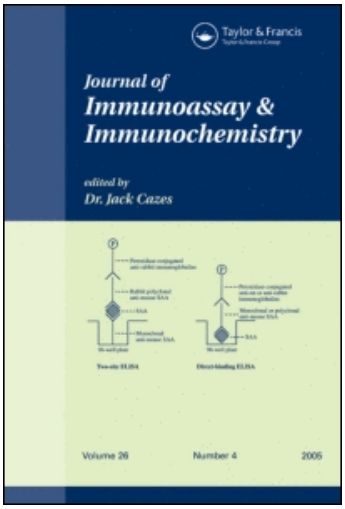

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Production of a High Affinity Antiserum to Benzodiazepines

R. Sherman-gold[a]; Y. Dudai[a]; L. Fogelfeld[a]; S. Fuchs[a]
[a] Department of Neurobiology and Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

To cite this Article Sherman-gold, R. , Dudai, Y. , Fogelfeld, L. and Fuchs, S.(1983) 'Production of a High Affinity Antiserum to Benzodiazepines', Journal of Immunoassay and Immunochemistry, 4: 2, 135 — 146
To link to this Article: DOI: 10.1080/15321818308057006
URL: http://dx.doi.org/10.1080/15321818308057006

JOURNAL OF IMMUNOASSAY, 4(2), 135-146 (1983)

PRODUCTION OF A HIGH AFFINITY ANTISERUM TO BENZODIAZEPINES

R. Sherman-Gold, Y. Dudai, L. Fogelfeld[+] and S. Fuchs[+]
Department of Neurobiology and [+]Department of Chemical Immunology
The Weizmann Institute of Science, Rehovot 76100, Israel

ABSTRACT

Antibodies specific for benzodiazepines were raised in rabbits by immunization with a conjugate of a benzodiazepine derivative, Ro 7-1986/1, with bovine serum albumin. The presence of anti-Ro 7-1986/1 antibodies in the sera was demonstrated by a radioimmunoassay using the radioligand [^{3}H]flunitrazepam ([^{3}H]FNZ). The antibodies displayed a high-affinity for [^{3}H]FNZ (K_D = 0.073 ± 0.003 nM) and cross-reacted with a broad spectrum of benzodiazepine derivatives. Benzodiazepine levels in samples of sera and urine of benzodiazepine-treated humans were determined. Due to the high sensitivity of the assay only minute volumes (microliter quantities) of body fluids are employed and, therefore, no extraction of the drugs is required. Nitrazepam and diazepam levels as low as 20 picograms can be easily observed. Intoxicating levels of benzodiazepines can be detected by a single measurement in less than 10 min. This radioimmunoassay is advantageous for pharmacokinetic studies, toxicological examinations and forensic medicine due to its high sensitivity, wide-range specificity and technical simplicity.

INTRODUCTION

Benzodiazepines are synthetic minor tranquilizers which are extensively used in medical practice for treating a wide range of disturbances, of both organic and psychosomatic origins. The evaluation of levels of benzodiazepines in body fluids is required

 0197-1522/83/0402-0135$3.50/0

for metabolic and pharmacokinetic investigations, therapeutic purposes, toxicological examinations and in forensic medicine. For these purposes a rapid, sensitive and technically simple method of screening for the presence of benzodiazepine derivatives is valuable. The currently proposed methods are based on chromatography (1-8), receptor assays (9-12) and immunoassays (13-22). In this communication we describe the preparation of antibodies which exhibit a high-affinity and specificity for a broad spectrum of benzodiazepines. We used these antibodies in a radioimmunoassay for determination of levels of the drugs in body fluids of benzodiazepine-treated patients.

MATERIALS

[^{3}H]Flunitrazepam ([^{3}H]FNZ), 84.3 Ci/mmol, was obtained from New England Nuclear (Boston, MA). Ro 7-1986/1 (5-(2-fluorophenyl)-1,3-dihydro-1-ethylamine-7-chloro-2H-1,4-benzodiazepin-2-one) and Ro 5-5492 (5-(2-fluorophenyl)-1,3-dihydro-1-(1-n-propylpiperazine)-7-chloro-2H-1,4-benzodiazepin-2-one), desmethyldiazepam and bromazepam were a gift from Drs. E. Gamzu and W.E. Scott (Hoffmann-La Roche, Nutley, NJ). Flunitrazepam and Ro 5-4864 (4-(4-chlorophenyl)-1,3-dihydro-1-methyl-7-chloro-2H-1,4-benzodiazepin-3-one) were donated by Dr. H. Mohler (Hoffmann-La ROche, Basel). Diazepam and medazepam were donated by Assia (Jerusalem). Nitrazepam, clonazepam, flurazepam and chlordiazepoxide were donated by Ikapharm (Kfar Saba). Bovine serum albumin (BSA) and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma (St. Louis, MO).

Activated charcoal powder was from BDH chemicals (Poole) and Dextran was from Pharmacia (Uppsala). All other chemicals were of analytical grade.

METHODS

Preparation of Ro-BSA conjugate was as follows: Ro 7-1986/1 (4 mg) was dissolved in 10 ml H_2O and the solution was kept in an ice bath. Succinic anhydride (10 mg) was added to the solution with stirring, pH was monitored during addition and up to 30 min after addition, and adjusted to pH 6.0 with 0.1 N NaOH, until no further change was observed. Incubation was continued for 3h at 4°C, and solution was then acidified to pH 5.0 with 0.5 N HCl. EDC (20 mg) and BSA (20 mg) were added successively to the succinilated-Ro 7-1986/1 solution and incubation proceeded for 16 h at 4°C, with stirring. The unreacted reagents and non-bound ligand were removed from Ro 7-1986/1-BSA conjugates by extensive dialysis of the reaction mixture against H_2O. This procedure yielded a coupling ratio of 18 moles Ro 7-1986/1 per mole BSA, as determined by light absorbance of Ro 7-1986/1 at 320 nm.

Rabbits were injected, intradermally in multiple sites, four times at two week intervals with 1 mg Ro 7-1986/1-BSA emulsified with complete Freund's adjuvant. Sera were collected two weeks after the fourth injection.

The presence of anti-Ro 7-1986/1 antibodies in the sera of immunized rabbits was determined by a radioimmunoassay (RIA) as follows: Anti-Ro 7-1986/1 antiserum was diluted 1:50 in phosphate buffered saline (PBS; 0.14 M NaCl, 0.01 M sodium phosphate, pH

7.2). Routinely, 50 l aliquots of the diluted antiserum were incubated with 0.4 nM [^{3}H]FNZ for 20 min at 4°C, in 0.5 ml final volume of PBS containing 0.01% BSA. In displacement experiments, non-radiolabeled benzodiazepine derivatives were included in the reaction mixture. The non-bound ligand was then removed by 0.2 ml dextran-coated activated charcoal solution in PBS, containing 0.01% BSA (1% activated charcoal, 0.1% dextran). The charcoal was spun down (3000 rpm, 3 min) and the supernatants were transferred into counting vials. Scintillation solution (5 ml Toluene-Triton) was added and radioactivity was counted by liquid scintillation spectrometry. The non-specific binding was measured either by the binding of 0.4 nM [^{3}H]FNZ to normal rabbit serum or by the binding of 0.4 nM [^{3}H]FNZ to anti-Ro 7-1986/1 antiserum in the presence of 1 μM diazepam. Non-specific binding did not amount to more than 5% of the total binding.

In binding isotherm experiments, 50 l aliquots of 1:50 diluted antiserum were incubated with 0.04-0.60 nM [^{3}H]FNZ for 1 h at 4°C, in 2 ml reaction volume, and the non-bound radioligand was subsequently removed by 1.5 ml activated charcoal solution. The level of non-specific binding was measured in the presence of 1 μM diazepam and was higher than in the routine assay due to a less efficient removal of non-bound radioligand when large reaction volumes were used.

The levels of benzodiazepines in sera and urine samples of benzodiazepine-treated patients were determined by measuring the binding of 0.4 nM [^{3}H]FNZ in the presence of various amounts of

body fluids (0.1-20 µl) added to the RIA reaction mixture. The concentrations of benzodiazepines in the samples were calculated from the linear parts of these inhibition curves (in the range of 25% to 75% inhibition of [^{3}H]FNZ binding) using standard inhibition curves with diazepam or nitrazepam.

RESULTS

Sera of rabbits which were immunized with a Ro 7-1986/1-BSA conjugate contained antibodies specific for benzodiazepines. Binding isotherms of 0.04-0.60 nM [^{3}H]FNZ revealed saturable binding sites (Fig. 1A), which displayed an apparent equilibrium dissociation constant, K_D, of 0.073 ± 0.003 nM (mean ± S.E.M., 3 determinations), as determined by Scatchard analysis (Fig. 1B). The number of [^{3}H]FNZ-binding sites amounted to ca. 100 pmol per ml antiserum.

The binding of [^{3}H]FNZ to anti-Ro 7-1986/1 antibodies was inhibited in the presence of various benzodiazepine derivatives. Twelve compounds were tested (Fig.2). The concentrations of the drugs that caused 50% inhibition in the binding of 0.4 nM [^{3}H]FNZ ranged from 0.2 nM when diazepam was used, to 0.2 µM when chlordiazepoxide was used. Micromolar concentrations of non-benzodiazepine compounds which bind to brain benzodiazepine-receptors, such as norharmane and caffeine (23,24), did not cause a significant inhibition of the binding of [^{3}H]FNZ in the RIA.

The levels of benzodiazepines in body fluids of benzodiazepine-treated patients (Table 1) are expressed in terms

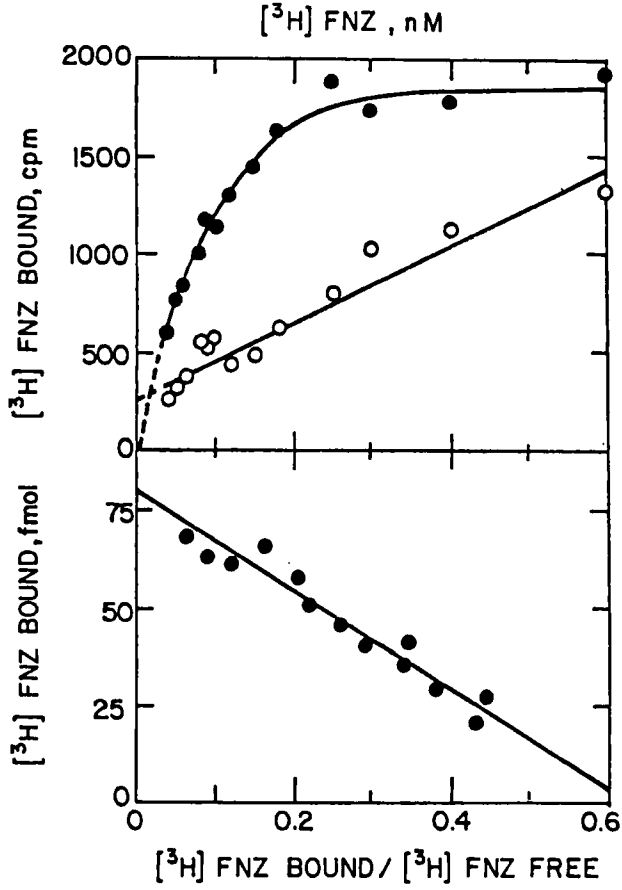

FIGURE 1.A. The binding of various concentrations of [^{3}H]FNZ to anti-Ro 7-1986/1 antiserum, after incubation of 1 h at 4°C. Specific binding (●——●) was calculated from the measured total binding by subtracting the non-specific binding (o——o), which was measured in the presence of 1 x 10^{-5} M diazepam.
B. Scatchard plot of the specific binding of [^{3}H]FNZ using the data presented in A.

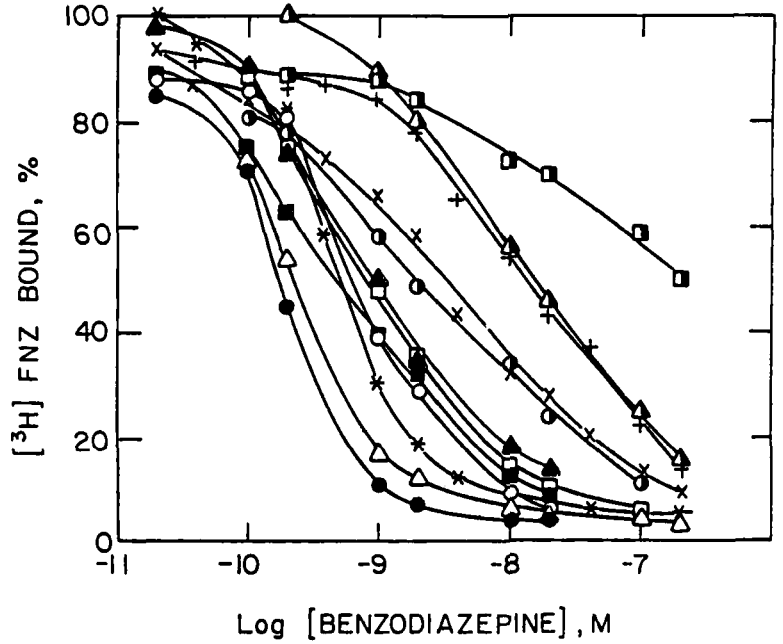

FIGURE 2. Displacement of [^{3}H]FNZ (0.4 nM) from anti-Ro 7-1986/1 antibodies by various concentrations of benzodiazepine derivatives: diazepam (●—●), Ro 5-5492 (Δ—Δ), nitrazepam (■—■), desmethyldiazepam (✱—✱), flunitrazepam (o—o), Ro 7-1986/1 (□—□), Ro 5-4864 (▲—▲), flurazepam (◑—◑), clonazepam (x—x), bromazepam (+—+), medazepam (◭—◭) and chlordiazepoxide (◨—◨).

of equivalents of nitrazepam or diazepam, using standard curves with these drugs (Fig.3). Control sera or urine of three untreated patients did not cause any inhibition of the binding of [^{3}H]FNZ to anti-Ro 7-1986/1 antibodies.

DISCUSSION

The specific high-affinity anti-Ro 7-1986/1 antibodies described in this report exhibited a high cross-reactivity with a broad spectrum of benzodiazepine derivatives and their metabolites

TABLE 1

Levels of Benzodiazepines in Sera and Urine Samples of Benzodiazepine-treated Patients as Determined by Anti-Ro 7-1986/1 Antibody RIA.

Patient	Benzodiazepine Treatment	Concentration, ng/ml serum	urine
1	10 mg nitrazepam [a]	42 (2.4)	42 (2.4)
2	10 mg nitrazepam [a]	100 (1.0)	83 (1.2)
3	10 mg nitrazepam [a]	13 (7.8)	8 (13.0)
4	10 mg oxazepam [a]	143 (0.7)	143 (0.7)
5	10 mg oxazepam [a]	50 (2.0)	50 (2.2)
6	4 mg diazepam [a]	25 (1.6)	15 (2.7)
7	5 mg diazepam [b]	44 (1.0)	n.d.
8	5 mg diazepam [c]	68 (0.65)	n.d.

[a] Received drug 8 orally h before sampling
[b] Received drug orally 5 h before sampling
[c] Received drug intramuscularly 2 h before sampling

Patients (adult females and males) received benzodiazepines as mild sedatives, not related to the treatment of their major illnesses. Values are given as equivalents of nitrazepam for nitrazepam- and oxazepam-treated patients, and as equivalents of diazepam for diazepam-treated patients. Numbers in parenthesis indicate the volume of body fluid (µl) which caused a 50% inhibition in the binding of 0.4 nM [^{3}H]FNZ.

which are present in urine. The RIA using these antibodies enables the screening of a wide range of clinically used benzodiazepines by a specific, sensitive and technically simple method. Most of the reported immunoassay methods for benzodiazepine determination allow the detection of a single derivative or closely related derivatives. A broad-spectrum RIA and its use for biological fluids was recently reported (16) using anti-oxazepam antibodies commercially available in Emit immunoassay kit from SYVA Co. (Palo Alto, CA). However, they used larger fluid volumes and an extraction step prior to the assay.

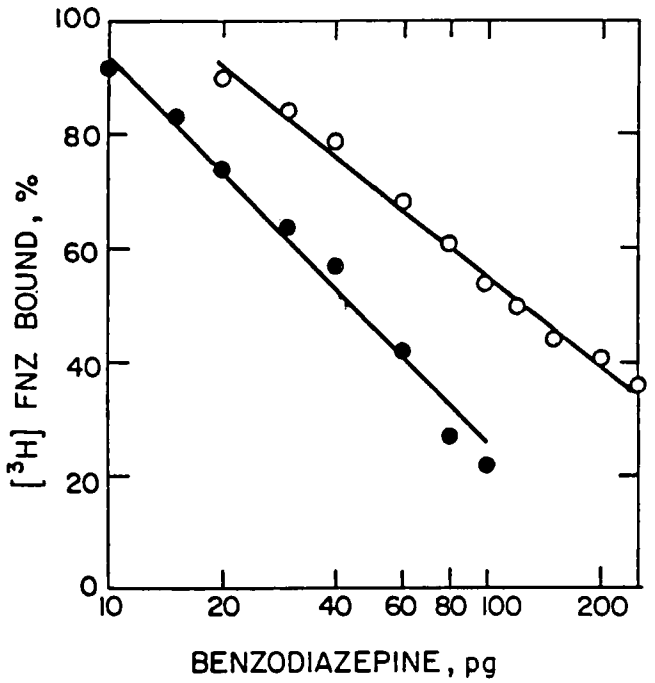

FIGURE 3. Standard curves for the displacement of [^{3}H]FNZ (0.4 nM) from anti-Ro 7-1986/1 antibodies by various amounts of diazepam (●—●) and nitrazepam (o—o).

High sensitivity in monitoring drugs is required in pharmacokinetic studies and in forensic medicine. Levels as low as 20 picograms of nitrazepam or diazepam can be detected under our standard conditions. The limit of detection can be lowered if smaller amounts of the radioligand are used and/or the volume of the reaction mixture is decreased. Due to the high sensitivity of the assay only minute volumes (microliter quantities) of biological fluids are employed for benzodiazepine determination. The presence of such small amounts of serum or urine does not interfere with radioactivity measurement by color-quenching, and there-

fore no extraction of the drugs is required. When large volumes of biological fluids are required for the determination (e.g., for the detection of low-affinity ligands such as medazepam or chlordiazepoxide), appropriate controls for measuring the color-quenching of a standard amount of the radioligand should be included.

The time duration of the test may be a crucial factor in cases of intoxication. The qualitative estimation of intoxicating levels of clinically used benzodiazepines in body fluids can be established in less than 10 min by a single measurement, using the RIA described here. When quantitative determinations are required, the assay gives a measure of the total level of the drugs, including their metabolites, in terms of equivalents of a benzodiazepine derivative which is employed as a standard. Using a diazepam standard gives the minimal value of equivalent benzodiazepine concentration.

It is concluded that the anti-Ro 7-1987/1 antibody RIA may be advantageous for various clinical purposes, since it permits both a rapid-qualitative evaluation and a sensitive-quantitative determination of benzodiazepines in biological fluids.

ACKNOWLEDGEMENTS

We wish to thank Dr. E. Gamzu and Dr. W.E. Scott for the generous gift of benzodiazepines, Prof. Meir Wilchek and Talia Miron for advice on the synthesis, and Irit Weiss for her technical assistance. Reprint requests should be addressed to R. Sherman-Gold, Dept. of Neurobiology, Stanford University Medical School, Stanford, California 94305, U.S.A.

REFERENCES

1. Zingales, I.A. Diazepam Metabolism during Chronic Medication Unbound Fraction in Plasma, Erythrocytes and Urine. J. Chromatog. 1973; 75:55-78.

2. Hailey, D.M. Chromatography of the 1,4-Benzodiazepines. J. Chromatog. 1974; 98:527-568.

3. de Silva, J.A.F., Bekersky, I., Puglisi, C.V., Brooks, M.A. and Weinfeld, R.E. Determination of 1,4-Benzodiazepines and -Diazepin-2-ones in Blood by Electron-capture Gas-liquid Chromatography. Anal. Chem. 1976; 48:10-19.

4. Greenblatt, D.J. Simultaneous Gass-chromatographic Analysis for Diazepan and its Major Metabolite, Desmethyldiazepam, with use of Double Internal Standardization. Clin. Chem. 1978; 24:1836-1841.

5. MacKichan, J.J., Jusko, W.J., Duffner, P.K. and Cohen, M.E. Liquid-chromatographic Assay of Diazepam and its Major Metabolites in Plasma. Clin. Chem. 1979; 25:856-859.

6. Cotler, S., Puglisi, C.V. and Gustafson, J.H. Determination of Diazepam and its Major Metabolites in Man and in the Cat by High-performance Liquid Chromatography. J. Chromatog. 1981; 222:95-106.

7. McCurdy, H.H., Lewellen, L-J., Cagle, J.C. and Solomons, E.T. A Rapid Procedure for the Screening and Quantitation of Barbiturates, Diazepam, Desmethyldiazepam and Methaqualone. J. Anal. Toxicol. 1981; 5:253-257.

8. Ratnaraj, N., Goldberg, V.D., Elyas, A. and Lascelles, P.T. Determination of Diazepam and its Major Metabolites using High-performance Liquid Chromatography. Analyst 1981; 106:1001-1004.

9. Hunt, P., Husson, J.M. and Raynaud, J.P. A Radioreceptor Assay for Benzodiazepines. J. Pharm. Pharmacol. 1978; 31:448-451.

10. Owen, F., Lofthouse, R. and Burne, R.C. A Radioreceptor Assay for Diazepam and its Metabolies in Serum. Clin. Chim. Acta 1979; 93:305-310.

11. Skolnick, P., Goodwin, F.K. and Paul, S.M. A Rapid and Sensitive Radioreceptor Assay for Benzodiazepine in Plasma. Arch. Gen. Psychiat. 1979; 36:78-80.

12. Lund, J. Radioreceptor Assay for Benzodiazepines in Biological Fluids using a New Dry and Stable Receptor Preparation. Scand. J. Clin. Lab. Invest. 1981; 41:275-280.

13. Peskar, B. and Spector, S. Quantitative determination of Diazepam in Blood by Radioimmunoassay. J. Pharmacol. Exp. Ther. 1973; 186:167-172.

14. Dixon, W.R., Earley, J. and Postma, E. Radioimmunoassay of Chlordiazepoxide in Plasma. J. Pharm. Sci. 1975; 64:937-939.

15. Gelbke, H.P., Schlicht, H.J. and Schmidt, G. Radioimmunological Screening and Gas Chromatographic Identification of Diazepam in Blood and Serum. Arch. Toxicol. 1977; 38:295-305.

16. Ko, H., ROyer, M.E., Hester, J.B. and Johnston, K.T. Radioimmunoassay of Triazolam. Anal. Lett. 1977; 10:1019-1040.

17. Glover, W., Earley, J., Delaney, M. and Dixon, R. Radioimmunoassay of Flunitrazepam in Human Plasma. J. Pharm. Sci. 1980; 69:601-602.

18. Robinson, K., Rutterford, M.G. and Smith, R.N. A Sensitive Benzodiazepine Radioimmunoassay of Broad Specificity. J. Pharm. Pharmacol. 1980; 32:773-777.

19. Wallace, J.E., Harris, S.C. and Shimek, E.L. Evaluation of an Enzyme Immunoassay for Determining Diazepam and Nordiazepam in Serum and Urine. Clin. Chem. 1980; 26:1905-1907.

20. Budd, R.D. Benzodiazepine Structure versus Reactivity with EMIT Oxazepam Antibody. Clin. Toxicol. 1981; 18:643-655.

21. Dixon, R., Glover, W. and Earley, J. Specific Radioimmunoassay for Flunitrazepam. J. Pharm. Sci. 1981; 70:230-231.

22. Hümpel, M., Nieuweboer, B., Milius, W., Henke, H. and Wendt, H. Kinetics and Biotransformation of Lormetazepam. Clin. Pharmacol. Ther. 1980; 28:673-679.

23. Sherman-Gold, R. and Dudai, Y. -Carboline Binding to Deoxycholate Solubilized Benzodiazepine Receptors from Calf Cerebral Cortex. Neurosci. Lett. 1981; 26:325-328.

24. Sherman-Gold, R. and Dudai, Y. Solubilization and Properties of a Benzodiazepine Receptor from Calf Cerebral Cortex. Brain Res. 1980; 198:465-490.