DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 17(17), 2391-2400 (1991)

A RADIOIMMUNOASSAY FOR COLCHICINE USING A HIGHLY SPECIFIC, HIGH AFFINITY MONOCLONAL ANTIBODY

S. K. Edmond Rouan[1,4], I. G. Otterness[2], D. J. Gans[3], C. T. Rhodes[1]

[1]Department of Pharmaceutics, University of Rhode Island
Kingston, RI 02881

[2]Department of Immunology and Infectious Diseases,
[3]Department of Clinical Research
Pfizer Inc, Central Research Division, Groton, CT 06340

[4]Present Address: Smith Kline and French Laboratories
709 Swedeland Road
King of Prussia, PA 19406

ABSTRACT

A sensitive and selective radioimmunoassay has been developed using a colchicine binding monoclonal antibody. The assay procedure uses a charcoal suspension to separate antibody bound and free colchicine and can be performed in less than two hours. Using the high affinity antibody, as little as 0.3 ng/ml of colchicine in serum can be detected. These results provide the framework for a fully validated clinical assay for therapeutic monitoring of colchicine.

INTRODUCTION

Colchicine is a potent drug with a narrow therapeutic range. Daily oral doses range from 0.5-2 mg for the treatment of gouty arthritis and other rheumatologic disorders such as familial Mediterranean fever and Bechet's disease[1]. Peak plasma concentrations of colchicine following oral administration of a 1 mg dose range from 1 to 2.5 ng/ml[2]. Assay methodologies for monitoring colchicine therapy must therefore be sensitive to nanogram levels of drug. Radioimmunoassays employing polyclonal colchicine binding antibodies have been developed to meet these sensitivity requirements[2,3,4,5,6]. However, these polyclonal antibodies cross-react to varying degrees with compounds structurally related to colchicine. Although colchicine metabolites have not been conclusively demonstrated in humans, ring A and C monodemethylations have been shown to occur in mammalian liver microsome preparations[7]. Results of pharmacokinetic studies of colchicine involving different polyclonal antisera may therefore be influenced by the presence of such metabolites. In addition to cross-reactivity with possible metabolites, several previously prepared antisera were 100% cross-reactive with β-lumicolchicine, a photodegradation product of colchicine.

We have recently prepared a high affinity colchicine binding monoclonal antibody[8,9]. In contrast to polyclonal antibodies, a stable hybridoma provides a source of large quantities of monoclonal reagent with consistent affinity and specificity for immunoassay purposes. In this communication we describe our investigation of the use of this monoclonal antibody for the radioimmunoassay of colchicine in serum.

METHODS AND MATERIALS

Preparation of the Antibody

As previously described[8], the hybridoma clone secreting antibody C44 was prepared by fusion of NS1 myeloma cells with spleen cells from mice immunized with a colchicine-keyhole limpet hemocyanin conjugate. The C44 hybridoma clone is a stable IgG_{2a} secreting cell line. This cell line is

maintained in culture in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 mM MEM nonessential amino acids solution. Frozen stocks of these cells have been prepared. For freezing, 2 x 10^6 cells are suspended in 1 ml of 50% fetal calf serum and 50% dimethylsulfoxide and, after a gradual 1°C per min freeze, cells are maintained in liquid nitrogen (-240°C). Cells stored under these conditions maintain viability and secretion characteristics after thawing. For radioimmunoassay, the C44 hybridoma clone was expanded in culture. The culture supernatant fluid was harvested by centrifugation and the immunoglobulin fraction precipitated by the addition of an equal volume of a saturated solution of ammonium sulfate. The precipitate was dialyzed against phosphate buffered saline (PBS, pH 7.4) and applied to a Protein A sepharose affinity chromatography column using a 50 mM Tris, 150 mM sodium chloride buffer (pH 8.4) and eluted with 0.1 M sodium acetate (pH 4). Sodium azide (0.1%) was added to the purified antibody and aliquots were stored at -20°C. The purity of the antibody preparation was verified using sodium dodecylsulfate polyacrylamide gel electrophoresis. The concentrations of antibody in tissue culture supernatant of the hybridoma cell line range from approximately 20-40 µg/ml. The C44 hybridoma has also been used to produce ascites fluid in BALB/c mice. Typical antibody concentrations in ascites fluid range from 2-4 mg/ml.

Radioimmunoassay of Colchicine in Serum

The purified antibody (0.03 mg/ml) was diluted to 0.4 µg/ml and 50 µl of the resulting preparation was added to borosilicate glass tubes (12 x 75 mm) containing 150 µl of normal human serum. This concentration of antibody bound approximately half of the radiolabeled ^{3}H-colchicine (Figure 1). ^{3}H-colchicine (24.2 Ci/mmol), labeled at the methoxy group of ring C, was purchased from NEN Research Products (Boston, MA); unlabeled colchicine was purchased from Sigma. Standard colchicine solutions were prepared in phosphate buffered saline (buffer) at concentrations ranging from 0.6 ng/ml to 154 ng/ml: 50 µl of either a standard colchicine concentration, an unknown

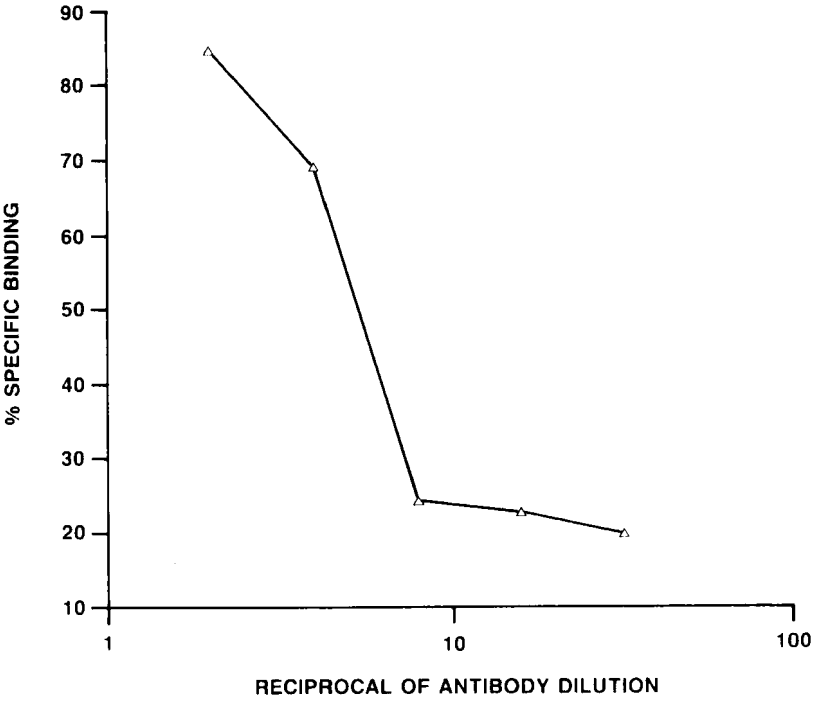

Figure 1: Relationship between antibody concentration and specifically bound counts as a percent of total radioactivity. Dilutions computed from 0.4 µg/ml stock unlabeled colchicine.

sample, or buffer was added first to each tube. 50 µl of ^{3}H-colchicine (4 ng/ml) was next added to all tubes. In all experiments the total counts and nonspecific counts were determined. Nonspecific binding was determined in the presence of a million-fold excess of cold colchicine to saturate specific ligand binding sites. The protocol used for the radioimmunoassay is outlined in Table 1. All tubes were set up in triplicate. After mixing antibody, ^{3}H-colchicine, and the unknown or standard colchicine sample together, the test tubes were incubated for 1 hour at room temperature in the dark. They were then placed on ice and 200 µl of a 1.5% charcoal (Norit A, Fisher) suspension, mixed well by magnetic stirring at 4°C, was added to each tube with the exception of those tubes used to measure total counts. Phosphate buffered saline (200 µl) was added to total

TABLE 1

Protocol for Colchicine Radioimmunoassay in Serum

Reagent	*Total Counts*	*Nonspecific Binding*	*Standard*
	Volume of Each Reagent Per Tube (ml)		
Normal Serum	0.15	0.15	0.15
PBS Buffer	0.05	- - -	- - -
Standard	- - -	- - -	0.05
Antibody	0.05	0.05	0.05
^{3}H-Colchicine	0.05	0.05	0.05
Colchicine (2 mM)	- - -	0.05	- - -
	Incubate 1 Hour at Room Temperature		
Charcoal Susp.	- - -	0.20	0.20
PBS	0.20	- - -	- - -

counts tubes. After charcoal addition the tubes were vortexed and allowed to settle for 5 min. The samples were centrifuged at 4°C for 10 minutes at 2000 x g; and then 0.3 ml of the supernatant was lifted off, added to 3 ml of scintillation fluid (Atomlite, NEN), and the radioactivity counted.

RESULTS AND DISCUSSION

After the assay conditions had been developed, the entire assay procedure for determining colchicine levels in serum was performed on three separate occasions to yield three distinct standard curves. On each occasion, labeled colchicine was added to a mixture of antibody and unlabeled colchicine in serum. The quantity bound (B) in the presence of a known concentration of unlabeled colchicine, as a percent of binding (B_0) in the absence of competing unlabeled colchicine, was determined at several unlabeled concentrations.

TABLE 2

Mean Percent Bound Values for Three Curves

Colchicine ng/ml serum	*Mean % B/B_0* I	II	III
0.2	81.0	90.9	
0.4	72.6	72.6	94.9
0.8	64.5	60.9	78.8
1.6	44.8	35.4	47.1
3.2	19.0	19.1	16.0
6.4	15.3	11.0	5.9
12.8	14.6	3.5	
25.6	8.8		
51.2	7.9		

N = 3 in all cases

% B/B_0 = Percent bound in the presence of a standard concentration of unlabeled colchicine as a fraction of that bound in the absence of competing ligand.

For each standard curve, triplicate percent bound readings were obtained from triplicate samples prepared independently at each concentration of unlabeled colchicine. Mean percent bound values at each concentration appear in Table 2. We took the individual percent bound readings and applied the probit transformation to each. (This necessitated dropping one of the three readings at the lowest concentration in curve III, as the estimated percent bound exceeded 100% for this reading and the probit could not be computed.) For each of the three curves, we then determined the linear regression of the individual probit values on the (natural) logarithms of the concentrations. Figure 2 presents the data and the fitted regression for curve II. A test for lack-of-fit[10] revealed no evidence of deviation of the regressions from linearity ($p > 0.10$ in all three cases). The r^2 ranged from 0.89 to 0.98.

Assuming now an unknown run in triplicate concurrently with the known samples, the mean of the three probits can be determined for this unknown.

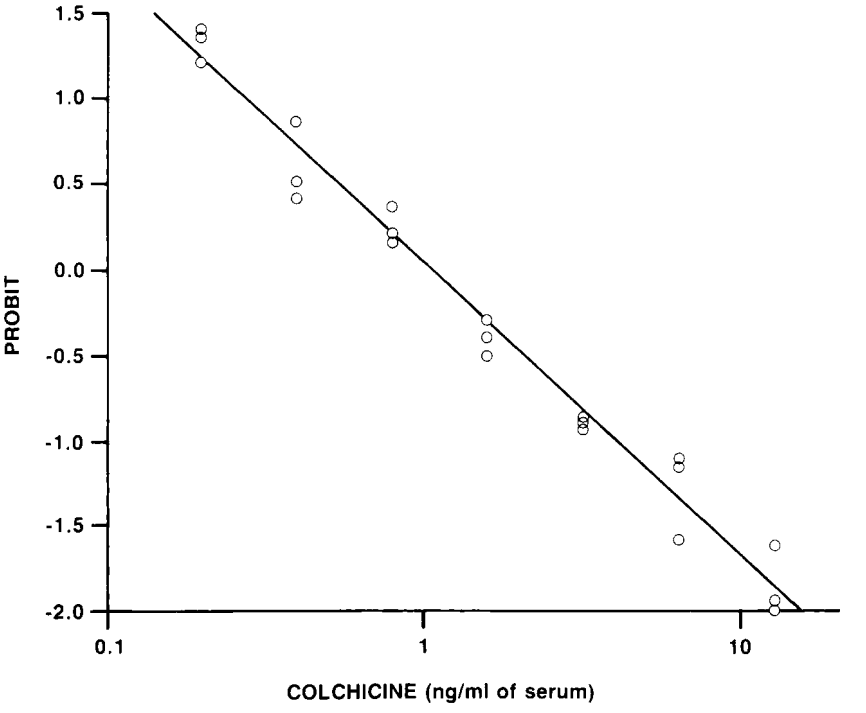

Figure 2: Standard curve (II) for Colchicine Radioimmunoassay.

The regression already described can then be used to invert this mean and obtain a point estimate for the unknown concentration. The accuracy of such decalibration can be predicted: regression theory provides not merely a point value, but an interval estimate for the decalibrated concentration[11]. For example, using regression information from curve II, it can be calculated that when the mean probit for the unknown yields a decalibrated point estimate of 1.00 ng/ml, this interval at the 95% level of confidence would range from 0.75 to 1.34 ng/ml. The semi-width of this (asymmetric) interval, as a percent of the estimated value 1.00, is 29.5%. This latter number gives a conservative figure for the decalibration error from this assay.

Some regression statistics, including this decalibration error estimate, appear in Table 3. The decalibration error is presented for a set of estimated concentrations expected in clinical practice.

TABLE 3

Regression Analyses

Curve	N	Estim. Coefficients b_0	b_1	LOF p	r^2	DEE at Estim. Conc. ng/ml 0.3	1.0	3.0
I	27	0.080	-0.468	>0.10	0.89	95.2%	90.5%	90.2%
II	21	0.047	-0.742	>0.50	0.98	31.7	29.5	29.8
III	14	0.456	-1.131	>0.10	0.98	22.9	18.6	18.6

Model: probit (% B/B_0) = $b_0 + b_1$ ln (conc)
LOF = Lack of fit (see text)
DEE = Decalibration error estimate: semi-width of interval (percent) at 95% confidence (see text)

This radioimmunoassay procedure showed variability in the nonspecific binding. The nonspecific binding is an indication of the efficiency of the technique used to separate bound and free radioligand. Nonspecific binding accounted for between 8.9-22.7% of the total counts, and its effect is subtracted out in the standard curves of the assay. The addition of dextran to the charcoal suspension may improve separation and reduce the level of nonspecific binding. Nonspecific counts of less than 1% using dextran coated charcoal (5% charcoal, 0.5% dextran) were reported previously in a colchicine radioimmunoassay using polyclonal antibody[4]. Alternate methods of separation of bound and free ligand, such as double antibody precipitation or the use of a protein A containing suspension, might also improve separation. This would be expected to improve the accuracy of the estimates of colchicine concentration and would likely extend the useful measurement range of this procedure.

The sensitivity of this assay system is dependent upon the high affinity of the C44 antibody for colchicine. The equilibrium dissociation constant for antibody colchicine interaction (K_d = 0.66 nM) was determined from Scatchard analysis of specific antibody binding to ^{3}H-colchicine[8]. This antibody has also

been shown to be highly specific for colchicine. In a competitive enzyme linked immunosorbent assay (ELISA) the cross-reactivity of the antibody with compounds structurally related to colchicine was examined. It was demonstrated that 0.1 mM trimethylcolchicinic acid was required to inhibit antibody binding to the solid phase antigen by 50% (IC_{50}), as compared to 3.6 nM colchicine. The IC_{50} measured by competitive ELISA exceeded 0.3 mM for podophyllotoxin, N-acetylmescaline, β-lumicolchicine, anhydrocotarnine acetone and 2-methoxynitrotropone.

The results of the present investigation provide a framework for the development of a fully validated assay for monitoring therapeutic plasma levels of colchicine. The antibody has the high affinity required for the sensitive determination of as little as 0.3 ng/ml of colchicine. This affinity, together with the high level of specificity compared to previously prepared polyclonal antisera, and the large quantities of reagent which can be produced, would provide a standardized assay procedure for monitoring therapeutic levels of colchicine. Such a standardized procedure would also permit greater compatibility among studies of the pharmacokinetic behavior of colchicine.

ACKNOWLEDGEMENTS

This research forms part of the doctoral dissertation of S. K. Edmond Rouan. Her work has been generously supported by the United States Pharmacopeial Convention Inc., Richardson Vicks Inc. and by a fellowship from the University of Rhode Island.

REFERENCES

1. S.L. Wallace, "Colchicine" in Textbook of Rheumatology, 2nd ed., Vol. 1, (Kelley, Harris, Ruddy, Sledge, eds.) W.B. Saunders Co. (PA), pp. 871-878 (1985).
2. H. Halkin, S. Dany, M. Greenwald, Y. Shnaps and M. Tirosh, Clin. Pharmacol. Ther., 28 (1), 82-87 (1980).

3. E.Z. Katz, M. Ehrenfeld, M. Levy and M. Eliakim, Arthritis Rheum., 25 (2), 227-231 (1982)
4. J.M. Scherrman, L. Boudet, R. Pontikis, Nguyen-Hoang-Nam and E. Fournier, J. Pharm. Pharmacol., 32, 800-802 (1980).
5. N.H. Ertel, J.C. Mittler, S. Akgun and S.L. Wallace, Science, 193, 233-235 (1976).
6. C. Boudene, F. Duprey, C. Bohuon, Biochem. J., 151, 413-415 (1975)
7. M. Schonharting, G. Mende and G. Sieber, Z. Physiol. Chem., 355, 1391-1399 (1974).
8. S.K. Edmond Rouan, I.G. Otterness, A.C. Cunningham and C.T. Rhodes, Hybridoma, 8, 435-448 (1989).
9. S.K. Edmond Rouan, I.G. Otterness, A.C. Cunningham and C.T. Rhodes, Am. J. Pathol. 137, 779-787 (1990).
10. N.R. Draper and H. Smith, Applied Regression Analysis, 2nd ed., New York: Wiley, pp. 33-40 (1981).
11. B. Ostle, Statistics in Research, 2nd ed., Ames, Iowa: Iowa State University Press, pp. 176-177 (1963).