *trans* with respect to the alkoxy group. In our compound this hydrogen appears as one singlet at 8.40 ppm suggesting that only one of the two isomers would be present.

This seemed confirmed by results of ^{13}C-NMR. The shift difference for the α-carbon (here 4-C) between *syn* and *anti* aldoxime isomers is of the order of 4 ppm (15). With compound (III) for which ^{13}C-NMR signals are easily assigned by analogy to the data obtained for 4-formylcolchicine (16) the carbon 4 appears as one singlet at 119.1 ppm.

M S fragmentation process of colchicine was first described by Wilson (17). The amino-hapten fragmentation

pattern showed a significant similarity to that of colchicine. The M S of acid derivatives (III and IV) exhibited numerous weak peaks. As with similar oxime-derivatives (18) the molecular ion of the hapten III (m/e 500) was not seen (the side chain broke at the N-O bond). With hemisuccinate derivative, except for a weak peak for the molecular ion, the fragmentation pattern is that of 4-hydroxymethyl colchicine (VII).

These three haptens were covalently linked to BSA to prepare the immunizing conjugates. Antibodies production in the immunized rabbits and goats was followed by monitoring the binding of (^{3}H) colchicine to the homologous antibodies. The preliminary assessment of the antisera is presented in Table 1. After 8 months all the rabbits produced operational titers (1/320 to 1/2200). Satisfactory titers were also obtained with goats, except for those immunized with V-BSA.

The RIA procedure will be described in depth in subsequent publication.

DISCUSSION

The position and the method of linkage of one hapten to a carrier molecule are important factors affecting the specificity of the resultant antibody. Considering that an ideal antigen would be one in which none of the important groups are linked to the protein (19) we have synthetized two modified colchicine derivatives at position 4. Furthermore, these haptens which result from O-carboxymethyloximation of an oxo group (III) or hemisuccination of an hydroxyl group (IV) should allow study of correlation between mode of conjugation (by simple (IV) or double (III) bonds) and specificity of antibodies.

The antigenic potency of the groups [$-NHCOCH_3$] and [$-COC(OCH_3)=$] should be tested with two antigens for

TABLE 1

ANTISERUM TITERS OF HAPTEN - PROTEIN CONJUGATES

lizing gen	Animals	Number of positives / Number tested	Number of boosters	Titers
-BSA	Rabbits	3/3	8	1/400
			-	1/800
			-	1/860
	Goats	2/2	6	1/170
			-	1/2200
-BSA	Rabbits	3/3	8	1/500
			-	1/540
			-	1/1100
	Goats	2/2	6	1/360
			-	1/1650
- BSA	Rabbits	4/4	6	1/320
			-	1/1000
			-	1/1900
			-	1/200
	Goats	0/2	-	-

which these functions are masked by linkage of the protein in these positions.

It is interesting to note that with the two conjugates actually reported the specificity varied according to the site of coupling of the carrier molecule on the hapten (ring B or C). Thus, lumicolchicine which is a modified ring C isomer of colchicine gives a total cross-reactivity with conjugate for which the point of attachment is the ring B (6) and a very low cross-reactivity (0.68%) with conjugate for which the couplage occurs on ring C (4). Our previous work has also shown the importance of the acetamido and methoxy functions. These results suggested that the immunoreactive groups are not the same or at least are of unequal importance according to the coupling technique. The comparative study of the cross-reactivities of these four antisera will be of interest for defining their characteristics against colchicine and its metabolites for the establishment of an accurate and specific RIA for colchicine.

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