Quantitation of Clobazam in Human Plasma Using High-Performance Liquid Chromatography

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Abstract

A rapid and simple reversed-phase high-performance liquid chromatographic (HPLC) method for the determination of clobazam concentrations in human blood samples is developed and validated. Solid-phase column extraction is performed to clean up blood samples before running the analytical HPLC system. The chromatography is isocratic with a mobile phase consisting of acetonitrile (20%, v/v), methanol (23%, v/v), and 0.1M potassium hydrogen phosphate buffer (pH 3.6; 57%, v/v) at a constant flow rate of 2 mL/min. Clobazam is detected at 226 nm. Chromatography is completed within less than 25 min. The recovery rate is greater than 95% and linear over a wide range of drug concentrations. The intra-assay coefficient of variation percentage varies between 4.3 and 12. This method is used for therapeutic drug monitoring in patients undergoing antiepileptic therapy with clobazam. Plasma levels of clobazam ranged from 21 to 663 ng/mL. Other antiepileptic compounds, such as clonazepam and phenobarbital, did not interfere with the detection of clobazam.

Introduction

Benzodiazepines exert clinically beneficial effects against insomnia, anxiety, elevated myotonus, and convulsions. The benzodiazepines diazepam, clonazepam, and clobazam (Figure 1) are currently used as antiepileptics. In contrast to other anticonvulsive drugs such as phenobarbital, benzodiazepines are relatively nontoxic and have modest untoward effects. The archetypal diazepam is the drug of choice for the acute treatment of grand mal-type seizures. It is also effective for short term prophylactic therapy but loses efficacy rather quickly. Hence, alternatives for prophylactic therapy of seizures have been sought. This quest led to the introduction of clobazam (a 1,5-benzodiazepine) and clonazepam (a 1,4-benzodiazepine) into the practice of medicine. Clobazam is an effective antiepileptic drug with most varieties of seizures and epilepsies for both short-term and long-term treatment (1). Clonazepam is commonly used for the treatment of various seizure types, including myoclonic and absence seizures, in both adults and children (2). The major metabolic pathway of clobazam in humans involves removal of the *N*-1 methyl group, resulting in the formation of the main pharmacologically active metabolite norclobazam (3) (Figure 1). Comedications and confounding diseases may necessitate therapeutic drug monitoring of benzodiazepines.

There is interaction between benzodiazepines and a number of compounds. Other antiepileptic drugs such as carbamazepine, phenobarbital, and phenytoin are all inducers of hepatic drug-metabolizing enzymes, thus enhancing the metabolism of benzodiazepines and lowering plasma levels. In contrast, the binding of cimetidine to cytochrome P-450 isoenzymes can inhibit the hepatic metabolism of benzodiazepines such as diazepam (4) or clobazam (5), increasing the plasma levels of benzodiazepines. In addition, a genetically determined lower activity of the metabolizing enzymes (mephenytoin polymorphism) or compounds whose metabolism cosegregates with that of mephenytoin (omeprazole) (6) can increase the plasma level of benzodiazepines, such as diazepam (7). Finally, liver disease was accompanied by reduced peak plasma levels of clobazam (8).

For therapeutic drug monitoring in a university teaching hospital, a simple and rapid reversed-phase high-performance liquid chromatographic (HPLC) method for the analysis of clobazam or clonazepam concentrations in patient sera was developed. Solid-phase column extraction (SPE) was used to clean up the blood samples prior to the running of the analytic HPLC system. An internal standard, nordiazepam (desmethyldiazepam; Figure 1), was employed to control the extraction procedure.

Table I. Recovery of Clobazam and Clonazepam from Human Serum

	Recovery of clobazam		Recovery of clonazepam	
Spiking level (ng/mL)	Mean ± SD (%)	CV (%)	Mean ± SD (%)	CV (%)
25	95.80 ± 8.44 (*n* = 5)	8.81	105.00 ± 2.65 (*n* = 3)	2.52
50	105.50 ± 7.82 (*n* = 6)	7.41	91.25 ± 7.09 (*n* = 4)	7.77
75	101.00 ± 4.18 (*n* = 5)	4.14	97.40 ± 8.14 (*n* = 5)	8.36
100	102.20 ± 6.53 (*n* = 5)	6.39	101.75 ± 9.91 (*n* = 4)	9.74

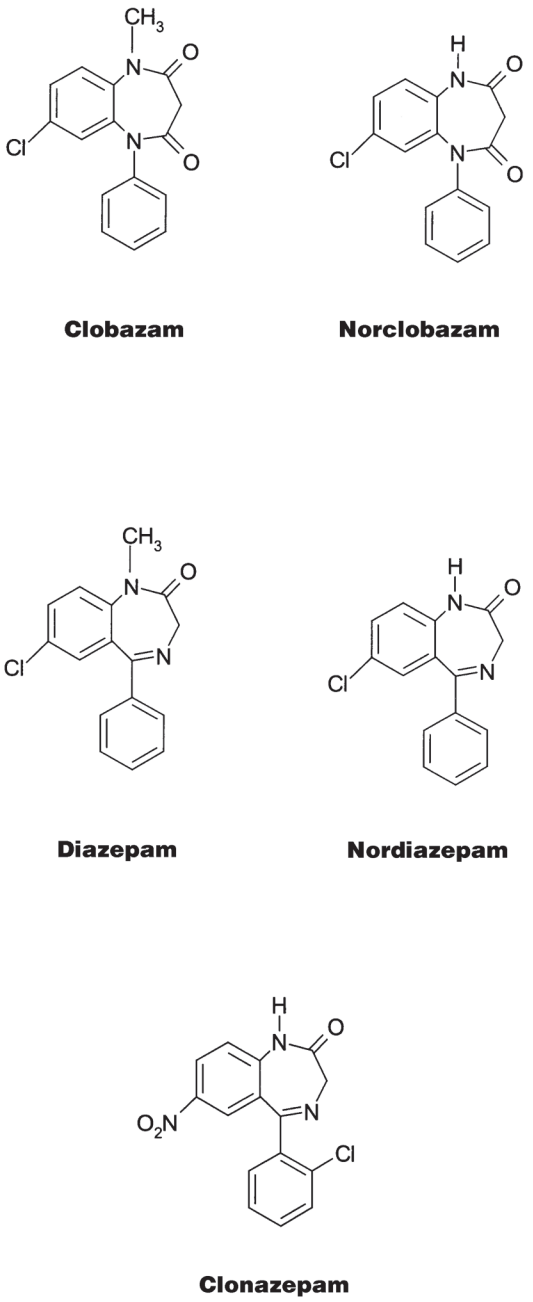

Figure 1. Structural formulas of clobazam, norclobazam, diazepam, nordiazepam, and clonazepam.

Experimental

Chemicals and reagents

Clobazam, norclobazam, diazepam, nordiazepam, and clonazepam were purchased from Sigma (Deisenhofen, Germany). HPLC-grade acetonitrile and methanol were supplied by Baker (Mallinckrodt-Baker, Deventer, Netherlands). All other chemicals were analytical reagent grade.

Apparatus

The HPLC system consisted of an isocratic pump (liquid chromatograph 655 A-11, Merck, Darmstadt, Germany), an inline detector (variable wavelength monitor 2141, Pharmacia, Freiburg, Germany), and an electronic integrator (Chromato-Integrator D 2500 A, Merck).

Chromatographic conditions

Chromatographic separation was achieved on a LiChrosorb C_{18} column (250 mm × 4-mm i.d., 10 µm, Merck) with a LiChrosorb C_{18} guard column (4 mm × 4-mm i.d., 5 µm, Merck) at ambient temperature. The mobile phase consisted of 200 mL methanol (23%, v/v), 180 mL acetonitrile (20%, v/v), and 500 mL 0.1M KH_2PO_4-buffer (pH 3.6, 57%, v/v). The mobile phase was pumped at a constant flow rate of 2 mL/min. The ultraviolet (UV) wavelength was set to 216 nm for the detection of clonazepam and 226 nm for the detection of clobazam. Aliquots (100 µL) of the extracts were injected into the HPLC system.

Standards

Stock solutions of 100 µg/mL for clobazam, norclobazam, clonazepam, and nordiazepam were prepared in methanol and stored at –20°C. Dilutions (1:100) of stock solution were freshly prepared in water prior to use. Aliquots of dilutions were added to drug-free serum to obtain standard curves.

Samples

Serum samples were obtained from patients undergoing antiepileptic therapy with clobazam or clonazepam. Serum samples or drug-free serum (900 µL) were carefully mixed with 100 µL methanol–H_2O (1:100) before SPE.

SPE

Solid-phase cartridges (Lichrolut RP-18, 100 mg, Merck) were mounted onto a sample collector (Adsorbex SPU), and methanol (2 × 1 mL) and H_2O (2 × 1 mL) were passed through the column with the help of a vacuum. After disconnecting the vacuum, 100 µL Na_2CO_3 (0.1M), 100 µL nordiazepam (internal standard, 1:100), and 1000 µL serum sample were carefully applied to the column. After connecting the vacuum, columns were washed with H_2O (2 × 1 mL) and methanol (50 µL). The elution of benzodiazepines was brought about using 500 µL methanol. Finally, the eluate was collected into a conical glass tube and evaporated to dryness under a gentle stream of nitrogen (40°C). The evaporate was reconstituted in 150 µL of mobile phase. An aliquot (100 µL) was injected into the HPLC column.

Table II. Precision and Accuracy for the Determination of Clobazam in Human Serum

		Clobazam (intra-assay)	
Amount of clobazam added (ng/mL)	Clobazam measured (mean ± SD)	Precision (CV [%])	Accuracy (%)
25	25.23 ± 3.05 (*n* = 3)	12.09	100.93 ± 12.20 (*n* = 3)
50	49.53 ± 2.10 (*n* = 3)	4.25	99.07 ± 4.21 (*n* = 3)
75	73.80 ± 5.27 (*n* = 3)	7.14	98.40 ± 7.03 (*n* = 3)
100	105.60 ± 4.97 (*n* = 3)	4.70	105.60 ± 4.97 (*n* = 3)

Recovery

Analytical recovery ranges were determined by comparing the peak heights of clobazam, clonazepam, and the internal standard nordiazepam. Drug-free pooled human serum (obtained by the blood transfusion center of the university hospital) was spiked with known amounts of clobazam, clonazepam, and the nordiazepam. Samples were processed as described in SPE. The peak heights obtained were compared with those obtained by direct injection of standard solution into the HPLC column. This procedure was repeated at different drug concentrations (25, 50, 75, and 100 ng/mL) for both clobazam and clonazepam (Table I).

Quantitation

Clobazam and clonazepam were quantitated by relating the peak height of nordiazepam and either clobazam or clonazepam in the unknown sample to the peak height of a known standard concentration.

Data analysis and statistics

Data are given as mean plus or minus standard deviation (SD). Precision was calculated as percent coefficient of variation (CV). Accuracy was expressed as the percent of clobazam measured in each sample relative to the known amount added to drug-free human serum.

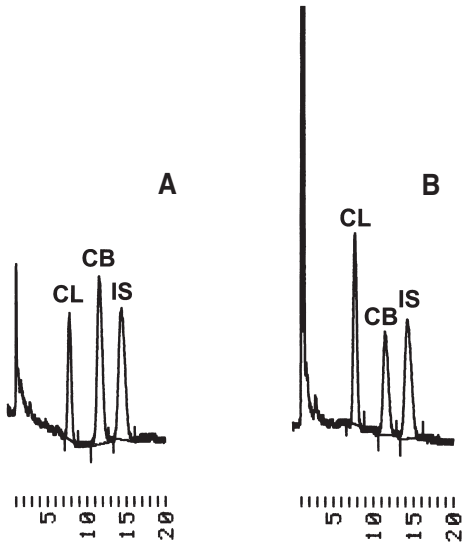

Figure 2. Representative chromatograms obtained by direct injection of 66 ng clobazam, 66 ng clonazepam, and 66 ng nordiazepam (internal standard) at 226 nm (A) and 216 nm (B). Peaks: clobazam (CB), clonazepam (CL), and nordiazepam (IS).

Results and Discussion

First, the recovery ranges of the benzodiazepines of interest were studied. These recoveries at 4 different concentrations of clobazam and clonazepam (Table I) amounted to 101 ± 7% (n = 21) and 98 ± 8% (n = 15), respectively. The recovery range for the internal standard nordiazepam amounted to 97 ± 2% (n = 6). Hence, the recoveries of the benzodiazepines and the internal standard were nearly complete. The precision of the clobazam assay was assessed by replicate analysis of drug-free pooled human serum containing various concentrations of clobazam (25–100 ng/mL) and the internal standard nordiazepam. Intra-assay variation was determined by analyzing 4 clobazam concentrations. The intra-assay coefficients of variation are depicted in Table II. The precision of the measurement, expressed as the coefficients of variation, ranged between 4 and 12%. The accuracy, expressed as the percent of concentration of clobazam measured in each sample relative to the amount of clobazam added, ranged from 98 to 106%. The interassay coefficient of variation for clobazam amounted to 8.6% (50 ng/mL; n = 7) with an accuracy of 103%. To summarize, these findings indicate that the method should offer reliable data.

Initial experiments revealed absorptivity maxima of 226 nm for clobazam and 216 nm for clonazepam. Nevertheless, both compounds can be detected at 226 nm and 216 nm. The maximum peak height of clobazam at 226 nm is approximately 100% greater than at 216 nm, whereas the peak height of norclobazam is not affected (data not shown). Clonazepam is monitored here at 216 nm, because under this condition, the maximum peak height is approximately 100% greater than at 226 nm (data not shown). This will obviously enhance the sensitivity of the assay

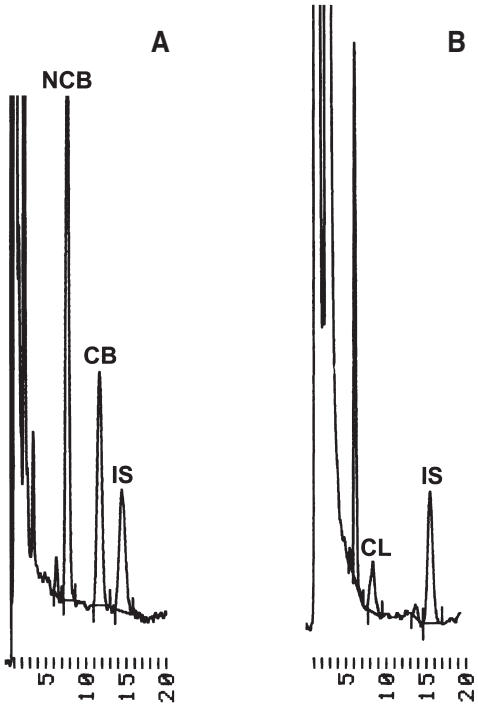

Figure 3. Representative chromatograms of blood samples obtained from two different patients undergoing antiepileptic therapy with clobazam (A) or clonazepam (B). Plasma concentrations for clobazam and clonazepam were measured at 226 nm (A) or 216 nm (B), respectively, and amounted to 80 ng/mL (clobazam) and 30 ng/mL (clonazepam). Peaks: clobazam (CB), norclobazam (NCB), clonazepam (CL), and nordiazepam (IS).

for clonazepam. Thus, the determination of clobazam and clonazepam is performed at these different wavelengths based on the information provided by the attending physician; otherwise, the experimental conditions are identical.

Figure 2 depicts typical chromatograms of direct injections of a mixture composed of clobazam, clonazepam, and nordiazepam at 226 nm (Figure 2A) and 216 nm (Figure 2B). These chromatograms indicate that the peak height of the internal standard is only slightly different at the two wavelengths used. Hence, nordiazepam is an appropriate internal standard for quantitation of clobazam and clonazepam.

The method described here offers a simple and rapid quantitation of clobazam and clonazepam in human blood samples. The chromatography is completed within 25 min, and the time from SPE to the final result lasts less than 1 h under optimal conditions. Thus, the method is useful for day-to-day alterations in drug therapy and also in cases of acute toxicity. Another advantage of the present work in comparison with that of others resides in its low requirements for instrumentation. The necessary equipment (isocratic pump, UV detector, and chart recorder) are available in most clinical laboratories. In contrast, previous works used radioimmunoassays (9), micellar electrokinetic capillary chromatography (10), capillary gas chromatography (11), gas chromatography (12), gas liquid chromatography (8), HPLC followed by DAD (13), or HPLC followed by an enzymatic assay (14).

An HPLC method (single wavelength detection) and SPE were used here. This procedure is fast, reduces the exposure of the technician to hazardous material, and leads to cleaner extracts, thereby protecting the columns and saving money and time. For instance, SPE methods can process 10 samples in approximately 45 min.

The detection limit (based on a signal-to-noise ratio of 3:1) is approximately 10 ng/mL for both clonazepam and clobazam. This is lower than the clinically relevant plasma concentrations of clonazepam, which are approximately 50 ng/mL (2), and probably also clobazam. However, the antiepileptic concentrations for clobazam have not been carefully studied and defined. Concentrations from 100 to 400 ng/mL have been reported (15), but an original report on how this was evaluated is not available in the published literature. A recent study on pharmacokinetics of a single oral dose of 20 mg clobazam revealed peak plasma concentrations of 350 ± 63 ng/mL in healthy volunteers, which were significantly higher in comparison with patients with viral hepatitis (239 ± 70 ng/mL) or cirrhosis (240 ± 113 ng/mL) (8). To summarize, it is obviously possible to measure therapeutically relevant steady-state concentrations of clobazam and clonazepam in patients.

This procedure was established upon the request of our colleagues in the department of neurology who were interested in assessing the compliance of patients in their outpatient clinic and determining the plasma concentrations of patients in the intensive care ward, where acute seizures in polytoxicated patients had to be treated. In some of these patients, co-medication with enzyme inducers such as phenobarbital raised the possibility of subtherapeutic levels of clobazam, despite adequate dosing. Using the method delineated previously, clobazam and clonazepam plasma concentrations in samples from 156 (clobazam) and 40 (clonazepam) patients were determined. Plasma levels could routinely be conveyed to the ward on the very same day. Figure 3 depicts two representative chromatograms of blood samples obtained from two different patients undergoing antiepileptic therapy with clobazam (Figure 3A) and clonazepam (Figure 3B). The values for clobazam and clonazepam plasma concentrations amounted to 80 ng/mL and 30 ng/mL, respectively. Values obtained for clobazam ranged from 21 to 633 ng/mL (mean ± SD = 145 ± 275 ng/ml) and from 23 to 268 ng/mL (mean ± SD = 50 ± 28) for clonazepam with mean daily doses of 14 and 2 mg, respectively. This indicates that the procedure described here is adequate for all routine requirements in a typical clinical setting.

It can be argued that if a patient is treated with both clobazam and clonazepam, the determination of clonazepam at 216 nm would be obscured because clonazepam exhibits the same retention time as norclobazam, which is the major active metabolite of clobazam. Nevertheless, under this condition, one would detect clobazam at approxi-

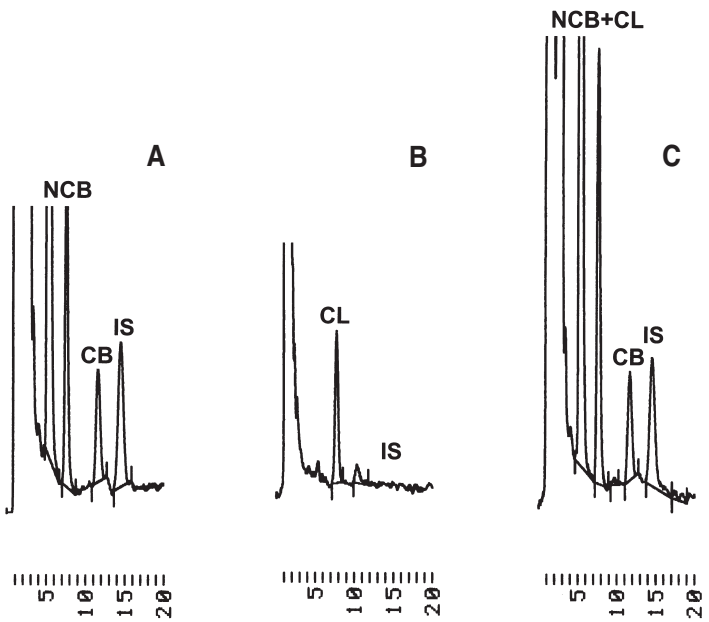

Figure 4. Representative chromatogram of a sample from a patient undergoing antiepileptic therapy with clobazam (A). This sample was spiked with 80 ng clonazepam before SPE to simulate a dual treatment of a patient with clobazam and clonazepam. For comparison, a representative chromatogram of drug-free pooled human serum spiked with 80 ng clonazepam is depicted (B). Additionally applied clonazepam increases the peak height of norclobazam, erroneously indicating a high concentration of the pharmacologically active metabolite of clobazam (i.e., norclobazam) (C). All depicted chromatograms were obtained at 226 nm. Peaks: clobazam (CB), norclobazam (NCB), clonazepam (CL), and nordiazepam (IS).

mately 12 min (data not shown), revealing the dual treatment. On the other hand, for instance, in an acute intoxication of clobazam, it might be helpful to determine the amount of the pharmacologically active metabolite norclobazam. Here, co-medication with clonazepam is a potential problem, because clonazepam has a noticeable absorptivity at 226 nm. Figure 4 illustrates a chromatogram of a blood sample measured at 226 nm that contains both clobazam and the metabolite norclobazam. This sample was spiked with clonazepam (Figure 4B). It is obvious that additional applied clonazepam erroneously mimics elevated norclobazam concentrations (Figure 4C). Nevertheless, the determination of the parent compound clobazam that is usually used for therapeutic drug monitoring and control of compliance is not affected by clonazepam. Thus, clonazepam might obfuscate norclobazam but not clobazam detection.

Finally, the internal standard nordiazepam is an active metabolite of the typical 1,4-benzodiazepine, namely diazepam. Hence, co-medication of diazepam would interfere with this determination of clobazam plasma levels. Specifically, someone would unknowingly underestimate the concentration of clobazam in these samples when correcting to the recovery of the internal standard nordiazepam. However, a sudden large increase in nordiazepam peaks (more than 100% recovery) would certainly arouse this suspicion. In this case, the monitoring time should be extended to at least 25 min, because a peak corresponding to the precursor (namely diazepam) would become prominent at approximately 22 min (data not shown).

These mentioned limitations arise from methodical concerns but not from clinical experience. To the best of our knowledge, none of these patients has intentionally been treated with clobazam and clonazepam or diazepam. Moreover, a dual treatment with benzodiazepines such as clobazam and clonazepam is very uncommon and rarely (if ever) indicated, because both share the same mechanism of action (activation of GABAa receptors and subsequent hyperpolarization of postsynaptic neurons). Typically, antiepileptic therapy is empirical. Only one drug after another will be tried until significant reductions in the severity and incidence of seizures are achieved. Usually, only one drug of a class of compounds sharing the same mechanism of action is tried. Thereafter, a drug from a class with an unrelated pharmacodynamic action is used. If monotherapy is unsuccessful, a drug from another group of compounds will be added to the regimen. Hence, combinations such as phenobarbital and clobazam are sometimes indicated. Fortunately, no chromatographic interferences were found by other antiepileptic drugs or commonly used benzodiazepines such as phenobarbital, flunitrazepam, and midazolam at the retention times for clobazam or clonazepam. Nevertheless, one should bear this possible analytical pitfall in mind.

Conclusion

In summary, a novel HPLC method that offers a rapid and easy technique for quantitating clobazam and clonazepam in human blood samples is described. This method was successfully used for therapeutic drug monitoring and is amenable to modification in order to quantitate other antiepileptic drugs.

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