A sensitive radioimmunoassay for colchicine

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Because of the low plasma colchicine concentrations required to produce pharmacological effects (Wallace et al 1973), it is essential that an antibody used in a radioimmunoassay (RIA) for colchicine must be of high affinity and specificity. Ertel et al (1976) reported the development of a sensitive RIA which can detect colchicine in small volumes of plasma and urine after therapeutic doses but the method is time-consuming. Previously, Boudene et al (1975) described an assay that lacked sensitivity to clinical or pharmacological concentrations of the drug.

We have developed a sensitive and rapid RIA using an antibody raised in goats after injection of *N*-desacetylthiocolchicine conjugated with bovine serum albumin (BSA). The incubation time is short and dextran-coated charcoal is used for the separation.

[ring A-4-^{3}H] Colchicine (7·7 Ci mmol^{-1}) was purchased from the Radiochemical Centre, Amersham (U.K.). Colchicine was supplied by Sigma (U.S.A.) and its derivatives were a gift from Roussel-Uclaf Laboratories (Paris, France). All solutions of colchicine and its derivatives were kept at 4 °C in the dark to prevent exposure to ultraviolet light. The antigen was a conjugate of *N*-desacetylthiocolchicine and BSA prepared by the method of Boudene et al (1975). The conjugate (4 mg) was dissolved in 2 ml of 0·15 M NaCl, emulsified in an equal volume of complete Freund's adjuvant and injected intramuscularly into 5 goats. Booster injections with half the amount of antigen were administered by the same route at weeks 4, 9, 14, 24 and 26. Blood was collected 10 days after each injection. Serum was separated by centrifugation at 2000 *g* for 15 min and the sera stored at −20 °C. For the detection of antibodies, sera or antisera were diluted in standard diluent buffer (0·01 M sodium phosphate buffer, pH 7·0) and 0·1 ml of various serum dilutions were added to polystyrene disposable tubes containing 0·5 ml of [^{3}H]colchicine (about 194 pg) in standard diluent buffer containing 1 mg ml^{-1} gelatin. The tubes were protected from light with aluminium foil and incubated at 0 °C (ice bath) for 1 h. Then, 0·25 ml of a dextran-coated charcoal suspension (5 g charcoal and 0·5 g dextran T 70 in 100 ml of diluent buffer), well mixed by magnetic stirring at 0 to + 4 °C, were added and the tubes were kept at 0 to + 4 °C for 5 min. The samples were centrifuged at + 4 °C for 10 min (2000 *g*) and 0·5 ml of the supernatants added to 10 ml Insta-gel (Packard Instrument Company, USA) and the radioactivity was measured.

For radioimmunoassay, the reagents were added to the assay tubes in the order given in Table 1. The antiserum was used at a final dilution (1:12000) which bound about 30% of the label. After incubation, the protocol used for the detection of antibodies was followed. In all experiments there were "total" and "non specific" tubes to measure the adsorption efficiency of the charcoal suspension. Both samples and standards were run in duplicate.

All 5 goats immunized produced antibodies, one giving an antiserum which, at a final dilution of 1:11000 bound 50% of 194 pg [^{3}H] colchicine. The average affinity constant (K) of the antiserum for colchicine was determined graphically from a Scatchard plot and found to be 5×10^{12} litre mol^{-1}. Concentrations of colchicine in the unknown samples were interpolated from the standard curve in the linear portion between 70 and 2500 pg of colchicine per assay tube (Fig. 1).

The limit of sensitivity defined as being the smallest amount which is significantly different from zero was calculated by measuring the background produced by 0·2 ml of normal human serum and found to be 70 pg per assay tube or 0·35 ng ml^{-1} at 95% confidence limits for 10 assays. More than 0·2 ml of urine or plasma alters the shape of the standard curve and the percentage binding, and so a sample volume of 0·05 to 0·2 ml was chosen. Colchicine added to serum, urine or buffer was recovered quantitatively (Table 2). Intra-assay variations for two plasma samples (mean values 2·71 and 7·0 ng ml^{-1} respectively) were 9 and 10·4% (n = 10). Two pools of plasma collected from patients receiving colchicine were assayed 10 times in two months

Table 1. Protocol for the colchicine radioimmunoassay in serum and urine

Reagent	Total counts tube	Non-specific binding tube	Standard tube	Sample tube
	Volume ml			
Normal serum or urine	0·15	0·15	0·15	—
Buffer	0·4	0·15	—	—
Standard	—	—	0·05	—
Sample	—	—	—	0·2
[^{3}H] Colchicine	0·5	0·5	0·5	0·5
Antiserum	—	—	0·1	0·1
		Incubate for 1 h at 0 °C		
Dextran-coated charcoal suspension	—	0·25	0·25	0·25

* Correspondence.

Table 2. Recovery of colchicine

	Amount of colchicine added (pg)	Recovery (%)
Serum	200	82·5
	1000	108·4
	2000	92·85
Urine	200	90
	1000	110·2
	2000	99·75
Buffer	200	91
	1000	97·6
	2000	105·4

and had interassay coefficients of variance of 14·5 and 8·15% (mean values 1·58 and 7·36 ng ml^{-1} respectively).

To determinate the specificity of the antiserum, colchicine was replaced by various colchicine analogues and other drugs. Results are shown in Table 3 and are expressed as the weight of cross reacting substance required for a 50% displacement of [^{3}H]colchicine and by the percentage of cross reactivity. Lumicolchicine produced by u.v. irradiation according to Ertel et al (1976) had a 100% cross reactivity in our assay and only 0·68% in the assay of Ertel et al (1976). These differences can be explained by the structure of conjugates: colchicine was conjugated with BSA through the ketone group on ring C in the Ertel technique and by the amino group on ring B of the *N*-desacetylthiocolchicine in our assay. The results in Table 3 indicate that our antiserum is more specific for ring A and B than for the tropolone ring and this is confirmed by the extent of cross reactivity of compounds with methoxy groups. Our antiserum was also characterized by a low cross reactivity with colchicine derivatives and the other drugs tested.

Table 3. The inhibition of antiserum binding of [^{3}H]-colchicine by colchicine and structurally related compounds

Compound	Amount required for 50% inhibition pM	% cross reactivity
Colchicine	0·66	100
N-Desacetylthiocolchicine	7·4	8·9
Thiocolchicoside	78	0·85
4-Formyl colchicine	240	0·275
Colchiceine	14	8·9
Lumicolchicine	0·66	100
Tropolone	94 500	< 0·01
1,2,3-Trimethoxybenzene	600	0·10
Papaverine	1500	0·044
Yohimbine	13 000	< 0·01
Emetine	16 000	< 0·01
Atropine	2800	0·023

Our procedure is better than the alternative assays since it is much quicker and more convenient. The procedures of Ertel et al (1976) and Boudene et al (1975) included two incubations, the first for 24 h and 18 h at 4 °C respectively and the second for a double antibody separation which lasts 16 h and 6 h respectively. We use only a 1 h incubation time because the kinetics of binding are dependent on temperature. We found that the extent of binding was the same in 1 h at 0 °C as in 24 h at + 4 °C. This led to a rapid assay with a simple charcoal separation rather than a double antibody precipitation. Non-specific binding in our assay was always less than 1%. In addition our assay is more sensitive than that of Boudene et al (1975) who used a similar antiserum. Their assay lacked sensitivity in the therapeutic range since they used an amount of [H^3]-colchicine (1600 pg as against 194 pg in our test) too high to be displaced by low concentrations of unlabelled drug and thus the sensitivity of their method was about 5000 pg.

When our method was applied to the measurement of the drug in the serum of a patient dosed with 1 mg by

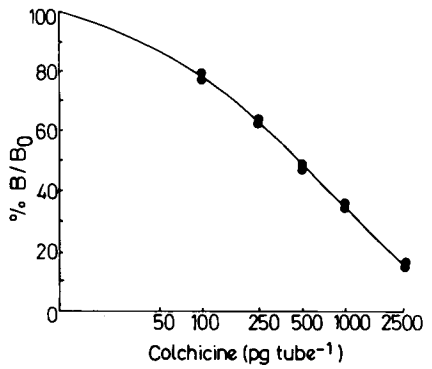

FIG. 1. A typical standard curve for the radioimmunoassay of colchicine.

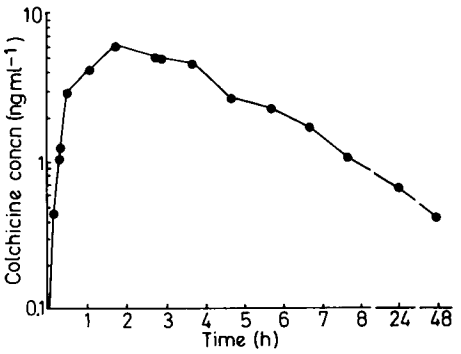

FIG. 2. Plasma colchicine concentrations after oral administration of 1mg to a patient.

mouth (colchicine, Houde Laboratories, Paris, France) the time-concentration curve was similar to those found by Wallace & Ertel (1973) after oral administration of a single [^{14}C]colchicine dose (Fig. 2). There was a peak 2 h after the drug had been taken (C_{max} of 6 ng ml^{-1}) and rapid distribution processes because the concentration decreased to <1 ng ml^{-1} by 8 h. The 24 and 48 h concentrations confirmed the prolonged excretion of colchicine and the existence of a long elimination half-time not found by Ertel et al (1976) who described a mean elimination half-time of 58 $\pm$ 20 min. Recently, Bourdon & Galliot (1976, 1979), with a fluorimetric technique and by the Sigma-Minus method, described a terminal half-time of 548 min for ten patients after an oral dose of 1 mg. Our findings agree with this observation and conflict with those of Jarvie et al (1979) who, using the pharmacokinetic data of Wallace & Ertel (1973), give a method for estimating the dose taken in cases of colchicine overdose.

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REFERENCES

Boudene, C., Duprey, F., Bohuon, C. (1975) Biochem. J. 151: 413–415

Bourdon, R., Galliot, M. (1976) Ann. Biol. Clin. 34: 393–401

Ertel, N. H., Mittler, J. C., Atgun, S., Wallace, S. L. (1976) Science 193: 233–235

Jarvie, D., Park, J., Stewart, J. M. (1979) Clin. Toxicol. 14 (4): 375–381

Galliot, M., Bourdon, R. (1979) Ier Congrès Hispano-Français de Biopharmacie et Pharmacocinétique. Barcelona, 2–6 April 1979

Wallace, S. L., Ertel, N. H. (1973) Metabolism 22: 749–753

LETTERS TO THE EDITOR

Why does sulpiride not block the effect of dopamine on the dopamine-sensitive adenylate cyclase?

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Sulpiride is a clinically-effective antipsychotic agent (Mielke et al 1977) which both resembles and differs from classical neuroleptics of the phenothiazine, thioxanthine and butyrophenone types (Spano et al 1979; Jenner & Marsden 1979). One of the major differences between sulpiride and the classical neuroleptics is that the former does not block the effects of dopamine on the dopamine-sensitive adenylate cyclase (Trabucchi et al 1975). This has led to the suggestion that there are two types of dopamine receptor in the brain, a D1 receptor linked to adenylate cyclase and unaffected by sulpiride, and a D2 receptor blocked by sulpiride but not linked to an adenylate cyclase (Kebabian & Calne 1979). Domperidone is a peripheral dopamine antagonist that is similarly a very weak antagonist on the dopamine-sensitive adenylate cyclase (Laduron & Leysen 1979). Domperidone has been used in binding studies as a D2 antagonist (Watling et al 1979).

We suggest an alternative explanation for the lack of effect of sulpiride and domperidone on the dopamine-sensitive adenylate cyclase. It is postulated that for a compound to act as a dopamine antagonist on the adenylate cyclase, a high degree of membrane penetration must be achieved. Thus only those drugs with a sufficiently high oil/water partition coefficient will function as dopamine antagonists in this system. Both sulpiride and domperidone penetrate poorly into the brain following peripheral administration (Honda et al 1977; Woodruff & Andrews 1979; Laduron & Leysen 1979). We point out that the poor penetration of these compounds into the brain might be linked to their poor penetration into membranes in the adenylate cyclase assay. In fact a direct estimate of the lipid solubility of sulpiride has shown this to be very low compared with classical neuroleptics. Thus Norman et al (1979) reported log P (n-octanol–aqueous buffer partition coefficient) values of $-0{\cdot}5$ for sulpiride and 4·25 for *cis*-flupenthixol. We do not envisage that high lipid solubility is the sole criterion for dopamine-blocking activity on the adenylate cyclase, since, for example, the (+)- and (−)-enantiomers of butaclamol have identical octanol–aqueous phase partition coefficients (Norman et al 1979), but differ greatly in their effects on the dopamine-sensitive adenylate cyclase. Rather we suggest that to block the effects of dopamine on the adenylate cyclase, a compound must be a dopamine receptor antagonist and have a sufficiently high degree of lipid solubility. In support of our hypothesis, the substituted benzamide *N*-(1-benzyl-3-pyrollidinyl)-5-chloro-2-methoxy-4-methylaminobenzamide (YM-08050) is chemically closely related to sulpiride, yet it is

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