J. Pharm. Pharmacol. 1979, 31: 448–451 Received September 28, 1978

A radioreceptor assay for benzodiazepines

PETER HUNT*, JEAN-MARC HUSSON AND JEAN-PIERRE RAYNAUD
Centre de Recherches Roussel Uclaf, 93230 Romainville, France

A simple, rapid and sensitive radioreceptor assay for determining benzodiazepines in serum is based on the displacement by the drug of specific [^{3}H]diazepam binding to a membrane fraction from rat brain. The limit of detection of the more active benzodiazepines is about 0·5 ng. Diazepam, nitrazepam, clobazam and HR 458† have been assayed in human serum after a single oral clinical dose. The results can be used for determining pharmacokinetic parameters. The technique measures not only the parent benzodiazepine but also clinically active metabolites.

The potency of various benzodiazepines in displacing [^{3}H]diazepam bound to a specific receptor in brain membrane preparations correlates well with their activity in pharmacological tests, especially those predicting anticonvulsant and muscle-relaxant properties (Squires & Braestrup 1977; Mohler & Okada 1977; 1978). It is also correlated with clinically recommended dosages in man (Mohler & Okada 1978; Braestrup et al 1977).

The high affinity which benzodiazepines show for the receptor and the specificity of binding, that is, the lack of cross-reactivity of other drugs from a wide range of pharmacological classes (Braestrup & Squires 1978), suggests that the receptor could be the basis of an assay for measuring their plasma concentrations. This paper describes the development of such a radioreceptor assay which could be of particular value as a straightforward and general method, since the existing methods such as radioimmunoassay, fluorimetry and gas chromatography are often technically difficult and usually have to be developed for each particular drug. Radioreceptor assays in human serum are applied to two anxiolytics, diazepam and a 1,5-benzodiazepine, clobazam (Barzaghi et al 1973) and to two sleep-inducers, nitrazepam and a novel imidazolobenzodiazepine (Ager et al 1977), HR 158†.

MATERIALS AND METHODS

Male rats (150–200 g), were Sprague Dawley strain. [Methyl l-^{3}H]diazepam (18 Ci mmol^{-1}) was supplied by the Radiochemical Centre (Amersham, U.K.).

The membrane preparation and incubation conditions were essentially as described by Mohler & Okada (1977). Rat cerebral cortex was homogenized in 20 vol 0·32 M sucrose and centrifuged at 1000 *g* for 10 min. The supernatant was centrifuged at 20 000 *g* for 20 min and the resulting pellet suspended in 50 mM Tris HCl pH 7·4 (20 vol). After a second centrifugation in the same conditions the pellets were either resuspended in the same buffer for incubation or stored at −30 °C. In the latter case, binding activity remained unchanged for periods of up to at least 1 month. [^{3}H]Diazepam (10^{-9}M) was incubated alone or in the presence of competitors at different concentrations with aliquots of the membrane suspension (2 ml containing ~1 mg protein) for 30 min at 0 °C. The incubation was terminated by rapid filtration under vacuum through Whatman GF/C filters. The filters were washed with ice-cold incubation buffer (2 × 5 ml) and then left to stand overnight in scintillation fluid containing 30% Triton X-100 before radioactivity measurement. Non-specific binding was estimated by incubation in the presence of 10^{-6} M unlabelled diazepam.

For the radioreceptor assay, benzodiazepines were extracted from serum samples which were first made basic by the addition of M NaOH (1/10 vol, except for HR 158 where 800 µl of serum was brought to ~ pH 9 by the addition of 16 µl of M NaOH). The total volume was made up to a minimum of 500 µl with water where necessary and shaken with ether (5 vol) for 15 min. The aqueous phase was frozen in dry-ice/methanol and the ether layer was decanted and evaporated to dryness by nitrogen. The residues were taken up in aliquots of membrane suspension and incubated with [^{3}H]diazepam as described.

Extraction yields for the parent benzodiazepines as well as for some of their major metabolites were checked either by using the radioactively labelled compounds (diazepam, HR 158) or by radioreceptor assay (clobazam, nitrazepam, oxazepam, *N*-des-

* Correspondence.

† 8-nitro-6-(*o*-chlorophenyl)-1,2-dihydro-2-(*N*-methylpiperazin-1-yl) methylene-1H,4H-imidazo [1,2α]-[1,4]-benzodiazepin-1-one methane sulphonate.

methyldiazepam, *N*-desmethylclobazam) and were in all cases greater than 80% after a single extraction.

Standard curves were constructed by carrying out the incubation in the presence of increasing quantities of the benzodiazepines assayed (0·5–50 ng for diazepam, nitrazepam and HR 158; 10–1000 ng for clobazam) which were added to control serum (same volumes as for test samples) and extracted in volumes as for test samples) and extracted in the same way as the test samples so that extraction yields were not corrected for. Ether extracts of control serum had no effect on specific [^{3}H]diazepam binding, i.e. no endogenous benzodiazepine-like factor was extracted in these conditions.

Human blood samples were taken from 6 male subjects (ages 28–39 yr) at various times after a single oral administration of each benzodiazepine at about 10 a.m. No special conditions were imposed. Serum was kept at −20 °C before assay.

Non-specific binding was very low and was not modified by ether extracts of human serum (100 μl). It was virtually the same for all 6 subjects (mean (s.d.) = 3·3 (0·2)% of total binding) and was not subtracted from total binding for the assay.

Serum kinetics were analysed using a computer program* which fits the experimental curve by the sum of a number of exponentials for which the individual parameters are determined. In cases (all except HR 158) where values were lacking in the absorption phase, a zero point was introduced.

Within-assay precision was determined by making 6 replicate assays at 4 points (approx. 2, 4, 8 and 18 ng) on the standard curve for diazepam after solvent extraction from human serum. The coefficient of variation (c.v.) was 8 to 10%. Day-to-day precision was estimated over a period of one week by 6 separate assays after solvent extraction from the same serum. The c.v. was 9 to 13%.

RESULTS

Displacement of [^{3}H]diazepam binding in vitro

The activities of the benzodiazepines and some of their metabolites, in displacing [^{3}H]diazepam in vitro, are shown in Table 1.

Diazepam, HR 158† and nitrazepam have very similar IC50 values, while that of clobazam is greater. Of the metabolites tested, *N*-desmethyldiazepam and oxazepam, which are amongst the principal metabolites of diazepam, both have strong activity and similarly the *N*-desmethyl derivative of clobazam, the main metabolite in man (Dr M. Volz, Hoechst AG, unpublished results), is also almost as active as the parent compound.

Table 1. Displacement of [^{3}H]diazepam binding to rat brain cortical membranes in vitro by various benzodiazepines. [^{3}H]diazepam (10^{-9}M) was incubated with membrane homogenate (2 ml containing 1 mg protein) for 30 min at 0 °C in the presence or absence of benzodiazepines, at varying concentrations, in triplicate. IC50 values were determined from log probability plots of benzodiazepine concentration against % displacement of specific [^{3}H]diazepam binding. Non-specific binding was estimated by an identical incubation in the presence of 10^{-6}M unlabelled diazepam. Values represent the average of two determinations differing by less than 10%.

	IC50 (10^{-9}M)
Diazepam	7·1
HR 158	7·2
N-Desmethyldiazepam	8·8
Nitrazepam	10
Oxazepam	20
Clobazam	170
N-Desmethylclobazam	210

The corresponding displacement curves for diazepam and for clobazam are shown in Fig. 1, where the displacer concentration is expressed in ng per 2 ml incubation. The curves (not shown) for HR 158 and nitrazepam are virtually super-imposable on that of diazepam. The range of detection theoretically possible is thus of the order of 0·5–50 ng for diazepam, HR 158 and nitrazepam and 10–1000 ng for clobazam taking ~10–15% displacement of binding as the lower limit.

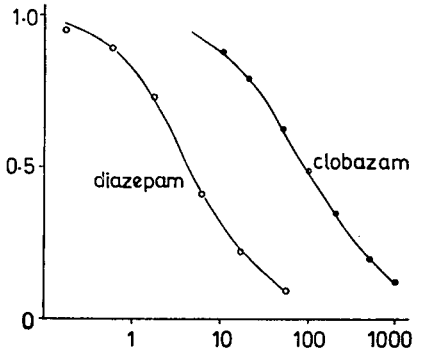

FIG. 1. Displacement of [^{3}H]diazepam binding in vitro —typical standard curves. [^{3}H]diazepam (10^{-9}M) was incubated as described for Table 1 in the presence or absence of unlabelled diazepam or clobazam, at various concentrations, in triplicate. Binding is expressed as a fraction of radioactivity bound in the absence of displacer (B_0). Ordinate: fraction of total binding B/B_0. Abscissa: concentration displacer (ng/incubation 2 ml).

* Developed at Roussel Uclaf by J. Lemoine and J. C. Milley.

† The *N*-desmethyl derivative of HR 158, a putative metabolite, has comparable activity (S. Clements-Jewery, personal communication).

Direct addition of rat or human serum to the incubation mixture strongly decreases specific [^{3}H]diazepam binding and effects are seen with as little as 10 μl serum (12% decrease with rat serum and 8% with human serum). For this reason, the assay described here has been carried out after solvent extraction of the benzodiazepines.

The standard curves (which can be linearized if required by using a log-probit plot) for the radioreceptor assay are constructed in the same way as the displacement curves above except that the benzodiazepine to be assayed is added in different concentrations to aliquots of control serum which are then extracted with ether exactly as for the treated serum.

Assay of benzodiazepines in human serum

Fig. 2 shows the concentrations of benzodiazepines (i.e. drug plus active metabolites) in human serum as a function of time after a single administration of an oral dose. For diazepam and nitrazepam (5 mg doses), 100 μl aliquots of serum were extracted and in these conditions the detection limit is 5 ng ml^{-1} for both drugs. The concentrations at 72 h after administration, 30 ng ml^{-1} for diazepam and 7·4 ng ml^{-1} for nitrazepam, are still above this limit.

With HR 158 and clobazam (2 and 10 mg doses respectively), larger aliquots of serum (500 μl or 800 μl) were extracted, so that the limits of detection are 2 ng ml^{-1} for HR 158 and 12·5 ng ml^{-1} for clobazam. At 48 h, clobazam could just be detected while HR 158 was no longer detectable. After an initial decline, a resurgence is seen in the levels of both nitrazepam and HR 158 (4 out of 6 subjects in each case) from about 3 to 4 h after administration. Similar effects have been described for both nitrazepam (Breimer et al 1977) and diazepam (Baird & Hailey 1972) and are attributed either to mobilization of the drug from lipid storage sites or to release from the enterohepatic circulation. In this case the formation of active metabolites may be a contributing factor as all receptor-active entities are measured.

A one or two compartment open model with 1st order absorption and 1st order distribution and/or elimination was assumed as a rough first approximation to the serum kinetics of the benzodiazepines.

The curve-fitting obtained with clobazam is shown in Fig. 3 with the absorption phase taken from 0 to 1 h and the distribution/elimination phases from 2 to 4 h and from 7 to 48 h. In view of the limited number of measurements (from 5 to 7 for periods up to 24–72 h after ingestion), the lag-time as well as absorption and elimination constants were not determined with precision and consequently are not reported. The serum clearances of the various compounds are shown in Table 2.

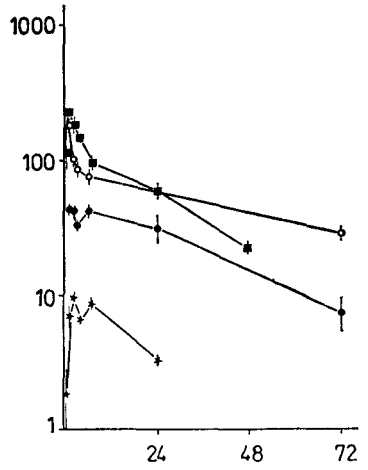

Fig. 2. Serum benzodiazepine values after a single oral dose in man. The benzodiazepine (5 mg diazepam (○) or nitrazepam (●), 2 mg HR 158 (✳), 10 mg clobazam (■)) was administered orally in a single dose to 6 subjects. Blood was taken at various times afterwards and aliquots of serum were extracted with ether for radioreceptor assay. A standard curve was constructed similarly by extraction from pooled serum taken just before drug administration. Values represent the means ± s.e.m. (diazepam, nitrazepam, HR 158, n = 6; clobazam n = 5). Ordinate: serum concentration (ng ml^{-1}). Abscissa: time after administration (h).

DISCUSSION

The radioreceptor assay is simple, convenient and of general application to active benzodiazepines. The sensitivity is higher for the more active compounds which are used clinically at lower dosages and is of the same order as that obtained in most of the assays currently used. It also approaches the sensitivity reported in a similar assay for neuroleptics using the

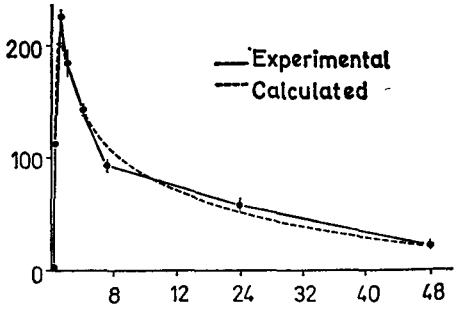

FIG. 3. Serum kinetics of clobazam. The experimental points were best fitted by the sum of 3 exponentials corresponding to an absorption phase (0 to 1 h) and 2 distribution/elimination phases (2 to 4 h and 7 to 48 h). A zero time point was added as insufficient measurements were made during the absorption phase. Ordinate: serum concn (ng ml^{-1}). Abscissa: time after drug (h).

Table 2. Clearance of benzodiazepines plus active metabolites from human serum.

Subject no	Clearance (litre h^{-1})			
	Diazepam	Nitrazepam	Clobazam	HR 158
1	0·93	3·98	2·97	6·8
2	0·79	2·89	2·43	7·3
3	0·77	3·29	2·46	—
4	0·98	2·63	2·99	12·1
5	0·60	1·78	2·49	12·0
6	0·85	1·83	—	9·6
Mean	0·82	2·73	2·67	9·6
	±0·05	±0·35	±0·13	±1·1

Serum clearance (Cls) is given by the formula

$$Cls = \frac{X_0}{\int_0^\infty Cdt}$$

where X_0 is the dose (assuming total absorption) and C is the serum concentration at time t. The individual values for the different subjects are shown together with the mean (± s.e.m.).

dopamine receptor (Creese & Snyder 1977). Another advantage of the technique is that it avoids the use of radioactively labelled compounds in man. It gives a measure not only of the parent benzodiazepine but also of any metabolites with an affinity for the receptor and which therefore have potential pharmacological or clinical activity. This latter aspect may be of particular interest for the correlation of benzodiazepine blood concentrations with their clinical or pharmacological effects (Garattini et al 1973).

In man, serum concentrations can be measured following the administration of usual clinical dosages, but prior extraction is necessary. We have found (unpublished results) that direct addition of even small volumes of serum to the incubation mixture strongly decreases specific [^{3}H]diazepam binding. Nevertheless, when relatively large doses of benzodiazepines are employed, such as in animal toxicological studies, direct assay is possible. For example, for volumes of rat serum up to 100 μl, the incubation conditions described here can still be used since the recoveries of added benzodiazepine are identical to those found after solvent extraction. The results with the four representative benzodiazepines chosen show that the technique can be successfully applied to pharmacokinetic studies. The serum clearances (Cls) found for diazepam (0·82) and nitrazepam (2·73 litre h^{-1}) are of the same order as reported values: for diazepam with a dose of 10 mg orally, the plasma clearance (Clp) = 1·75 litre h^{-1} (Klotz et al 1973) and after the same dose i.v., Clp = 2·1 litre h^{-1} (Andreasen et al 1976) which confirms that absorption after oral administration is good, as assumed; for nitrazepam with a 5 mg dose orally, Cls = 3·6 (Iisalo et al 1977) or 4·1 litre h^{-1} (Kangas et al 1977). When active metabolites, such as those of diazepam and clobazam (the principal metabolites of nitrazepam are not pharmacologically active (Randall & Kappell 1973), but their inactivity with respect to the benzodiazepine receptor in vitro has not been confirmed), are formed in sufficient quantity, the overall serum clearance would be expected to be lower than that of the parent compound alone. Although after a single dose the metabolites are unlikely to add very much to the overall activity, their contribution may be more important after chronic administration.

Acknowledgement

The technical assistance of Mr J. P. Stepniewski is gratefully acknowledged.

REFERENCES

Ager, I. R., Danswan, G. W., Harrison, D. R., Kay, D. P., Kennewell, P. D., Taylor, J. B. (1977) J. Med. Chem. 20: 1035–1041

Andreasen, P. B., Hendel, J., Greisen, G., Hvidberg, E. F. (1976) Eur. J. Clin. Pharmacol. 10: 115–120

Baird, E. S., Hailey, D. M. (1972) Br. J. Anaesth. 44: 803–808

Barzaghi, F., Fournex, R., Mantegazza, P. (1973) Arzneim.-Forsch. 23: 683–686

Braestrup, C., Albrechtsen, R., Squires, R. F. (1977) Nature (London) 269: 702–704

Braestrup, C., Squires, R. F. (1978) Eur. J. Pharmacol. 48: 263–270

Breimer, D. D., Bracht, H., De Boer, A. G. (1977) Br. J. Clin. Pharmacol. 4: 709–711

Creese, I., Snyder, S. H. (1977) Nature (London) 270: 180–182

Garattini, S., Marcucci, F., Morselli, P. L., Mussini, E. (1973) in: Davies, D. S., Prichard, B. N. C. (eds), Biological Effects of Drugs in Relation to their Plasma Concentrations, Macmillan, London, pp. 211–225

Iisalo, E., Kangas, L., Ruikka, I. (1977) Br. J. Clin. Pharmacol. 4: 646–647

Kangas, L., Allonen, H., Lammintausta, R., Pynnonen, S., Salonen, M. (1977) Acta Pharmacol. Toxicol. 41: Suppl. 4, 56

Klotz, U., Avant, G. R., Wilkinson, G. R., Hoyumpa, A., Schenker, S. (1973) Gastroenterology 65: 552

Mohler, H., Okada, T. (1977) Science 198: 849–851

Mohler, H., Okada, T. (1978) Life Sci., 22: 985–996

Randall, L. O., Kappell, B. (1973) in: Garattini, S., Mussini, E., Randall, L. O. (eds), The Benzodiazepines, Raven Press, New York, pp. 27–51

Squires, R. F., Braestrup, C. (1977) Nature (London) 266: 732–734