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Note

Simple high-performance liquid chromatographic assay for the routine monitoring of clonazepam in plasma

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The benzodiazepine derivative clonazepam is an effective oral anticonvulsant for all forms of generalized epilepsy. Used parenterally, clonazepam has been found to be most effective against status epilepticus [1]. However, clonazepam has been one of the least-monitored anticonvulsants owing to the more difficult nature of its assay. Its concentration in the plasma is in the order of 100 to 1000 times lower than the level of most other anticonvulsants, resulting in a sensitivity problem for the measurement of clonazepam by chromatographic techniques. Other anticonvulsants are often administered concomitantly with clonazepam and as these are present in higher concentrations in plasma, adequate chromatographic separation is essential.

Although clonazepam can be measured by gas—liquid chromatography (GLC) using electron-capture detection [2], it chromatographs far more readily using high-performance liquid chromatography (HPLC). However ultraviolet (UV) detection for the HPLC assay is not as sensitive as electron-capture detection used in GLC. A previously reported HPLC assay for measuring clonazepam employed the normal-phase mode [3]. We have developed an HPLC assay for clonazepam in the reversed-phase mode using hexane—ethyl acetate (90:10, v/v) as the extraction solvent. The assay produced clean chromatography with sufficient sensitivity for plasma clonazepam assays to be carried out with UV detectors that have a sensitivity limit of 0.01 absorbance units full scale (a.u.f.s.).

EXPERIMENTAL

Reagents

Pure standard samples of clonazepam (CLON), flunitrazepam (internal standard I.S.), 7-aminoclonazepam and 7-acetamidoclonazepam were obtained from Roche Products (Dee Why, Australia). Seronorm Pharmaca AED,

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which is a lyophilized reference serum of animal origin for the quality control of serum analysis of antiepileptic drugs, was obtained from Nyegaard (Oslo, Norway). The solvents hexane, ethyl acetate, acetonitrile and methanol were all of HPLC grade from Waters Assoc. (Milford, MA, U.S.A.). All other reagents were of analytical reagent grade.

Standard solutions

A stock solution of clonazepam was prepared with a concentration of 1.0 μg/ml in methanol. Aliquots of this solution were then accurately syringed into extraction tubes and carefully evaporated to dryness under a gentle stream of nitrogen. Drug-free plasma (1 ml) was added to each of these tubes to give standard plasma solutions ranging in concentration from 10 to 100 ng/ml. A solution of the internal standard, flunitrazepam, was prepared with a concentration of 5 μg/ml in methanol.

Instrumentation

Analyses were performed on a high-performance liquid chromatograph consisting of a Waters Assoc. Model 6000A solvent delivery system and a Model 450 variable-wavelength UV detector. Samples were injected by means of a WISP Model 710B autosampler. The assays were carried out in the reversed-phase mode using a Perkin-Elmer analytical C_8 (10 μm) column, 25 cm × 4.6 mm. The mobile phase consisted of acetonitrile—0.05 mol/l sodium acetate (adjusted to pH 7.5) (38:62, v/v), with a flow-rate of 1.8 ml/min. The wavelength of the UV detector was set at the λ_{max} for clonazepam of 306 nm and the range set at 0.01 a.u.f.s.

Assay procedure

To a screw-capped pyrex tube (16 × 125 mm) containing 1.0 ml of plasma sample or standard were added 0.5 ml of 1 mol/l ammonia solution (adjusted to pH 9.5 with hydrochloric acid), 20 μl of the internal standard (5.0 μg/ml flunitrazepam in methanol) and 10 ml of extraction solvent (hexane—ethyl acetate, 90:10, v/v). The tube was capped with a PTFE-lined screw cap and the contents were shaken vigorously for 1 min. After centrifugation (1 min at 1000 *g*) the organic phase (top layer) was transferred to a clean dry quick-fit test tube and the solvent removed in vacuo using a Buchi Rotovapor. The dried extract was then redissolved in 200 μl of the mobile phase and 180 μl were injected onto the column. Retention times for clonazepam and flunitrazepam were 6.5 and 7.9 min, respectively. A calibration curve was obtained from the standard solutions by plotting the ratio of the peak height of clonazepam to that of the internal standard against the concentration of clonazepam. The calibration curve obtained from three replicate determinations for the concentrations 10, 20, 40, 70 and 80 ng/ml and ten replicate determinations for the concentrations 50 and 100 ng/ml, was linear over this range of standard solution concentrations. The regression equation was $Y = 0.0102X - 0.0132$ and the correlation coefficient (r) was 0.998. The nominal therapeutic range for plasma clonazepam concentration employed by this hospital is 25—75 ng/ml. The calibration curve adequately covers this range. The precision of the assay was 2.8% (coefficient of variation, C.V.) at 50 ng/ml and 1.5% (C.V.) at 100 ng/ml

(n=10). The limit of detection was 3 ng/ml. The concentrations of clonazepam in the samples were determined from the standard calibration curve.

RESULTS AND DISCUSSION

The use of hexane—ethyl acetate (90:10) as the extraction solvent yielded clean extracts with greater than 75% recovery of clonazepam without any interference in the chromatography. Although diethyl ether gives excellent recovery as an extraction solvent, other endogenous substances are also extracted from the plasma resulting in a relatively impure extract. Dissolution of this extract with the mobile phase required a tedious filtering step using an aqueous sample clarification kit obtained from Waters Assoc. (Part Number 26865) with some loss of sample prior to injection onto the LC column. Rovei and Sanjuan [4] used chloroform as the extraction solvent to extract clonazepam from plasma that had been alkalinised with phosphate buffer (pH 9.1). However, we found the use of hexane—ethyl acetate (90:10) yielded a cleaner extract than chloroform or dichloromethane. In addition the hexane—ethyl acetate (90:10) solvent extracted a smaller quantity of the acidic anticonvulsant drugs such as phenytoin and carbamazepine.

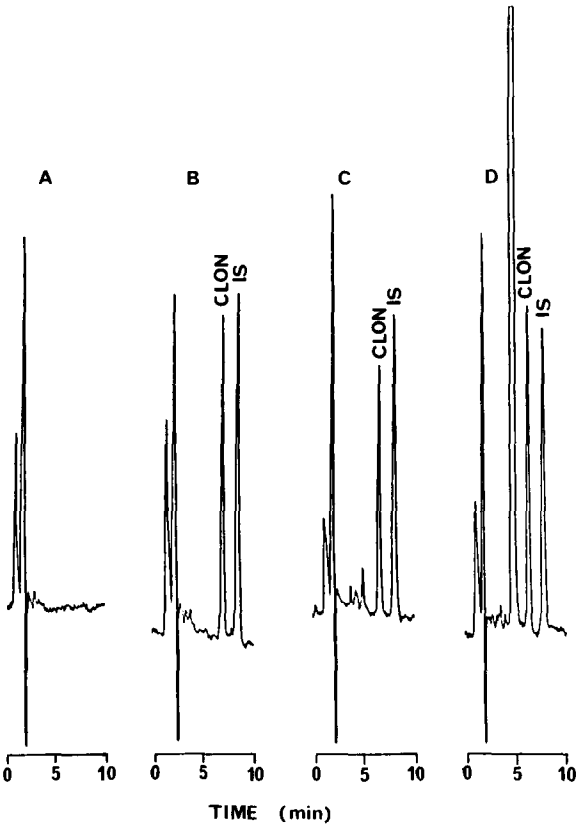

Fig. 1. Chromatograms of plasma extracts of a blank plasma (A), spiked standard (B), a typical sample (C), and an external quality control (Seronorm Pharmaca AED) (D). Peaks: CLON = clonazepam, IS = internal standard (flunitrazepam).

The chromatogram of a blank plasma extract (from a control subject who has not taken clonazepam) is shown in Fig. 1A. The chromatogram of the extract from a spiked plasma standard of clonazepam is shown in Fig. 1B, and that from a patient receiving clonazepam in Fig. 1C. Fig. 1D shows the chromatogram from an extract of an external quality control serum (Seronorm Pharmaca AED) which contains carbamazepine, clonazepam, ethosuximide, phenobarbitone, phenytoin, primidone and valproic acid. As the column slowly deteriorated after more than 500 injections, the sensitivity and resolution of clonazepam from carbamazepine was gradually reduced and the column eventually required replacement after approx. 800 injections. Thiopentone was found to co-chromatograph with clonazepam and as there are occasional clinical situations requiring simultaneous administration of thiopentone and clonazepam, e.g. status epilepticus, thiopentone interference can be eliminated by an acidic back-extraction of the thiopentone from the organic phase. None of the other commonly used anticonvulsants were found to interfere with the assay (see Table I).

TABLE I

RETENTION TIMES FOR VARIOUS OTHER DRUGS AND METABOLITES USING THE CLONAZEPAM ASSAY CONDITIONS

Drug	Retention time (min)
Valproic Acid	Approx. 2*
Ethosuximide	Approx. 2*
Primidone	2.8*
Carbamazepine epoxide	2.9
Phenobarbitone	2.9*
7-Acetamidoclonazepam	3.0
7-Aminoclonazepam	3.2
Bromazepam	4.4
Carbamazepine	4.8
Phenytoin	4.9*
Oxazepam	5.4
Lorazepam	5.8
Nitrazepam	5.8
Clonazepam	6.5
Chlordiazepoxide	6.6
N-Desmethyldiazepam (nordiazepam)	7.4
Flunitrazepam	7.9
Diazepam	10.9
Prazepam	21
Medazepam	24

*These drugs were measured at λ = 215 nm as they were not detected in our clonazepam assay at λ = 306 nm.

The main metabolites of clonazepam in plasma are 7-aminoclonazepam and to a lesser extent 7-acetamidoclonazepam [1] which had retention times of 3.0 and 3.2 min, respectively and were well resolved from clonazepam. In general the body metabolizes a drug by converting it into more polar compounds to facilitate excretion. These more polar metabolites are eluted earlier than the parent drug in the reversed-phase mode of chromatography. In our

assay these two metabolites are barely detectable because of their low absorbance at wavelength 306 nm.

The benzodiazepine chlordiazepoxide had a retention time of 6.6 min and was not able to be resolved from clonazepam under these chromatographic conditions. It is possible that on changing to a smaller-particle-size packing material (e.g. 5 μm) these two substances may be resolved. Using the same mobile phase we obtained good separation of clonazepam and chlordiazepoxide on a 5-μm C_{18} column obtained from Waters Assoc. It is unlikely that this would be of importance in clinical practice when an alternative benzodiazepine could be substituted for chlordiazepoxide. None of the other benzodiazepines tested interfered with the assay (see Table I). However, if large quantities of the diazepam metabolite, N-desmethyldiazepam (nordiazepam), were present it could interfere with the internal standard, flunitrazepam, the difference in retention time being only 0.5 min.

The described clonazepam assay has the distinct advantages of clean chromatograpy, adequate sensitivity, and routine monitoring application, thereby being a useful addition to the comprehensive monitoring of antiepileptic drugs used in clinical practice.

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