DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 8(6), 811-817 (1982)

A DOUBLE ANTIBODY ASSAY FOR THE DIRECT DETERMINATION OF [N-(4-AMINO)PHENYL] AMINOPHENYL IMINODIACETIC ACID AND ITS DERIVATIVES IN SERUM

Fausta K. Teracostu and Michael C. Theodorakis*

University of Illinois at Urbana-Champaign
College of Veterinary Medicine
Division of Nuclear Medicine and Radiopharmacology
Urbana, Illinois 61801

ABSTRACT

A double antibody assay for the determination [N-(4-amino)-phenyl] aminophenyl iminodiacetic acid (I) and a number of its derivatives in serum has been developed. The same antiserum and tracer were used for the determination of the serum levels of derivatives of (I). The minimum detectable concentration of I that was detectable was 6 ng/ml. The precision error was less than 1% and the accuracy as indicated by the recovery ratios was satisfactory for all derivatives of I.

INTRODUCTION

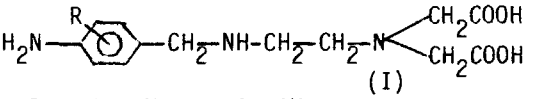
Several investigators have developed radioimmunoassays for the determination of endogenous and exogenous compounds in plasma and serum. These assays are characterized by the fact that they

---

*To whom inquiries should be directed.

have been developed solely for the determination of one compound only. Extensive cross-reactivity in these assays is undesirable. In this sense, the development of radioimmunoassays for the determination of compounds depends on the development of highly specific antiserums (4) and tracers of high specific activity. Although extensive cross-reactivity in most instances is detrimental to the assay, there are instances where extensive cross-reactivity may be desirable. This paper describes the development of such an assay for the direct determination of [N-(4-amino)phenyl] aminophenyl iminodiacetic acid (I) and its derivatives in serum.

The derivatives of (I) are good chelators of Fe^{3+} which localize in the hepatobiliary system and therefore can be used for removing iron deposits in cases of transfusional siderosis (1-3,9). These agents are administered orally to hypertransfused rabbits in order to determine their effectiveness for removing iron deposits from the liver. A direct radioimmunoassay for the determination of (I) and its derivatives has been developed. The innovation in this assay is that one antiserum and one tracer is used for all different derivatives of I. This assay has adequate sensitivity, it is highly reproducible, and extremely simplified.



(I)

R = H-, CH_3—, CH_3CH_2—,
I II III

EXPERIMENTAL

Preparation of Immunogen. Bovine serum albumin (BSA) was conjugated to (I) through the tyrosyl residues of BSA via a diazotization and condensation procedure (5,6). Compound I (200 mg) was dissolved in 3 ml of distilled water and the pH was adjusted to 9 with 1 N NaOH. Sodium nitrite (60 mg) and 2 ml of 1 N HCl was added. The diazotized I solution was added to 50 ml solution of 2% w/v BSA in ice cold 0.04 N NaOH. The solution was stirred at 5 C for 24 h and the pH was adjusted at 8.5. The immunogen was dialyzed against distilled water for 3 days and it was lyophilized. Spectrophotometric analysis of the conjugate indicated that a molar ratio of BSA to I ranging from 18-24.

Preparation of Iodinated Tracer. The conjugate (BSA-I) was iodinated using the chloramine-T procedure (7). Conjugate (2 mg in 0.4 ml of phosphate buffer, pH 7.5) and 1.5 mCi of carrier-free $Na^{125}I$ were mixed with 2 mg of chloramine-T in phosphate buffer, pH 7.5. After 90 seconds, 4 mg of sodium metabisulfite was added and then carrier potassium iodide (5 mg). The mixture was chromatographed by gel-filtration in a 0.9 x 15 cm G-25 Sephadex column. The specific activity was about 1.8 to 1.9 Ci/mmole of I.

Preparation of Antiserum to I. The conjugate (BSA-I) was used to immunize male albino rabbits according to a procedure previously described (8). After 6 months, antiserum dilution of 1:1200 yielded 50% binding of 6 ng of I.

Second Antibody. Goat anti-rabbit immunoglobin G was purchased from Miles Research Products, Elkhart, Indiana.

Radioimmunoassay. Standards of the three derivatives were prepared by dilution of a 0.4 mg/ml stock solution in 0.055 M phosphate buffer, pH 7.5. Standards ranging from 0 to 250 ng were prepared in 0.055 M phosphate buffer, pH 7.5. The tracer (labeled immunogen) was diluted in 0.055 M phosphate buffer to an activity of 30,000-40,000 cpm/0.1 ml. The antiserum was diluted with buffer to yield 40-60% binding with tracer and 0.1 ml was used for the assay. The radioimmunoassay protocol was as follows. The standards and unknowns were mixed with the radioiodinated tracer, buffer and the antiserum in 6 x 75 mm disposable tubes. All tubes were vortexed for approximately 5 seconds and incubated for 3.5 hr at 25 C. Then 0.1 ml of the second antibody was added and the tubes were incubated for 2 hours at 5 C. The tubes wre centrifuged for 20 minutes at 3000 rpm, and the free (supernatant) and bound (precipitated) fractions were separated and counted. The percent bound activity was plotted versus concentration.

RESULTS AND DISCUSSION

This radioimmunoassay provides a degree of flexibility and convenience over other radioimmunoassays because the same antiserum and labeled antigen can be used in developing assays for three different compounds that their chemical structures are closely related. The I and its derivatives are low molecular

Table 1. Cross-reactivity of the antiserum with I and its derivatives

Compound	Concentration for 50% binding ($M \times 10^{-5}$)	Concentration ratio[a]	Molecular weight
I	0.020	1.00	281
II	0.020	1.00	295
III	0.022	1.10	309

[a]Compared to I

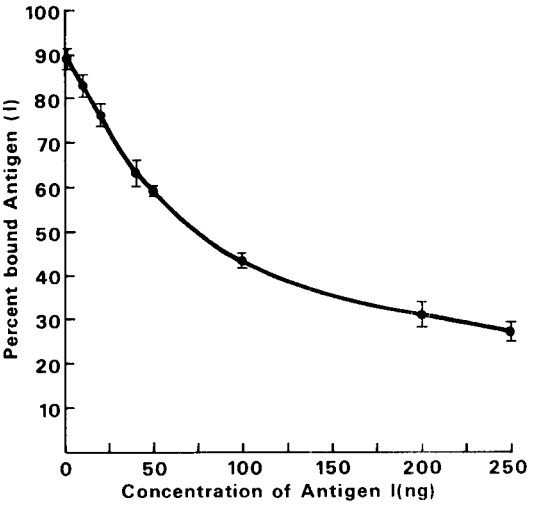


FIGURE 1. Plot of the standard curve for I.

Table 2. Precision and Accuracy

Added Antigen[a] (ng)	Measured[b] I (ng) Mean ± SE	Ratio	Measured[b] II (ng) Mean ± SE	Ratio	Measured[b] III (ng) Mean ± SE	Ratio
10	9.90±0.100	0.99	9.20±0.14	0.92	11.3±0.54	1.2
20	19.8±0.0115	0.99	19.1±1.2	0.95	18.9±1.10	0.94

[a]Antigen I or II or III [b]Replicates of three

weight compounds and their structural differences are on the aromatic ring. The immunogen was synthesized by diazotization of I which was then conjugated mainly through the tyrosyl residues of BSA by a diazotization and condensation reaction (5). Thus, the antiserum which was formed in response had antigenic determinants which recognized the iminodiacetic moiety of the molecule which was common to all three derivatives. The cross-reactivity studies indicated that the affinity of the antiserum for all three antigens was very high (Table 1).

The antibody did not recognize the structural differences of the three derivatives; therefore, the same antiserum preparations were used to assay all three compounds in serum. All three compounds gave standard curves which showed adequate linearity over concentrations ranging from 0-60 ng of compound/ml (Fig. 1). Therefore, the unknown serum samples were diluted adequately to fall within this concentration range. The diluting process also decreased the degree of nonspecific binding (4). Corrections for nonspecific binding were made for both standards and unknowns. The standard error of replicates for all three derivatives (Table 2) was less than 1% of the mean value for each concentration measured, indicating that the precison of the assay was good. The accuracy of the assays was acceptable as it was indicated by the recovery ratios (Table 2).

REFERENCES

1. B. N. Figgis and G. B. Robortson. Nature 205:694 (1965).

2. F. A. Cotton and G. Wilkinson. "Advanced inorganic chemistry." Interscience Publishers, New York, N.Y., p. 861 (1966).

3. M. C. Theodorakis, W. C. Groutas, A. J. Bermudez, D. Magnin, and S. V. S. Stefanakou. J. Pharm. Sci. 69:581 (1980).

4. P. Mojaverian and G. D. Chase. J. Pharm. Sci. 69:721 (1980).

5. G. E. Means and R. E. Feeney. "Chemical modification of proteins." Holden-Day, San Francisco, CA, pp. 144-193 (1977).

6. E. A. Kabat and M. M. Mayer. "Experimental immunology." Charles C. Thomas, Springfield, IL, pp. 789-802 (1971).

7. A. E. Bolton. "Radioiodination techniques." Amersham Corporation, Arlington Heights, IL, p. 45.

8. M. C. Theodorakis and S. V. S. Stefanakou. Drug Dev. Ind. Pharm. 7:411 (1981).

9. C. G. Pitt, G. Gupta, W. E. Estes, H. Rosenkrantz, J. J. Metterville, A. L. Grumbliss, R. A. Palmer, K. W. Nordquest, K. A. Sprinkle, Hardy, D. R. Whitcomb, B. R. Byers, J. E. L. Arceneaux, C. G. Gaines and C. V. Sciortino. J. Pharmacol. Exp. Ther. 208:12 (1979).