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# Radioimmunoassay of diazepam in samples of forensic interest

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The radioimmunoassay of diazepam in biological fluids is described. The method is particularly applicable to samples encountered in forensic toxicology that may be haemolysed, decomposed and of small volume. The antiserum and tritiated diazepam that are used are commercially available. Polyethylene glycol (mol. wt 6000) is used to separate the bound and free fractions, and the free fraction is counted by extracting the tritiated diazepam into a toluene-based scintillant. Blood samples are extracted with ethyl acetate before analysis but urine samples may be assayed directly. The assay is convenient, quick and virtually specific for diazepam. The results correlate well with those obtained by gas chromatography.

Radioimmunoassay (RIA) is a useful method for determining drugs and their metabolites in biological fluids, particularly blood. The assays that are commercially available (either as kits or separate items) enable a broad range of drugs to be analysed, and the sensitivity of RIA allows minimal sample volumes. In addition, samples can be analysed in batches, and so RIA can be used as a 'drug-screen' in forensic toxicology (Smith 1979).

Blood samples submitted for forensic examination are often haemolysed, decomposing and of small volume. The state of such samples and the need to conserve as much sample as possible to confirm positive RIA results by other methods mean that conventional RIA methods, especially those involving tritium-labelled compounds, usually require modification for forensic use.

This paper describes an assay that is virtually specific for diazepam, a drug that is widely prescribed and encountered in a significant number of toxicological and road traffic cases. Only 50 $\mu$l of blood are required and the results are not affected by haemolysis or decomposition. The assay is therefore suitable for general toxicological work or for extensive surveys of diazepam use.

## MATERIALS AND METHODS

### *Buffer*

A 0·067 M phosphate buffer of pH 7·4 containing 0·2% bovine $\gamma$-globulin (Cohn Fraction II, Sigma Chemical Co., Poole, Dorset, U.K.) and 0·1% sodium azide is used throughout the assay.

### *Antiserum*

The antiserum was purchased in ampoules each containing the residue from 1 ml of freeze-dried antiserum (Guildhay Antisera, Department of Biochemistry, University of Surrey, Guildford, Surrey, GU2 5XH, U.K.). Before use, the contents of a vial are reconstituted with 1 ml of distilled water and diluted to 10 ml with buffer. 100 $\mu$l aliquots of this solution are dispensed into 5 ml polypropylene tubes and stored at −20 °C. For an assay, an aliquot is allowed to thaw and buffer is then added to give the required dilution, usually about 1 : 300.

### *Tritiated diazepam*

[*N-methyl*-$^3$H]diazepam (30–60 Ci mmol$^{-1}$; 1·1–2·2 TBq mmol$^{-1}$) was purchased from the Radiochemical Centre (Amersham, Bucks., U.K.) in 250 $\mu$Ci lots dissolved in 250 $\mu$l of ethanol. A stock solution of 50 $\mu$l (50 $\mu$Ci) made up to 3·5 ml with ethanol is stored at −20 °C in a polypropylene tube. For an assay, 50 $\mu$l (0·7 $\mu$Ci) of this stock solution are diluted to 5 ml with buffer.

### *Diazepam standard*

12 mg of diazepam are dissolved in a little ethanol and made up to 100 ml with distilled water. 500 $\mu$l aliquots of this solution are dispensed into polypropylene tubes and stored at −20 °C. It has been shown that solutions of diazepam in plasma do not deteriorate under these conditions for at least a year (Howard 1978) and our standards proved to be equally stable. For an assay, an aliquot is thawed and diluted with buffer to give standard solutions containing 1·25, 2·5, 5·0, 10·0 and 20·0 ng ml$^{-1}$. The zero standard is buffer.

### *Sample preparation*

Urine can be analysed directly. Blood samples (50 $\mu$l) are buffered to about pH 9 with an equal volume of any suitable buffer and extracted with 500 $\mu$l ethyl acetate on a vortex mixer for 30 s.

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After centrifugation (1 min, 12 000 *g*), a 400 $\mu$l aliquot of the ethyl acetate layer is transferred to a polypropylene tube and evaporated to dryness under a compressed air flow at room temperature (20 °C). The residue is taken up in 160 $\mu$l of buffer which corresponds to a 1 : 4 dilution of the original sample. 25 $\mu$l of this solution are made up to 250 $\mu$l with buffer to give a 1 : 40 dilution, and an aliquot of this is further diluted to 1 : 400. In most cases, these three dilutions are sufficient to ensure that a result lies on the standard curve.

*Scintillant*

The scintillant consisted of 4 g 2,5-diphenyloxazole (PPO) and 0·2 g 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) per litre of reagent grade toluene.

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50 $\mu$l each of standard or sample, [$^3$H]diazepam and antiserum are pipetted into duplicate sets of micro-centrifuge tubes. 50 $\mu$l of [$^3$H]diazepam and 100 $\mu$l of buffer are put in another pair of tubes to measure the total radioactivity per tube. All the tubes are vortexed and incubated at 2 °C for 1 h. 475 $\mu$l of 23% w/v polyethylene glycol of molecular weight 6000 (PEG-6000) in buffer containing no bovine $\gamma$-globulin are then added to each tube. The tubes are vortexed thoroughly and allowed to stand for 5–10 min at room temperature before centrifuging (2 min, 12,000 *g*). 400 $\mu$l aliquots of the supernatants are then transferred to 5 ml polypropylene liquid scintillation tubes and 4 ml of scintillant are added. After shaking vigorously for 10 min to extract [$^3$H]diazepam into the scintillant, the tubes are placed in a liquid scintillation counter and left in the dark for 15–30 min to allow the phases to separate and any luminescence of the samples or tubes to decay to zero. The tubes are then counted for 2–5 min each and the results are plotted.

## DISCUSSION

The diazepam antiserum used in the assay is, as far as we are aware, the only one that is commercially available. Its preparation and use for assaying plasma diazepam have been described briefly (Bourne et al 1978). Other radioimmunoassays (Peskar & Spector 1973; Gelbke et al 1977) and radioreceptor assays (Hunt et al 1979; Owen et al 1979) for diazepam have been developed, but all differ considerably from the assay described here which was designed to be unaffected by haemolysis or decomposition and to require very little sample.

A sensitive assay covering a relatively narrow concentration range was chosen in preference to a less sensitive assay with a wider range since the former is not only more economical but is preferable in cases where it is necessary to demonstrate the absence rather than the presence of diazepam.

The assay conditions were developed empirically and were subsequently compared with the conditions that, in theory (Ekins et al 1968), give the optimum standard curve for an equilibrium assay. The empirical conditions were selected by using an amount of [$^3$H]diazepam (approx. 50 pg) per assay tube that was small enough to give a sensitive assay while its activity (approx. 7 pCi) was sufficient for about 5000 counts to be accumulated in 2 min. An antiserum dilution curve was then constructed by incubating 50 $\mu$l each of [$^3$H]diazepam, buffer and increasing antiserum dilutions under the conditions described in the previous section. 50% of the [$^3$H]diazepam was bound by an initial antiserum dilution of 1 : 300, which was used in the assay. Over a period of months, the zero point of the standard curve drops from 50% B/T (antibody-bound activity/total activity per tube). This appears to be due to decomposition of the [$^3$H]diazepam rather than the frozen aliquots of antiserum, and so it is advisable to replace the [$^3$H]diazepam at approximately 6 monthly intervals.

The theoretical optimum assay conditions, i.e. [$^3$H]diazepam and antibody binding site concentrations equal to 4/K and 3/K respectively where K is the antibody affinity constant, were calculated from a Scatchard plot (Scatchard 1949) of the data contained in a typical standard curve. The best straight line was calculated by the least squares method. From the slope of the line, the average value of K was found to be $1{\cdot}17 \times 10^9$ litre mol$^{-1}$, and the intercept with the abscissa defined the antibody binding site concentration as $1{\cdot}29 \times 10^{-9}$ mol litre$^{-1}$. The concentration of [$^3$H]diazepam in the assay tubes ($0{\cdot}91 \times 10^{-9}$ mol litre$^{-1}$) was calculated from the specific activity of the [$^3$H]-diazepam. The figures showed that the antibody binding site and [$^3$H]diazepam concentrations that were derived empirically were about 50 and 25% respectively of their optimum theoretical values. A standard curve was constructed using the optimum theoretical values and this is compared with an empirical standard curve in Fig. 1. The empirically derived assay conditions gave acceptable sensitivity and precision in practice and were adopted for routine use since the theoretical conditions were less economical in terms of the antiserum and [$^3$H]diazepam required.

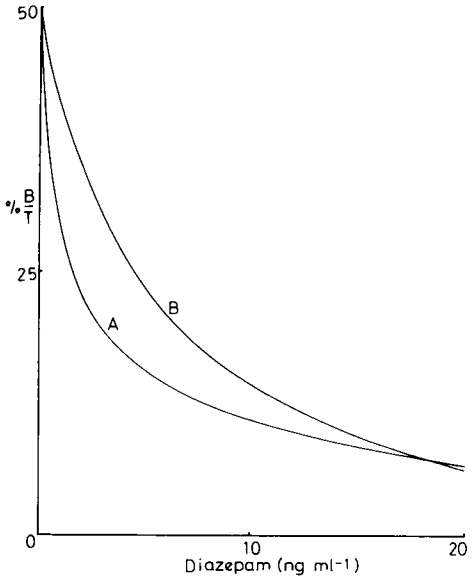


FIG. 1. Diazepam standard curves. B = bound activity; T = total activity. Curve A: empirically derived conditions, [Ab] = 1·5/K and [Ag*] = 1/K. Curve B: optimum theoretical conditions, [Ab] = 3/K and [Ag*] = 4/K. [Ab] = antibody binding site concentration; [Ag*] = [$^3$H]diazepam concentration; K = antibody affinity constant.

The incubation time was standardized at 1 h as equilibrium was found to be reached within this time. Incubation at room temperature resulted in a shallower curve than that obtained after incubation at 2 °C and there was also some loss of precision. 2 °C was therefore used routinely. It has been shown (Keane et al 1976) that, for RIA systems in which there is a significant enthalpy component in the binding, the sensitivity is maximum at the lowest possible incubation temperature.

PEG-6000 was used to separate the bound and free fractions because it is quick, effective and economical (Desbuquois & Aurbach 1971). The other commonly used separation methods—charcoal adsorption, double-antibody and solid-phase methods all have disadvantages. PEG-6000 precipitation of the bound fraction does not appear to disturb the equilibrium, nor does it depend on precise timing or temperature control. The resulting precipitates are compact and waxy on centrifugation and not easily disturbed. The 23% PEG-6000 solution adopted is not too viscous to be pipetted quickly and accurately. The procedure was arranged to give a final concentration of 17·5% PEG-6000 in the assay tubes since experiments showed this to be the minimum concentration for virtually complete precipitation of the bound fraction. The bovine $\gamma$-globulin in the buffer acts as a carrier protein. It is not necessary to cool the 23% PEG-6000 solution to the incubation temperature before adding it to the assay tubes, nor is the time interval before centrifugation critical. The sole disadvantage of PEG-6000 precipitation is that the supernatants from the assay tubes are immiscible with relatively inexpensive scintillants. For this reason the supernatants are shaken with the scintillant to extract the [$^3$H]diazepam from the aqueous phase. A laboratory shaker fitted with home-made racks taking 70 tubes proved adequate, extraction efficiency being 96%. Although it involves an additional step, the extraction procedure has the advantage that substances liable to cause quenching in conventional liquid scintillation counting may be retained in the aqueous phase and so be prevented from affecting the counting rate. This was so when haemolysed blood samples were analysed directly.

Little or no [$^3$H]diazepam is bound to the walls of the polypropylene tubes used but, when blood samples were analysed directly during the development of the assay, a significant amount of the [$^3$H]diazepam was non-specifically bound, presumably by proteins in the sample, and erroneously low results were obtained. The magnitude of this could be measured by incubating the sample with [$^3$H]diazepam in the absence of antibody, and a correction factor could then be applied to the assay results. This is tedious and required an additional sample, and so the problem was avoided by solvent extraction of the sample before assay. Ethyl acetate is a good solvent for extracting 1,4-benzodiazepines and their metabolites from body fluids (Clifford & Smyth 1974) and it can be pipetted with reasonable accuracy. The extraction was 88% efficient when tested on blood samples containing 0·5 $\mu$g ml$^{-1}$ diazepam. Ethyl acetate was also the most suitable solvent for use with a benzodiazepine assay of wide cross-reactivity (Rutterford & Smith 1980) and so the same extraction was used in the assay described here. Non-specific binding does not occur with urine samples so prior treatment is unnecessary.

The cross reactions of various benzodiazepines with the antiserum were investigated. These were negligible up to 1 $\mu$g ml under the conditions of the assay, for chlordiazepoxide, clonazepam, flurazepam, desalkylflurazepam, lorazepam, nitrazepam and oxazepam. Desmethyldiazepam, flunitrazepam, 3-hydroxydiazepam (temazepam) and medazepam cross-reacted to varying extents (see Fig. 2). For blood the principle effect of the cross-reactivity

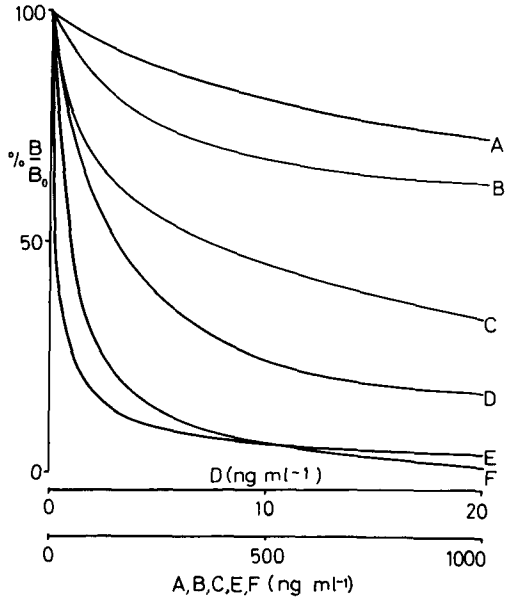


FIG. 2. Standard curves of benzodiazepines. B = bound activity; $B_0$ = activity bound in absence of unlabelled drug. A = desalkylflurazepam; B = desmethyldiazepam; C = Medazepam; D = diazepam; E = 3-hydroxydiazepam (temazepam); F = flunitrazepam.

pattern is to confer upon the assay virtual specificity for diazepam, since its metabolites or other benzodiazepines are normally present in blood at concentrations too low to be detected, particularly when the sample is diluted sufficiently for the diazepam concentration to lie within the range of the standard curve. With urine samples the assay gives high positive results following ingestion of diazepam, despite the fact that unmetabolized diazepam is rarely found in urine on gas chromatographic (g.c.) analysis. Since 65–75% of ingested diazepam is excreted in the urine as conjugated metabolites (Greenblatt & Shader 1974), the inference is that the results are primarily due to a cross-reaction of desmethyldiazepam glucuronide with the antiserum. This has not been investigated, since benzodiazepines in urine are conveniently detected by hydrolysis and analysis of the resulting benzophenones.

Positive RIA results for diazepam in blood are confirmed routinely by g.c. of a hexane extract using prazepam as an internal standard. Regression analysis was applied to the results of 51 cases in which of different operators carried out the RIA and g.c. A regression line, $y = 0{\cdot}98x + 55$, was obtained, where y and x represent the RIA and g.c. results respectively, with a correlation coefficient of 0·85. In 17 of these cases, the g.c. analysis was by a single operator while the RIA was by several operators. The results of these cases were examined and the correlation coefficient was 0·97 while the regression line was virtually unchange ($y = 0{\cdot}96x + 63$). The RIA method thus correlates well with g.c. but the results, about 60 ng $ml^{-1}$ higher, probably reflect differences in extraction.

The inter-assay coefficient of variation of the RIA method determined by analysing a blood sample containing 50 ng $ml^{-1}$ diazepam was 11·5%. Aliquots of the sample were stored at −20 °C and analysed at intervals over a period of months by operators of varying skill.

The potential sensitivity of the assay is proportional to the reciprocal of the affinity constant of the antibody and is therefore about $10^{-9}$ mol $litre^{-1}$ or 0·3 ng $ml^{-1}$. In practice, blank bloods gave an apparent diazepam concentration of just over 1 ng $ml^{-1}$ on average, with a coefficient of variation of about 120%. There is thus no problem in distinguishing blanks from diazepam concentrations likely to have a clinical effect.

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