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Short communication

Optimised determination of clobazam in human plasma with extraction and high-performance liquid chromatography analysis

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Abstract

The analysis of clobazam by high-performance liquid chromatography and UV detection is described herein. After adding an internal standard, 600 μl of plasma were extracted under basic conditions onto disposable cartridges packed with celite. The organic extract was then evaporated to dryness and the residue reconstituted in 200 μl of mobile phase. A 20 μl aliquot was injected into chromatograph. The HPLC system was equipped with an Ultrasphere C8 analytical column coupled with an UV detector set at 235 nm. The mobile phase was an acetate buffer 20 m*M*, pH 5.5, containing acetonitrile and triethylamine 70:30:0.01 (v/v); the flow-rate was 1.8 ml/min. Using this method, clobazam can be detected with a sensitivity limit of 6 ng/ml and the RSD% intra- and inter-assay were lower than 5%. For its ruggedness and reliability, the proposed method is particularly suitable for therapeutic drug monitoring in epilepsy. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Clobazam

1. Introduction

Clobazam is an anticonvulsant drug that has already been used for some time in epilepsy treatment. It is particularly useful when used together with other antiepileptic drugs, since it helps to prevent dependency. In other applications clobazam is used as a covering drug when there is a change in therapy. The drug's action is very quick (usually effective within a couple of hours but no longer than a few days) and the monitoring of the drug's haematic levels is of great clinical interest in order to determine its correct use since, up until this date, the exact therapeutic ranges have not been decided.

Several chromatographic analysis methods for the analysis of the drug in human plasma have been proposed, amongst which GLC [1] and HPLC [2–8]. Almost all of the methods were based on the preliminary extraction of clobazam from the sample using organic solvents.

The liquid–liquid extraction and purification techniques with organic solvents are tedious, sometimes hard to carry out and dangerous for the technician. In this study the conditions for purifying clobazam through the solid–liquid technique in disposable cartridges packed with celite were examined and optimised. These allow for the drug's extraction from the watery matrix in a simple and repeatable

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way thus simplifying an otherwise complicated phase separation step.

The extracts were then analysed using HPLC with ultraviolet detection (HPLC–UV). Both of the mobile and stationary phases and the detection conditions were studied with the aim of perfecting the accuracy and the analytical sensitivity. The optimised method, for its praticability, proved a useful instrument in therapeutic monitoring of clobazam.

2. Experimental

2.1. Reagents

All reagents were obtained from Farmitalia (Milan, Italy). Acetic acid, dicloroethane, sodium hydroxide, triethylamine and tris buffer were of analytical grade; acetonitrile, isopropyl alcohol and methanol were of HPLC grade. Clobazam (7-Chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4-[3H,5H] dione) and methoxycarbamazepine (5H-Dibenz[b,f]azepine-5-methoxycarbamide) were provided free of charge by Hoechst Pharmaceuticals (Hounslow, Middlesex, UK) and Ciba Laboratories (Horsham, West Sussex, UK), respectively. The columns Chem Elut CE packed with 1 g of celite, were supplied from Varian (Harbor City, CA).

2.2. Chromatographic instrumentation

The chromatographic separation was performed with a Beckman HPLC apparatus (Beckman Instruments, Berkeley, CA). A 110B solvent delivery pump and an analytical column Ultrasphere Octyl (4.6×250 mm, 5 μm spherical 80 Å pore) were coupled with a System Gold 166 UV detector. Samples were introduced with a model 7725 injector (Rheodyne, Cotati, CA) with 20 μl loop. The recording was performed with a Omniscribe D5000 analogical recorder (Houston Instruments, Gistel, Belgium).

2.3. Operating conditions

The mobile phase was obtained by mixing acetate buffer 20 m*M*, pH 5.5, acetonitrile and triethylamine 70:30:0.01 (v/v); the flow-rate was 1.8 ml/min. The solvent was degassed before use by filtering through a 0.45 μm nylon filter (Lida, Kenosha, WI) under vacuum. The column effluent was monitored at 235 nm and sensitivity of 0.005 AUFS (output 10 mV). The recorder was set at 0.5 cm/min chart speed and 10 mV input: the results were calculated by determination of the peak height ratios by dividing the clobazam value by that of the internal standard.

2.4. Preparation of standards

Starting from stock solutions of clobazam 1 mg/ml in methanol, standards were prepared using a pool of drug-free human sera as diluent. The calibration curve was performed with standards at concentrations of 0, 25, 50, 100, 200, 300, 400 and 500 ng/ml. Aliquots of the standards were stored at −20°C until needed.

Solutions of methoxycarbamazepine 2500 ng/ml in methanol were prepared by dilution from a stock solution at 1 mg/ml, were stored in the same way and used as internal standard (IS). All the stored diluted standards are stable at least for 1 month.

2.5. Extraction procedure

600 μl of calibration standard, quality control or sample were dispensed into 10 ml conical glass tubes. Then, 500 μl of tris-buffer 0.8 *M*, pH 10.9, and 100 μl of IS working solution were added and the tubes were vortex mixed. One ml of the mixtures was loaded onto a Chem Elut CE cartridge and left to absorb for 3–5 min. Finally, 3 ml of the extraction mixture dichloroethane:isopropyl alcohol 97.5:2.5 (v/v) were added and the eluate collected into a 10 ml conical glass tube. The extraction operation was repeated twice. The extracts were dried under nitrogen, redissolved in 200 μl of mobile phase and injected into the chromatograph (20 μl).

3. Results and discussion

The good purification and separation conditions allow for an accurate determination of clobazam without any interference from drugs that are frequently used together with this drug in antiepileptic

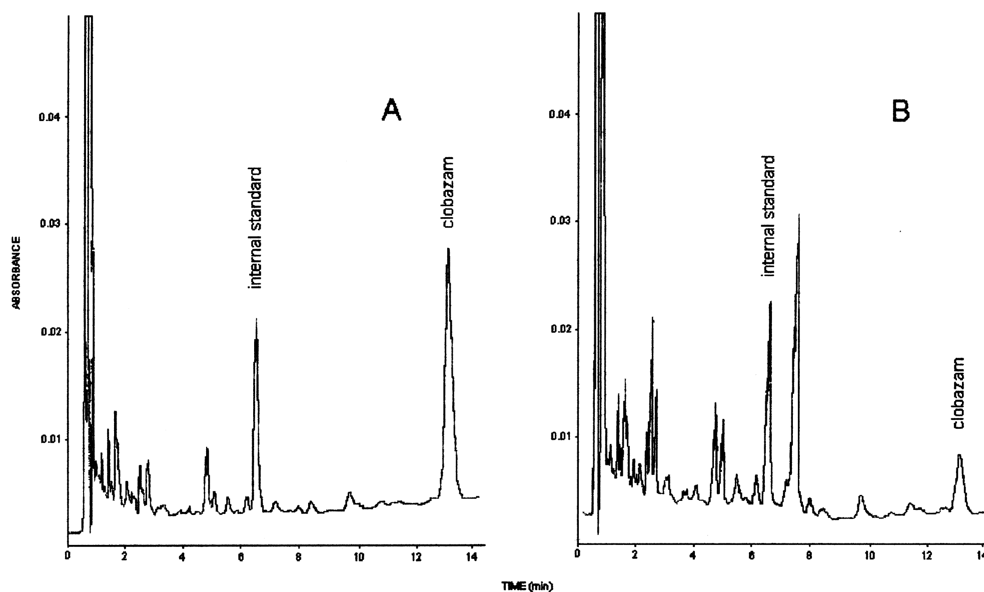

Fig. 1. Representative chromatograms of a drug-free plasma added with 300 ng/ml of clobazam (A) and of a plasma coming from a patient in therapy (B).

treatment (Fig. 1). Extracts from plasma containing carbamazepine, ethosuccimide, phenobarbital, phenitoin, primidone, lamotrigine and vigabatrin at therapeutic concentrations did not demonstrate significant interference.

For the recovery test, plasma samples added with clobazam 50, 100, 200 and 400 μg/l were extracted with the above mentioned procedure and then analysed through HPLC–UV. A comparison with similar solutions prepared in mobile phase showed an average recovery of 75.0±2.1% and 74.3±1.8% (mean±SD) for clobazam and for IS respectively.

The method's linearity was studied by analysing in triplicate the calibration curve (range from 0 to 500 ng/ml) and determining the peak height ratios by dividing the value of clobazam peak height by that of the internal standard. A graph was then constructed of peak height ratios versus concentration. This was linear (y=0.0032, x=0.0084) with very good correlation coefficients of r=0.9995. (Fig. 2)

The intra- and inter-assay precision were calculated by replicate analysis of control sera (n=6) with a known clobazam concentration. The results obtained showed a RSD% less than 4% for the intra-assay precision (Table 1) and less than 5% for that of inter-assay (Table 2). The accuracy calculated during these tests were within the range of ±5% compared to the expected value.

The method's sensitivity as signal-to-noise ratio was equal to 6 ng/ml (S/N=2).

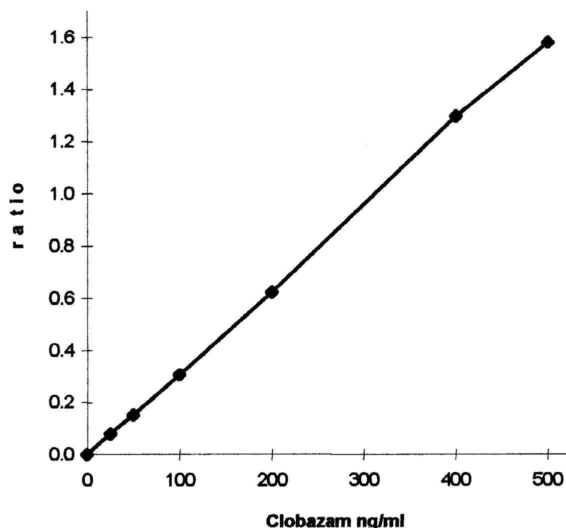

Fig. 2. Linearity of the clobazam HPLC–UV determination.

Table 1
Within-run precision for clobazam analysis at two different concentration levels in six replicates

Replicates	Control low (80 ng/ml)	Control high (240 ng/ml)
1	82	242
2	81	216
3	77	230
4	81	233
5	82	243
6	81	236
mean	80.7	233.3
SD	1.86	9.90
RSD%	2.3	4.2
accuracy%	+ 0.9	−2.8

Table 2
Between-run precision for clobazam analysis at two different concentration levels in six different days

Replicates	Control low (80 ng/ml)	Control high (240 ng/ml)
1	80	233
2	81	210
3	77	226
4	73	226
5	80	243
6	75	229
mean	77.7	227.8
SD	3.20	10.80
RSD%	4.1	4.74
accuracy%	−2.9	−5.1

4. Conclusion

The described method demonstrates a satisfying analytical performance comparable to other methods previously proposed and showed itself to be particularly useful for its practicability. The simple solid-phase extraction together with the method's reliability mean that it is possible to analyse clobazam even in numerous sample series. Consequently, the assay has been routinely successfully used in our laboratory to measure clobazam in plasma from epileptic patients; during the last 3 years, no major problems have occurred with this analysis.

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