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Short communication

Development of a rapid RP–HPLC method for the determination of clonazepam in human plasma

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Abstract

A rapid and sensitive high-performance liquid chromatography method with UV detection was developed for the determination of clonazepam in human plasma using 3-methylclonazepam, as internal standard. A one-step extraction of both compounds was performed with a mixture of hexane/ethyl acetate (90:10, v/v). The HPLC analysis was carried out on a Nova Pak® C_{18} reversed-phase column with a mobile phase of acetonitrile–0.01 M sodium acetate adjusted to pH 7 with dilute acetic acid (40:60, v/v). A linear response was observed over the concentration range 5–100 ng/mL. Intra- and inter-day assay precision and accuracy fulfilled the international requirements. The lower limit of quantification was 5 ng/mL without interference of endogenous components.

For analytical purpose, the stability of clonazepam in bidistilled water and plasma has been studied. A rapid degradation was noticed when clonazepam was stored in bidistilled water at the daylight following a first-order kinetic rate with a 87 min half life whereas no significant degradation was observed in plasma.

This method was applied to measure plasma concentrations of clonazepam either in patients receiving therapeutic doses or in poisoning cases.

Keywords: Clonazepam; Gas chromatography; Liquid chromatography

1. Introduction

Clonazepam, 5-(2-chlorphenyl)-1,3-dihydro-7-nitro-2H-1,4-benzodiazepin-2-one is an anticonvulsant agent widely used in the treatment of epilepsy in adults and children [1–3]. As the drug presents a narrow therapeutic range from 10 to 50 ng/mL [4], the monitoring of clonazepam plasma concentrations in epileptic patients is recommended [5]. Moreover, some intoxications involving clonazepam have been described [6–8]. Hence, it is necessary to develop a specific and rapid method for the determination of clonazepam in biological fluids. Several methods were already published, involving gas chromatography with or without derivatization [9,10] and liquid chromatography (LC) [11–18]. Most of the LC methods suffer from either extensive sample preparation involving two extractions [11], long-time analysis [12,13] or expensive equipment [14–16] and therefore, they are not suitable for routine work. Moreover, as described in some LC techniques, the use of another psychotropic drug as internal standard [11,17,18] could be detrimental in case of polymedication.

The aim of this study was to develop a rapid and selective RP–HPLC method for the measurement of clonazepam in human plasma. The quantification of plasma levels was performed using a single liquid–liquid extraction procedure in the presence of 3-methylclonazepam as internal standard (I.S.). Under the described chromatographic conditions, clon-

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azepam and I.S. were well separated and resolved from endogenous plasma compounds and from co-prescribed anticonvulsivant drugs.

Observations concerning a degradation of clonazepam in different aqueous media have already been published [19,20]. All these results prompted us to study the clonazepam stability in bidistilled water and plasma.

Finally, the described method was applied for therapeutic monitoring of clonazepam in routine hospital and in case of poisoning.

2. Experimental

2.1. Chemicals

Clonazepam and 3-methylclonazepam (Fig. 1) as internal standard were gifts from Roche (Neuilly/Seine, France). Acetonitrile, hexane and ethyl acetate were HPLC grade and were obtained from VWR (Strasbourg, France). Sodium acetate and acetic acid were obtained from Aldrich (Saint Quentin Fallavier, France) and VWR (Strasbourg, France), respectively. Water was deionized and glass-distilled prior to use. Drug-free human plasma was obtained from Aquitaine Establishment of Blood Transfusion (Bordeaux, France).

2.2. HPLC apparatus

The chromatographic apparatus Waters (Milford, USA) was equipped with a constant flow pump Model 501, a Model 2487 ultraviolet detector and a 746 Data Module Integrator. The chromatographic separation was performed with a Nova Pak® C_{18} analytical column (3.9 mm × 150 mm; 4 μm particle size). The mobile phase, acetonitrile–0.01 M sodium acetate buffer (pH = 7)–(40: 60, v/v) was run at a flow rate of 1 mL/min at +20 °C in an air-conditioned room. The buffer pH was adjusted with diluted acetic acid. The column effluent was monitored using ultraviolet detector at 320 nm. The mobile phase was filtered through a 0.5 μm filter and degassed prior to use.

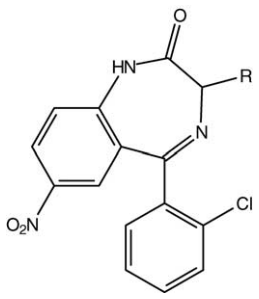

Fig. 1. Chemical structures of clonazepam (R=H) and 3-methyl clonazepam (R=CH_3; internal standard: I.S.).

2.3. Preparation of stock and standard solutions

Stock standard solutions of clonazepam and 3-methylclonazepam were prepared at concentrations of 1 mg/mL in methanol and stored at −20 °C until analysis. The internal standard stock solution was diluted daily in bidistilled water to yield a 1 μg/mL working solution stored in a brown flask.

From the stock solution of clonazepam, a working solution (100 ng/mL) was made-up in bidistilled water in a brown flask and the calibration standards were prepared freshly from this working solution. Calibration standards of clonazepam in drug-free human plasma were made by spiking with an appropriate volume of the working solution, giving final concentrations of 5, 10, 20, 50 and 100 ng/mL. In the same manner, plasma quality controls (QC) spiked with 12, 24 and 72 ng/mL were prepared to measure the accuracy and the precision of the method.

2.4. Sample preparation

One milliliter specimens were pipetted into polypropylene round-bottom tubes and spiked with 75 μL of 1 μg/mL internal standard solution. After adding 0.2 mL of NaOH 0.1 M and 8 mL of hexane–ethyl acetate (90:10, v/v), the sample was shacked for 30 min. The two phases were separated through centrifugation at 1400 × *g* for 20 min. The upper organic layer was transferred into a glass tube and completely evaporated under a steam of nitrogen. The dry residue was then reconstituted with 200 μL of mobile phase and centrifuged at 16,110 × *g*. 60 μL were injected onto the RP–HPLC column.

Least squares linear regression analysis (weighting factor = 1) was used to characterise the calibration curve.

2.5. Recovery

Extraction recoveries from human plasma were determined by comparison of HPLC responses from extracted samples, containing known amounts (5, 10, 20, 50 and 100 ng/mL) of clonazepam, to those from unextracted and directly injected standards, spiked with the same amounts.

2.6. Analytical method validation

The method was validated by the QC samples prepared at three concentrations spanning the concentration range. Each of the QC samples were replicated (n = 6) and calibration samples were analysed on three consecutive days. Precision and accuracy were determined. The precision was calculated as the coefficient of variation (CV) within a single run (intra-day) and between different assays (inter-day).

Accuracy was expressed as the percentage (%) of bias: [(found concentration − spiked concentration)/spiked concentration] × 100.

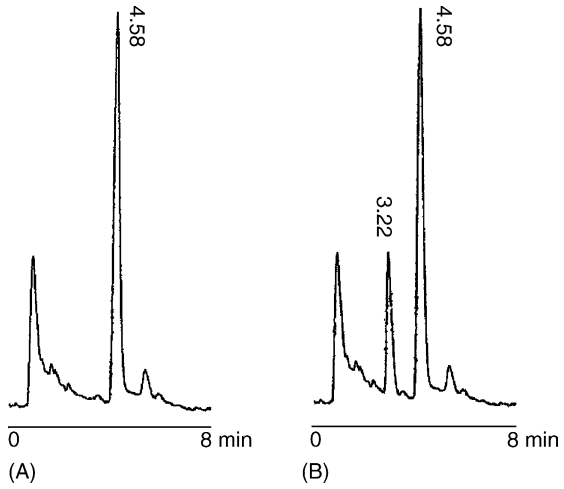

Fig. 2. Representative chromatograms of (A) blank plasma with internal standard (I.S.); (B) plasma spiked with 24 ng/mL of clonazepam. Clonazepam: 3.22 min; 3-methylclonazepam (I.S.): 4.58 min.

The limit of quantification (LOQ) was defined as the lowest clonazepam concentration that could be determined with a precision below 20% and with an accuracy between 80 and 120%, as determined in the inter-day analytical runs.

The limit of detection (LOD), defined as the lowest concentration of the analyte that can be clearly detected above the baseline signal, is estimated as three-times the signal-to-noise ratio.

3. Results and discussion

3.1. Chromatography

The objective of this work was the development of a rapid, specific and easy RP–HPLC assay with a total run time <8 min while maintaining suitable sensitivity and selectivity.

Under the described RP–HPLC procedure, clonazepam and 3-methylclonazepam (I.S.) were sufficiently resolved from endogenous plasma compounds and their retention times were 3.22 min for clonazepam and 4.58 min for I.S., respectively. Representative chromatograms of plasma samples are illustrated in Fig. 2.

3.2. Precision and accuracy

Tables 1 and 2 show a summary of intra- and inter-day accuracy and precision expressed as CV (%) and percent bias, respectively. These results indicate that the method is precise:

Table 1
Intra-day precision and accuracy (n = 6)

C_{added} (ng/mL)	12	24	72
Mean C_{found} ± S.D. (ng/mL)	12.8 ± 0.5	23.4 ± 1.4	72.8 ± 2.5
CV (%)	4.1	6.0	3.5
Bias (%)	6.6	−2.4	1.1

Table 2
Inter-day precision and accuracy (n = 18)

C_{added} (ng/mL)	12	24	72
Mean C_{found} ± S.D. (ng/mL)	12.7 ± 0.3	23.9 ± 0.5	72.1 ± 3.1
CV (%)	2.3	2.1	4.2
Bias (%)	6.3	−0.1	0.1

intra-day precision was less than 6.0% and inter-day precision was less than 4.2%. The method is accurate (bias ranged from −2.4 to 6.6%).

3.3. Linearity

The calibration curve for clonazepam was linear over the concentration range of 5–100 ng/mL in human plasma. The equation $y = ax + b$ was fitted to the data, where y represents the ratio of clonazepam area to internal standard area, x is the spiked clonazepam concentration, a is the slope of the regression, and b is the y intercept. The (mean ± S.D.) regression equations from six replicate calibration curves on different days for human plasma: $y = (0.0151 \pm 0.0006)x + (0.0109 \pm 0.0026)$ showed significant linearity ($r = 0.995 \pm 0.003$).

3.4. Sensitivity

The LOQ was determined (n = 6) by injection of spiked plasma with clonazepam. The LOQ was found to be 5 ng/mL for human plasma: the mean percent accuracy value was 91% with CV of 7%.

The LOD was determined around 2 ng/ml. The mean percent accuracy value was 88% with CV of 12.16%.

3.5. Specificity

Potential interferences were investigated by assaying different blank plasma spiked with drug. The ability to separate all the compounds from clonazepam and I.S. was demonstrated by assessing the relative retention time between the peak of clonazepam or I.S. and the peaks corresponding to various substances. The proposed method was considered adequate since the peaks of clonazepam and I.S. are well separated from the other anticonvulsivant drugs that are eluted before and after (Table 3).

3.6. Stability

Previous stability studies have reported a degradation of clonazepam in plasma or in phosphate buffer (pH = 7.4) [19,20]. So, before the validation of our LC assay, we studied the potential degradation of clonazepam in bidistilled water and plasma in terms of precision and accuracy.

First the working solutions (100 ng/mL) prepared in bidistilled water (pH = 6.5) were placed in glass tubes at daylight at +20 °C. During four hours, at suitable time interval, the tubes were taken away and stored in a dark place. The in-

Table 3
Compounds studied for possible interferences

Compound	tr_1	tr_2
10-Hydroxycarbamazepine	0.25	0.18
Carbamazepine	0.73	0.53
Carbamazepine 10,11-epoxide	0.74	0.53
Clobazam	1.41	1.01[a]
Desmethylclobazam	0.26	0.18
Diphenylhydantoïne	NR	NR
Ethosuccimide	NR	NR
Felbamate	0.74	0.53
Lamotrigine	0.47	0.33
Oxcarbazepine	0.57	0.74
Phenobarbital	NR	NR
Primidone	0.48	0.34
Stiripentol	3.68	2.64
Vigabatrin	NR	NR

tr_1: Relative retention time of clonazepam (3.22 min); tr_2: relative retention time of I.S. (4.58 min); NR: no response.
[a] Compound not extracted in our chromatographic conditions.

ternal standard prepared in bidistilled water was stocked in the dark and added just before the analysis. Three stability studies were achieved. The (mean ± S.D.) regression equations from three replicate calibration curves on the different days of experiment for bidistilled water were y = (0.0059 ± 0.0012)x + (0.0262 ± 0.0014) showing significant linearity (r = 0.9986 ± 0.0017). The mean percentage of intact clonazepam was calculated for each experiment. As depicted in Fig. 3a, a decrease of clonazepam versus time was observed in compliance with a first-order rate reaction model.

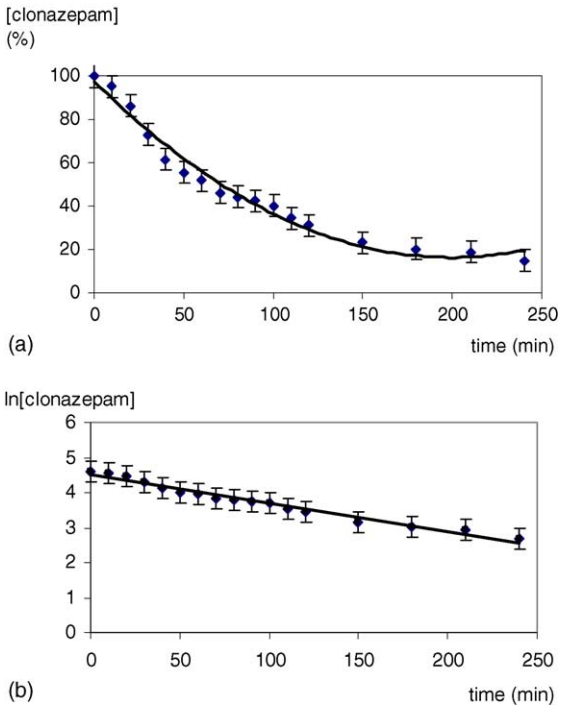

Fig. 3. Study of the clonazepam stability in bidistilled water (pH = 6.5) at + 20 °C at daylight: clonazepam degradation rate versus time in (a); degradation kinetic of clonazepam in (b).

The semilogarithmic plot: ln[Clonazepam] = $f(t)$ is straight line (Fig. 3b) and with a slope k (rate velocity): 0.0079 ± 0.0003 min^{-1}. The half-life ($t_{1/2}$) of the degradation was determined from the equation $t_{1/2}$ = ln2/k and arised 87 min showing a rapid degradation of clonazepam in the selected conditions.

In similar conditions, no significant degradation was noticed with bidistilled water solutions of clonazepam when stored in the dark at +20 °C. Methylclonazepam was found stable in similar conditions. Hence, the working solutions of clonazepam and (I.S.) were prepared in bidistilled water and stored in brown flasks.

In a second time, the influence of temperature and light on the stability of the clonazepam prepared in human plasma was studied. Two QC samples spiked with clonazepam (12 and 72 ng/mL, n = 4) were stored under different conditions; at −20 °C during a month; at +4 °C during 48 h; at +20 °C for 24 h in daylight for one group of samples, and in the dark for another group. No decomposition was noticed in the frozen samples after 1 month. Storage for 48 h at +4 °C produced no significant decrease of the drug concentration. Storage for 24 h at +20 °C in daylight or in the dark showed a good stability with CV and bias less than 12%. The stability behaviour of this highly protein-bound compound (around 83%) [21] could be related to stabilization effects attributed to plasma proteins [22].

In summary, this stability study confirms the previously reported results concerning the clonazepam degradation observed in bidistilled water. As we measured a short half-life of the degradation of the preparation, water clonazepam working solutions should be made carefully and stored in the dark.

3.7. Clinical cases

By using the described method, we measured plasma concentrations of clonazepam for therapeutic monitoring (Table 4: patient numbers 1–5) or after poisoning (Table 4: patients numbers 6–10). Fig. 4 shows the chromatogram of a patient sample following a therapeutic dosage (patient number 1).

Table 4
Concentrations of clonazepam for therapeutic monitoring (patient nos. 1–5) and after poisoning (patient nos. 6–10)

Patient no.	Clonazepam (ng/mL)
1	10
2	14
3	57
4	27
5	55
6	105
7	229*
8	405*
9	435*
10	418*

For quantification, appropriate dilutions of these plasma samples were made in drug-free human plasma.

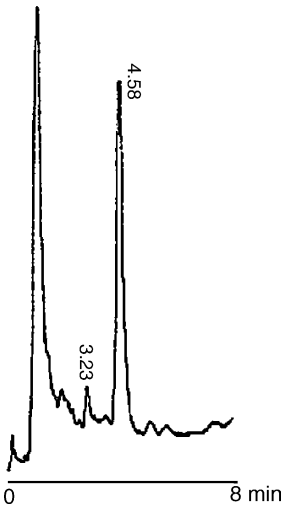

Fig. 4. Chromatogram obtained from a patient sample following a therapeutic management (10 ng/mL) (clonazepam tr = 3.23 min.; I.S. tr = 4.58 min.).

According to the literature, clonazepam is generally well tolerated at therapeutic dosages [4]. In overdoses, the clinical signs generally encountered are somnolence and lethargy in minor cases, ataxia, hypotony, hypotension, respiratory depression and coma in serious cases [4]. All these symptoms should have been increased when the patient had taken simultaneously either alcohol or antidepressive drugs. Fig. 5 shows the chromatogram of patient number 6 with a concentration two-fold above therapeutic concentration: no medical symptoms of intoxication were noted. Conversely, important breathing troubles were noticed by the physicians for the patient numbers 7–10 with high toxic doses.

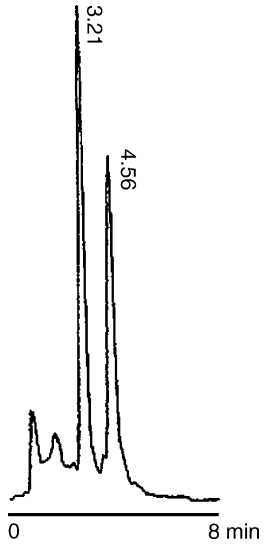

Fig. 5. Chromatogram obtained from a patient sample following an overdose of clonazepam (105 ng/mL) (clonazepam tr =3.21 min.; I.S. tr = 4.56 min.).

4. Conclusion

A reliable, fast chromatographic isocratic RP–HPLC method for the determination of clonazepam in human plasma has been developed and validated. It involves a simple liquid–liquid extraction with UV detection and provides a good separation of clonazepam and 3-methylclonazepam (I.S.). Moreover, the process allows the detection of clonazepam with high selectivity. For analytical purpose, the stability of clonazepam has been studied in bidistilled water at daylight. The degradation of clonazepam is rapid with a 87 min half-life. This method is extensively used for clonazepam therapeutic drug monitoring and is also useful for intoxication.

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