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Determination of lormetazepam and its main metabolite in serum using micellar electrokinetic capillary chromatography with direct injection and ultraviolet absorbance detection

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Abstract

The use of micellar electrokinetic chromatography for the determination of lormetazepam and its metabolite, lorazepam, in serum samples at a concentration range of therapeutic interest was investigated. The separation was carried out at 30 °C and 25 kV, using a 15 m*M* borate–phosphate buffer (pH 8) with 30 m*M* sodium dodecyl sulfate as the separation electrolyte and 15% methanol as organic modifier. The analyses were carried out in 20 min under these conditions. Detection limits of 0.5 mg l^{-1} were achieved for both benzodiazepines in serum. This method was employed for the quantitative resolution of both drugs (at different concentration ratios) in serum with very good recoveries.

Keywords: Lormetazepam; Lorazepam

1. Introduction

Lormetazepam and its metabolite, lorazepam, are benzodiazepines employed in psychiatric disturbances associated with stress situations. They have no significant, dangerous side effects at low doses, but the use of these drugs and related compounds may produce dependence. Sudden withdrawal of treatment after prolonged use can trigger status epilepticus, a life-threatening condition, and their use must be controlled since drug abuse may occur.

The structures of the two compounds are shown in Fig. 1.

The use of micellar electrokinetic chromatography (MEKC) in clinical and forensic analysis has the important advantages of versatility, low cost, efficiency, specificity and the rapid setting up of equipment. Furthermore, the presence of sodium dodecyl sulfate (SDS) in the background electrolyte is particularly attractive as it allows the direct injection of serum into the capillary. Proteins are soluble in SDS and migrate with the micelles, competing with the drugs for protein binding sites. Thormann et al. [1] used direct injection of plasma and serum in MEKC for the determination of different drugs. Further studies [2–13] have been published on the use of MEKC for the determination

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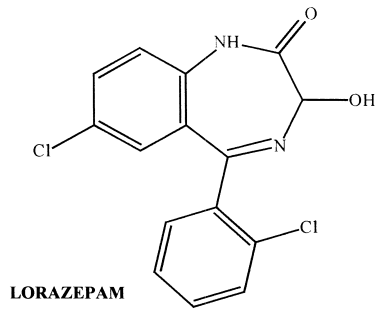
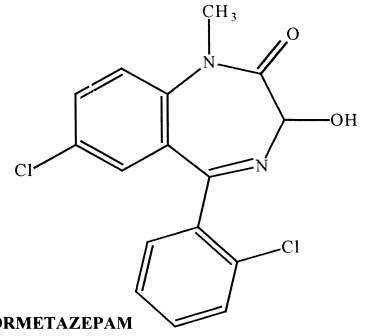


LORAZEPAM



LORMETAZEPAM

Fig. 1. Chemical structures of lorazepam and lormetazepam.

of benzodiazepines in synthetic mixtures [2] and in body fluids (urine, blood) [3–8]. In this way, the determination of benzodiazepines and their metabolites [4] or only of benzodiazepines in human urine by using stepwise solid-phase extraction are described [5]. The determination of lorazepam in plasma has been used for therapeutic drug monitoring [6]. Other studies include the use of capillary electrophoresis in its capillary zone electrophoresis (CZE) mode [9,10] or high-performance liquid chromatography (HPLC) [11–14]. Acid hydrolysis of lorazepam was studied by Panderi et al. [15] and Boonkerd et al. used MEKC to study the chiral separation of lorazepam and lormetazepam in pharmaceutical products [16].

Detection by UV absorption has remained very popular due to the ease of its implementation and wide applicability, particularly for the detection of organic and biologically active compounds. However, its limitations include relatively poor detection limits. Thus, a number attempts have been made to achieve lower detection limits by increasing the absorption path length [17] or by using a photodiode array detector [18].

In this paper, we describe a MEKC method using the direct injection of serum which, in combination with a bubble cell and electropherogram treatment, allows the simultaneous monitoring of lormetazepam and its metabolite (lorazepam) in serum within a therapeutically relevant concentration range.

2. Experimental

2.1. Apparatus

A Beckman P/ACE 5510 (Fullerton, CA, USA) capillary electrophoresis system equipped with a diode-array detector was used. The system was controlled by a Dell Dimension P133V with P/ACE Station Software.

Electrophoretic separation was carried out in a 57 cm (50 cm to the detector)×75 μm I.D. fused-silica capillary housed in a cartridge with a 200×100 μm detector window and with a bubble in the detection window.

A Beckman (Fullerton, CA) DU-70 spectrophotometer equipped with 1.0 cm quartz cells and connected to an IBM-PS 2 Model 30 computer, fitted with Beckman Data Leader software was also used.

A Crison (Barcelona, Spain) MicropH 2002 pHmeter was used for the pH measurements.

2.2. Chemicals

Lorazepam and lormetazepam were obtained from Sigma (St. Louis, MO, USA). Standard solutions were prepared in ethanol–water (50:50, v/v).

Methanol and acetonitrile (HPLC grade) were purchased from Scharlau (Barcelona, Spain). All chemicals and solvents used were of analytical-reagent grade. Milli-Q water was used throughout the study.

The background electrolyte was prepared using a 100 m*M* sodium tetraborate–sodium phosphate stock buffer solution, a 150 m*M* SDS stock solution and HPLC-grade methanol. A 0.1 *M* solution of sodium hydroxide was used for conditioning the capillary between separations.

2.3. Stability of the solutions

Standard solutions of lorazepam and lormetazepam were obtained by dissolving appropriate amounts of these drugs in ethanol–water (50:50, v/v) (334 mg l^{-1} for lorazepam and 361 mg l^{-1} for lormetazepam). Solutions were protected from light and stored at 4 °C.

The stability was controlled by the daily preparation of fresh solutions. The stock solution was diluted with water in a calibrated flask and the spectrophotometric (spectra recorded between 190 and 315 nm at a scan speed of 300 nm min^{-1}) and electrophoretic (injection time 5 s at 25 °C at a potential of 20 kV, 25 m*M* borate–phosphate buffer, pH 8, 50 m*M* SDS and 10% acetonitrile) measurements were carried out over a period of 7 days. Lorazepam and lormetazepam were assumed to be stable under these operating conditions. Although this test is often considered to be part of the ruggedness test of the procedure, it should be carried out at the beginning of the validation procedure since it determines the validity of the data of the other tests.

2.4. Pretreatment serum samples

The blood samples were obtained in the early morning from healthy persons. This blood was introduced into a sterile tube (Autosep, Gel+Clot Act, silicone coated Z) and was centrifuged for 10 min at 1921 *g*. The serum was collected in separate 1-ml vials and which were frozen for storage. Finally, an aliquot of 200 μl of drug-free serum was spiked with the standard solutions of the benzodiazepines before injection into the capillary.

2.5. Operating conditions

The capillary was conditioned by flushing, first with 0.1 *M* NaOH for 20 min, then with water for 10 min, and finally, with the background electrolyte solution for 10 min.

The benzodiazepine samples were placed in 200-μl polypropylene vials, and the background electrolyte and NaOH solution in 4-ml glass vials. The rinse step was carried out using different vials from the separation in order to maintain a constant buffer level in the anodic separation vial.

A clean up step was performed before each separation. The capillary was rinsed with 0.1 *M* NaOH for 2 min, followed by the separation buffer for 2 min and, finally, a 3 s hydrodynamic injection of the sample. The separation was performed at 25 kV for 20 min (with a 125 kV min^{-1} ramp voltage) at a temperature of 30 °C. Under the selected conditions the current intensity was 104.7 μA. The separation vial set was changed after every four runs.

The data generated from the first two injections in a sequence were rejected on account of the need for system equilibration.

3. Results and discussion

3.1. Preliminary studies

Two different capillaries were used in this study. First, the tests were carried out using a 57 cm×75 μm I.D. capillary with a 100×800 μm detection window; however, this was later replaced by a different capillary of the same length but with a bubble in the 100×200 μm detection window. This increased sensitivity of the assay and thus improved the limits of detection and quantification. The study was continued under these conditions, testing different buffers at different pH values in an electrolyte containing micellar additives and organic modifiers for the separation of the benzodiazepines from the blood proteins and other blood components. The initial background electrolyte was borate–phosphate buffer, pH 8 (25 m*M*), SDS (50 m*M*) and acetonitrile (10%). All testing was carried out at a temperature of 25 °C and the voltage was maintained at 20 kV with a voltage ramp of 40 kV min^{-1}.

3.1.1. Influence of the pH in the separation

The pH value of the running electrolyte has a significant impact on the electrophoretic mobilities of the benzodiazepines. Separations at different pH values (6.5, 7, 8 and 9.2) with borate and phosphate buffers, acetonitrile and with/without SDS in the separation electrolyte have been tested. A temperature of 25 °C and voltage of 20 kV were used. Using these separations, it can be shown that SDS is

necessary for the separation of the benzodiazepines from the serum proteins which migrate with the electroosmotic flow (EOF). The borate–phosphate buffer (pH 8) mixture was chosen as optimum in order to minimize analysis times whilst maintaining good resolution between the peaks.

3.1.2. Influence of the borate–phosphate buffer concentration

The ionic strength of the buffer has significant effects on solute mobility and separation efficiency [18]. If the capillary temperature is controlled, the EOF decreases as the buffer concentration is increased, leading to longer analysis times [19]; a low buffer concentration will give shorter analysis times but could lead to broadened, asymmetric peaks [20]. The effects of the buffer concentration on migration time, benzodiazepine resolution and the EOF are shown in Fig. 2. When the buffer concentration was higher than 30 m*M*, the peaks did not appear in the electropherogram registered for 45 min. In contrast, the resolution improves with an increasing concentration of borate buffer. A 15 m*M* buffer solution was selected in order to guarantee good peak shapes and low current, minimising the noise and baseline aberrations. Temperature and voltage parameters were 25 °C and 20 kV, respectively.

3.1.3. Influence of organic modifiers

Acetonitrile, methanol and ethanol were tested at concentrations between 5 and 25%. Taking into account the resolution between peaks, the run time and the peak shape, 15% methanol was selected as the optimum organic modifier. This solvent alters the retention mechanism of the micellar solutions by changing the polarity of the aqueous phase, the electrolyte viscosity and the zeta potential.

3.1.4. Influence of surfactants

These studies were carried out by using a 20 kV separation potential at 25 °C. The surfactants tested were SDS, sodium cholate, sodium desoxycholate and mixtures of both bile salts. The experiments showed that SDS provides greater resolution between benzodiazepine peaks than other surfactants or mixtures. Fig. 3 shows the effect of different concentrations of SDS in the buffer (15 m*M* borate–phosphate, pH 8; 15% methanol). As can be seen, migration times and current intensity increase as the SDS concentration increases. The resolution was similar in the range studied (from 2 to 2.4). 30 m*M*

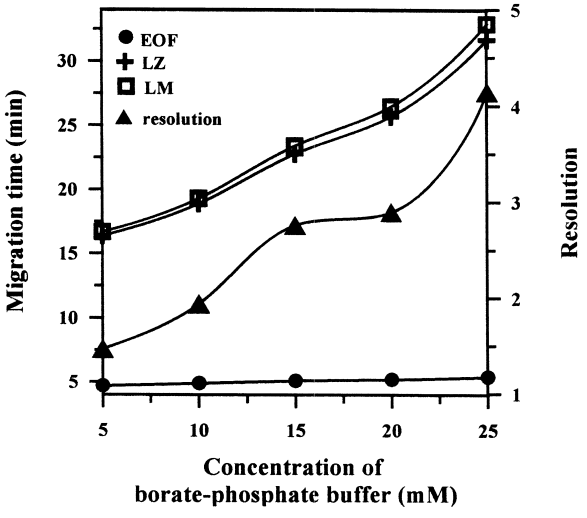


Fig. 2. Influence of the concentration of borate–phosphate buffer on migration times and resolution (● EOF, □ LM, + LZ and ▲ resolution). Background electrolyte: 50 m*M* SDS, 10% acetonitrile, 25 °C and 20 kV.

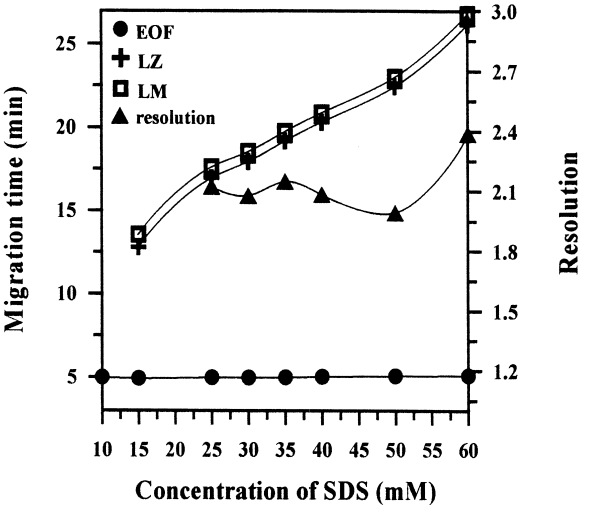


Fig. 3. Influence of the concentration of SDS on the migration times and resolution (● EOF, □ LM, + LZ and ▲ resolution). Background electrolyte: 15 m*M* borate–phosphate buffer, 10% acetonitrile, 25 °C and 20 kV.

SDS was selected as the optimum concentration since it gave narrow, high peaks with good resolution and an acceptable analysis time and baseline noise.

3.1.5. Effect of temperature

The effect of the temperature on the separation was studied between 20 and 40 °C. Temperatures below 20 °C were not tested as the surfactant is only able to form micelles at temperatures above the Kraft point (16 °C for SDS) and because temperature regulation is not efficient at greater than 4 °C below room temperature.

Contributions of Joule heating and temperature gradients become more noticeable at temperatures exceeding 35 °C, resulting in band broadening. A 30 °C temperature was selected as a compromise for resolution, run time, current intensity and acceptable levels of baseline noise.

3.1.6. Influence of the running voltage

The EOF as well as the rate of benzodiazepine migration is proportional to the applied voltage. The application of a high voltage reduces the analysis time, but may lead to significant loss of resolution and of peak efficiency if excessive heating occurs within the capillary (Joule heating). The maximum voltage can be determined from an Ohm's law plot. Running voltages in the range 5–30 kV were tested using the experimental conditions selected above (15 m*M* borate–phosphate buffer, pH 8, 30 m*M* SDS, 15% methanol; 30 °C and 25 kV). The graph was linear up to 20 kV. Control of the negative effects (high current intensity and Joule heating) allowed an optimum voltage of 25 kV to be selected. As expected, reductions in the migration times were obtained with increases in the applied voltage.

At 25 kV, the voltage ramp was varied from 0.2 to 1 min (0.42–2.1 kV s^{-1}); 0.2 min was selected as it provided the best analysis times (Fig. 4a).

3.1.7. Injection time

This study was carried out by injecting solutions of both benzodiazepines in a time range between 2 and 10 s ($n=5$) corresponding to 11–60 nl, at constant concentrations of 35.58 and 36.10 mg l^{-1} for lorazepam and lormetazepam, respectively. All results were obtained using corrected peak areas

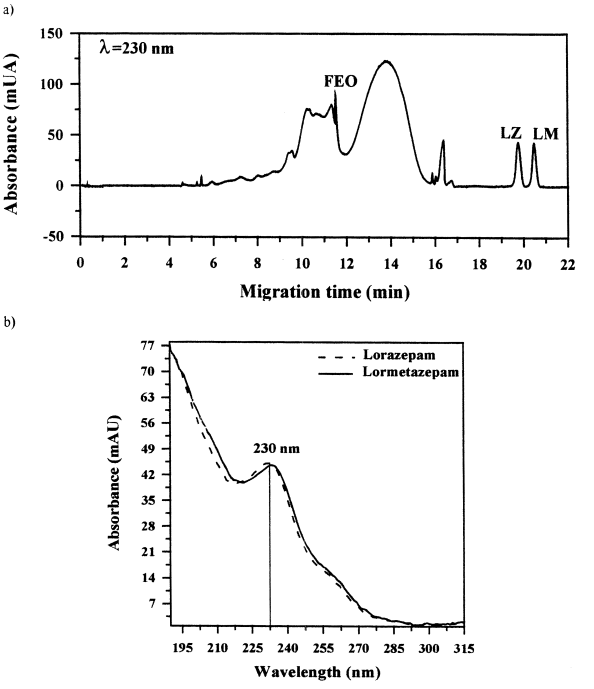


Fig. 4. (a) An electropherogram obtained under the selected conditions (15 m*M* borate–phosphate buffer, pH 8, 30 m*M* SDS, 15% methanol, 25 kV and 30 °C); (b) spectra of lormetazepam and lorazepam.

(CPAs) at 230 nm (Fig. 4b). To calculate the CPA, the peak area for each benzodiazepine was divided by its corresponding migration time [21]. Three seconds was selected as the optimum injection time.

3.1.8. Optimisation of the rinse and wash steps

It is important to maintain a constant EOF within a sequence since any variation may result in poor migration time accuracy [22]. Components of the samples may be adsorbed onto the capillary surface and the effective charge on the wall may vary, altering the EOF. To avoid difficulties due to adsorption and to ensure a constant EOF, the capillary was flushed between injections with 0.1 *M* NaOH solution which strips off the top surface of the capillary. A 2 min wash step with 0.1 *M* sodium hydroxide, followed by a 2 min buffer wash, was considered adequate to restore the capillary wall surface and re-equilibrate the capillary between sample injections.

Table 1
Intra-day mean and RSD values of CPA for lorazepam and lormetazepam at the different concentrations used in the linearity experiments

Concentration of analyte (mg l^{-1})	Lorazepam		Lormetazepam	
	Mean	RSD (%)	Mean	RSD (%)
5 (n=5)	3569.8	2.7	4936.8	2.3
25 (n=5)	15 202.6	2.4	22 076.4	1.6
50 (n=5)	31 750.8	2.3	44 172.2	2.1
75 (n=5)	41 090.0	2.6	66 282.0	1.4

3.2. Performance evaluation

3.2.1. Linearity of the response and precision

Intra-day precision was evaluated on the basis of five linearity experiments (each at four different concentrations) carried out on the second day of the study. Individual linear regression equations were calculated for each of the five linearity experiments, with confidence levels of 95%:

Lorazepam: CPA=1077.8±(1311.1)+559.4±(27.9)×[LZ] (mg l^{-1}); r^2=0.9950; CPA=1827.1±(2158.8)+532.7±(46.1)×[LZ] (mg l^{-1}); r^2=0.9852; CPA=1696.2±(2247.4)+549.9±(47.9)×[LZ] (mg l^{-1}); r^2=0.9850; CPA=2315.2±(2511.4)+530.8±(53.6)×[LZ] (mg l^{-1}); r^2=0.9800; CPA=1431.11±(2130.7)+566.9±(45.5)×[LZ] (mg l^{-1}); r^2=0.9872.

Lormetazepam: CPA=−114.7±(584.9)+890.9±(12.5)×[LM] (mg l^{-1}); r^2=0.9996; CPA=720.6±(338.8)+860.5±(7.23)×[LM] (mg l^{-1}); r^2=0.9998; CPA=−127.7±(979.8)+881.0±(20.9)×[LM] (mg l^{-1}); r^2=0.9988; CPA=721.1±(527.8)+869.6±(11.3)×[LM] (mg l^{-1}); r^2=0.9997; CPA=625.4±(196.0)+885.3±(4.2)×[LM] (mg l^{-1}); r^2=0.9999.

Standardized residuals were randomly distributed around zero and the RSD values within the slopes were 2.9% for lorazepam and 1.4% for lormetazepam. Table 1 shows the intra-day mean and RSD values of CPA for the drugs at the different concentrations employed in linearity experiments.

The results of the calibration graphs carried out on 5 different days (each at five concentrations) confirm that the inter-day precision within the slopes (RSD=3.2 and 1.5% for lorazepam and lormetazepam, respectively) is acceptable. The higher RSD value is still acceptable for biological applications and supports the ruggedness of MEKC with the direct injection of serum. Table 2 shows the inter-day mean and RSD values of the CPAs obtained on each of the 5 days.

Finally, serum samples spiked with lorazepam and lormetazepam at different concentration ratios were injected in order to establish the maximum errors expected for different concentrations of these benzodiazepines in serum. CPAs were used to calculate the corresponding concentration using a regression equation obtained as the mean of the five injections achieved in 1 day. Errors associated with these estimations were always less than 4%.

Table 2
Mean and RSD values of CPAs obtained on each of the 5 days

Concentration of analyte (mg l^{-1})	Lorazepam		Lormetazepam	
	Mean	RSD (%)	Mean	RSD (%)
5 (n=5)	3195.4	3.8	4308.4	3.9
10 (n=5)	6480.4	2.6	9214.8	2.6
25 (n=5)	14 424.8	3.3	20 556.4	3.3
50 (n=5)	29 068.2	3.1	42 296.8	1.8
75 (n=5)	41 334.4	2.6	66 428.8	1.3

3.2.2. Limits of detection (LODs) and quantitation (LOQs)

LODs between 1 and 2 mg l^{-1} and LOQs between 3 and 6 mg l^{-1} were estimated for the two benzodiazepines. These limits were calculated on the basis of the baseline noise. The baseline noise was evaluated by recording the detector response over a period of about 10 times the peak width. The LOD was obtained as the sample concentration which produced a peak with a height three times the level of the baseline noise [23].The LOQ was calculated as 10 times this height. In order to improve the LOD and the LOQ, we resorted to the addition of 10 electropherograms recorded from 225 to 234 nm. Taking into account that the noise signals are random, the intensity of the drugs peaks will increase and the baseline noise will decrease [24]. By using this method, new LODs and LOQs were established at 0.5 and 1.7 mg l^{-1}, respectively.

3.2.3. Peak purity

Co-migration of peaks is possible in MEKC as in any other separation technique. It is therefore useful to investigate the purity of separated peaks. Several techniques have been proposed in the literature for validating peak purity [25]. In this study, the technique used for validating peak purity was normalized, using the comparison of spectra from various peak sections. The captured spectra were overlayed at the apex, upslope and downslope; no interference was detected in the lorazepam and lormetazepam peaks.

4. Conclusion

MEKC makes the direct injection of serum possible and is, therefore, simple in its execution and yields accurate results. This method gave satisfactory results in terms of the precision of corrected peak areas and linearity of the responses as a function of the concentration. The method is sufficiently accurate for the collection of reliable bioanalytical data. Compared with HPLC the method is relatively cheap, requiring only a few milliliters of buffer and inexpensive capillaries. As evidenced by the results obtained in these studies, MEKC is proving to be an attractive alternative to HPLC in therapeutic drug monitoring and in other bioanalytical applications.

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