ASSAY OF CLOBAZAM IN HUMAN SERUM BY A RADIORECEPTOR TECHNIQUE

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- 1 Clobazam has been assayed in human serum using a radioreceptor technique.
- 2 The assay is simple and sufficiently sensitive for detecting serum levels up to 48 h after oral administration of a usual clinical dose. It has the advantage of measuring the parent compound plus active metabolites.
- 3 Serum clearances and elimination half-lives have been determined for five subjects.

Introduction

A SIMPLE and convenient radioreceptor assay for determining benzodiazepines in serum has been developed (Hunt, P., Husson, J.M. & Raynaud, J.P., submitted for publication) based on the displacement by benzodiazepines of [^3H]-diazepam binding to a rat brain membrane fraction *in vitro*. The more active benzodiazepines compete with [^3H]-diazepam for the receptor at concentrations in the nanomolar range (Squires & Braestrup, 1977; Möhler & Okada, 1977a), which means that the sensitivity of the assay is high. The specificity is also high, as other drugs from a wide range of pharmacological classes have little or no activity (Braestrup & Squires, 1978). The potency of benzodiazepines in displacing [^3H]-diazepam binding has been correlated not only with their pharmacological activity (Squires & Braestrup, 1977; Möhler & Okada, 1977b, 1978) but also with their clinically recommended doses (Braestrup, Albrechtsen & Squires, 1977; Möhler & Okada, 1978). As the radioreceptor assay measures overall receptor activity, it therefore gives an estimate of the serum levels of all potential clinically active compounds, which thus includes active metabolites.

Here the technique has been applied to the assay of clobazam in human serum following a single administration of a usual therapeutic dose.

Methods

Male rats (150–200 g), Sprague–Dawley strain, were used for all experiments. Methyl-1-[^3H]-diazepam (18 Ci/mmol) was supplied by the Radiochemical Centre (Amersham, U.K.). Radioactivity measurements were made by liquid scintillation.

The preparation of a membrane fraction from rat cerebral cortex was as described by Möhler & Okada (1977a). Membrane pellets were either immediately resuspended in 50 mM Tris-HCl pH 7.4 for incubation or stored at -30°C in which case binding activity

remained unchanged for periods of up to at least 1 month.

For the assay of clobazam aliquots of serum (500 or 800 μl) in triplicate were first made basic by the addition of 1N NaOH (1/10 volume) and extracted by shaking with ether (5 volumes) for 15 minutes. The aqueous phase was frozen in dry-ice/methanol and the ether layer was decanted and evaporated to dryness by a stream of nitrogen. The residue was taken-up directly in the membrane suspension (2 ml containing about 1 mg protein) and incubated with [^3H]-diazepam (10^{-9}M) 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 ($2 \times 5\text{ ml}$) and then left to stand overnight in scintillation fluid containing 30% Triton X-100, before radioactivity measurement. Non-specific binding was estimated by an incubation in the presence of a high concentration (10^{-6}M) of unlabelled diazepam.

The standard curve was also constructed by extraction from serum in exactly the same way so that extraction yields were not corrected for. The recoveries of clobazam and *N*-desmethyl-clobazam were checked by radioreceptor assay and were respectively 88 and 80% after a single extraction.

Blood samples were taken from five male volunteers (aged 28–36 yr) at various times after a single oral administration of clobazam (10 mg) at about 10.00. No special conditions, such as fasting, were imposed. Serum was conserved at -20°C before assay.

As non-specific binding was very low and virtually the same for each subject (mean $\pm$ s.e.m. = $3.3 \pm 0.1\%$ total binding), it was not subtracted from total binding for the assay.

Serum kinetics were analysed using a computer program (developed at Roussel Uclaf by J. Lemoine and J.C. Milley) whereby the experimental curve is fitted by the sum of a number of exponentials for

which the individual parameters are determined.

Results

Typical standard curves for diazepam and clobazam are shown in Figure 1, in which the concentrations of

Figure 1 Displacement of [^3H]-diazepam binding by unlabelled diazepam and clobazam. [^3H]-diazepam 10^{-9}M was incubated with the membrane suspension (2 ml containing 1 mg protein) for 30 min at 0°C in the presence or absence of unlabelled diazepam ($\circ$) or clobazam ($\bullet$) at varying concentrations in triplicate. Binding is expressed as the fraction of radioactivity bound in the absence of displacer (B_0).

the displacing compounds are expressed as ng per 2 ml incubation. The corresponding IC_{50} values (that is, the concentrations displacing 50% of specific [^3H]-diazepam binding) are 7.1 nM and 170 nM for diazepam and clobazam respectively. In the same conditions, *N*-desmethyl-clobazam ($\text{IC}_{50} = 210$ nM), the main metabolite of clobazam in human serum (Volz *et al.*, 1979) has almost the same activity as the parent compound. The range of detection theoretically possible for clobazam is thus of the order of 10–1000 ng, taking 10–15% displacement of binding as the lower limit.

Figure 2 shows the level of clobazam (or clobazam equivalents) in human serum as a function of time after a single oral administration of a 10 mg dose. Peak serum levels of 200–260 ng/ml were observed from 1–2 h after ingestion. At 48 h the level (mean = 22 ng/ml) approached the limit of detection, which was 12.5 ng/ml when 800 μl aliquots of serum were extracted.

The serum kinetics could be described by a two compartment open model with first-order absorption and first-order distribution and/or elimination. The

Figure 2 Serum levels of clobazam after a single oral dose in man. Clobazam 10 mg was administered as a single oral dose to five subjects. Blood was taken at various times afterwards and aliquots of serum (500 μl or for the 24 h and 48 h samples, 800 μl) were extracted with ether for assay. A standard curve (---) was constructed similarly by extraction from pooled serum taken just before drug administration. Values represent the means $\pm$ s.e.m. for the five subjects. The experimental points (—) were best fitted by the sum of three exponentials corresponding to an absorption phase (0–1 h) and two distribution/elimination phases (2–4 h and 7–48 h). A zero time point was introduced as insufficient measurements were made during the absorption phase.

curve-fitting obtained for clobazam is shown in Figure 2, in which the absorption phase was considered to be from 0–1 h and the two distribution/elimination phases from 2–4 h and from 7–48 hours. The curves for the individual subjects were fitted in the same way and the respective total serum clearances as well as the half-lives for the latter elimination phase are shown in Table 1. The average serum clearance was 2.67 ± 0.13

Table 1 Serum clearance and elimination half-life of clobazam plus active metabolites in human serum

Subject	Serum clearance (l/h)	Elimination half-life (h)
1	2.97	20.0
2	2.43	27.7
3	2.46	12.6
4	2.99	19.3
5	2.49	22.2
Mean $\pm$ s.e.m.	2.67 ± 0.13	20.4 ± 2.4

Serum clearance (Cl_s) is given by the formula:

$$Cl_s = \frac{X_0}{\int_0^\infty C dt}$$

where X_0 is the dose (assuming total absorption) and C is the serum concentration at time t . The elimination half-life corresponds to the latter phase of elimination from 7–48 hours.

1 per h and the average elimination half-life, determined for the period from 7–48 h, was 20.4 ± 2.4 hours.

Conclusions

The radioreceptor assay is a rapid and straightforward technique for measuring clobazam levels in human serum. It is sufficiently sensitive for detecting the drug following the single oral administration of a usual clinical dose, so that pharmacokinetic parameters can be determined.

As the technique takes into account all receptor-

active compounds, thereby including any pharmacologically active metabolites as well as the parent compounds, the *N*-desmethyl derivative of clobazam would also be expected to contribute to some extent to the pharmacokinetic profile. This contribution is likely to be more important when the drug is used chronically and the metabolites attain steady-state levels. In these conditions the radioreceptor assay may be particularly useful for correlating blood levels of receptor-active compounds with clinical or pharmacological effects.

The technical assistance of Mr J.P. Stepniewski and the participation of the volunteers is gratefully acknowledged.

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Discussion

DR M. LADER (London) asked whether Dr Hunt had been able to detect clobazam in saliva in humans with his receptor assay. Dr Lader's group had been using the benzodiazepine receptor assay and had found it useful as a semi-quantitative estimate of benzodiazepine usage by monitoring the drug in saliva.

DR HUNT replied that they had carried out some preliminary investigations but had been unable to detect clobazam in saliva (after administering an oral dose of 20 mg and extracting 500 μl saliva). He suggested that the problem may lie with the method of extraction.

DR LADER commented that his group were able to measure diazepam in saliva without difficulty. Even though the plasma binding is high, there seems to be rather more in saliva than would have been predicted from the plasma-free drug concentrations.

DR W. RUPP (Frankfurt) added that clobazam is only about 10% non-protein-bound in plasma. As only free drug appears in saliva, any assay would have to have a limit of detection of less than 10 ng/ml before any clobazam could be detected in saliva following a single oral dose of 10 or 20 mg.

PROFESSOR D. LAWSON (Glasgow) asked whether, in view of the fact that clobazam is approx-

imately 85% protein-bound, any data were available on possible interactions with highly protein-bound drugs such as anticoagulants.

DR M. VOLZ (Frankfurt) replied that no such studies had been carried out.

DR B.S. MELDRUM (London) commented on Dr Hunt's finding that clobazam is at least ten times less potent than diazepam at displacing labelled diazepam from binding sites in brain membrane preparations. Mohler & Okada (1977a) have demonstrated that a wide range of 1,4-benzodiazepines show a good correlation between their potency at displacing diazepam and their potency at blocking Metrazol (leptazol)-induced seizures. Clobazam does not seem to fit this pattern.

DR HUNT replied that he could not give a definitive answer to this question. One possible explanation may be that the receptor studies were carried out *in vitro* and could not take into account any possible modification of the pharmacological effects *in vivo* related to the distribution and metabolism of the drug. It may be that the same receptor in different brain regions is associated with different pharmacological effects, and the distribution of the drug is therefore very important. PROFESSOR H. VOGEL (Frankfurt) added that we