# A VALIDATED LC METHOD FOR DETERMINATION OF CARBAMAZEPINE IN SALIVA

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**ABSTRACT**

Carbamazepine (CBZ) is a drug used as anticonvulsant, especially in the treatment of epilepsy. For adequate control disease, treatment must achieve adequate blood concentrations, therefore, it is very important to control the levels of anticonvulsant drugs in biological matrices with suitable analytical methods. A simple and fast liquid chromatographic method with UV detection was developed and validated for the determination of CBZ in saliva. Chromatographic separation was achieved with a RP-18 column, using acetonitrile and water with triethylamine at pH 7,3 as mobile phase in isocratic elution mode, at a flow rate of 1 mL/min. The detection was done at 230 nm. and bromazepam at 1,5 µg/mL was used as IS. The run time was 5 min. The described method was linear over a range of 0,5 - 5,5 µg/mL. The intraassay and interassay precision, expressed as the RSD, were in the range of 0,67 - 2,93 % and 0,17 - 9,33 %, respectively, the extraction recoveries were between 100,67 – 100,69 %, the accuracy values ranged from -3,33 to 0,69 % and - 2,67 to 0,72 in intraday and interday analysis, respectively, and the assay demonstrated adequate selectivity and specificity. The LLOQ is below the therapeutic level, demonstrating an adequate sensitivity of the method. The results showed that the proposed method was found to be suitable for quantitative determination of CBZ in saliva.

**Keywords:** Carbamazepine, saliva, validation.

## 1. INTRODUCTION

Carbamazepine (CBZ) (Figure 1) is an iminostilbene derivative that is used as both an anticonvulsant and for the relief of pain associated with trigeminal neuralgia as well as for psychiatric disorders. It is one of the most widely used anticonvulsants for the treatment of epilepsy. For adequate control disease, treatment must achieve adequate blood concentrations and maintain stable levels to protect the patient all day. The clinical effect of CBZ correlates better with blood levels than with doses, since it presents a low aqueous solubility, and a metabolism induced by other drugs and by autoinduction, which generates a poor dose-response relationship and large differences between individuals [1,2]. Besides, it presents a narrow therapeutic blood concentration range and a high plasma protein binding (75 %), being able to displace or be displaced by other drugs with the same characteristics, and thus cause toxicity or therapeutic failure [3]. In view of these factors, monitoring blood levels is one of the strategies enabling clinical results to improve, allowing to individualize drug posology [3,4].

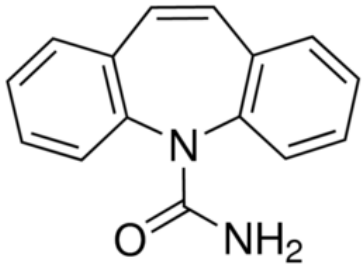


**Figure 1.** Chemical structure of Carbamazepine.

Saliva is an alternative biological fluid to serum or plasma, easier to collect and handle besides the non-invasive sampling, the concentrations in this sample correspond to the non-protein drug bound fraction, which is the pharmacologically active and, consequently better correlated with clinical effects [2,5,6]. The therapeutic CBZ concentrations in saliva range from 1,2 -3,5 µg/mL [2, 6-8]. There is evidence that shows a high correlation between CBZ levels in saliva and blood, demonstrating the usefulness of saliva for the evaluation of anticonvulsant therapy [5,7-11].

CBZ concentrations are mainly determined in plasma or serum by HPLC [7, 9-17] and ELISA [8, 19, 20]. There are few analytical methods that are reported in literature that quantify CBZ in saliva. Among these, some use methodologies not common in many laboratories, such as UHPLC [18, 21], and HPTLC [2]. Other studies show the application of the HPLC technique but do not describe the development and validation of the method [7, 9, 10]. Other authors report HPLC methods with UV or DAD detection, but the extraction methodologies are complex and time consuming, and with incomplete validation protocols or long retention time [6, 11, 22]. Therefore, the aim of this study was to develop and validate a simple, fast, sensitive, accurate, precise, and selective HPLC method for the quantification of CBZ in saliva which can be easily applied in numerous laboratories. CBZ can be quantified in a short period, improving analysis throughput, and thus representing an alternative analytical tool.

## 2. EXPERIMENTAL

### 2.1 Materials and reagents

CBZ standard (≤ 100 % purity), and bromazepam standard (internal standard, ≤ 100 % purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN) and methanol (MeOH) LC grade, triethylamine (TEA) and phosphoric acid, p.a. grade, were obtained from Merck (Darmstadt, Germany). Milli-Q grade water was used for the preparation of mobile phase (Milli Q® system, Merck/Millipore, Molsheim, France).

### 2.2 Instrumentation and chromatographic conditions

Chromatographic analysis was performed on a Merck Hitachi Elite LaChrom HPLC system (Merck, Darmstadt, Germany). The system consists of a quaternary L-2130 pump, a programmable L-2400 absorbance detector, a Rheodyne 7725 manual injector system (Cotati, CA, U.S.A.), a 20 µL loop, and a Varian Star 800 interface. Instrument control and data collection were carried out using Varian Star Chromatography Workstation software (version 6.2).

The separation was performed on a Purospher ® STAR RP-18 endcapped column (125 mm x 4 mm, 5 µm; Merck, Darmstadt, Germany). The mobile phase consisted of water with TEA (0,05 %) pH adjusted to 7,3 with phosphoric acid (98 %) and ACN, 65:35 v/v, in isocratic elution mode, and at a flow rate of 1 mL/min. The detection was done at 230 nm. and bromazepam at 1,5 µg/mL was used as IS. All analyses were performed at room temperature (23 ± 2 ºC).

### 2.3 Preparation of standards and quality controls

The stock solution of CBZ and IS were individually prepared in MeOH at 1.0 mg/mL. The intermediate standards were prepared from the stock solutions after adequate dilution with ACN / water (35:65 v/v) at 100 µg/mL. These solutions were used to spike the aliquots of drug-free saliva samples and obtain six calibration standards at concentrations of 0,5 - 1,5 - 2,5 - 3,5 - 4,5 - 5,5 µg/mL for CBZ and 1,5 µg/mL for IS.

The quality controls (QC) samples were independently prepared similarly to calibration standards to produce four different concentration levels (LLOQ, low, medium, and high): 0,5 - 1,5 - 2,5 - 4,5 µg/mL for CBZ. The stock and intermediate solutions were stored in glass vials at -20 °C. The calibration standards and QC samples were freshly prepared before each analysis.

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### 2.4 Collection and sample preparation

Drug-free saliva samples were collected from healthy volunteers after written consent. Saliva was collected using Salivettes® system (Sarstedt, Nümbrecht, Germany). Volunteers were required to not eat or brush their teeth for at least 1 hour prior to sampling and to rinse their mouths with water 10 min before taking the sample.

The swab is removed from the Salivette® and placed in the mouth and chewed for about 2 minutes to stimulate salivation. The swab with the absorbed saliva is returned to the Salivette® and the stopper is replaced, then it is centrifuged at 2500 rpm for 5 min. to yield a clear saliva sample in the conical tube. The samples are further processed or stored at -20 °C until analysis.

### 2.5 Method validation

The method was validated according to the Food and Drug Administration, Bioanalytical Method Validation guidance (FDA) [23] and European Medicines Agency guideline (EMA) [24]. Linearity, sensitivity, accuracy, precision, extraction recovery, carryover, selectivity, specificity, stability, and robustness were used as the validation parameters.

#### 2.5.1 Linearity and sensitivity

The range was selected according to reported therapeutic CBZ concentrations in saliva (1,2 -3,5 µg/mL) [2, 6-8]. Calibration curves were constructed using six non-zero calibration standards at concentrations of 0,5 - 1,5 - 2,5 - 3,5 - 4,5 - 5,5 µg/mL for CBZ and 1,5 µg/mL for IS. The linearity was determined by plotting the peak area relation of CBZ to the IS versus the corresponding nominal concentration of CBZ. Each solution was injected three times and three independent calibration curves were analyzed. The back calculated standard concentrations were determined to meet the acceptance criteria: the calibration standards should be ± 15 % of nominal (theoretical) concentrations, except at LLOQ where the calibrator must be within ± 20 %. This criterion should be met for at least 75 % of calibration standards. Linearity was evaluated statistically by linear regression using analysis of variance (ANOVA). The LLOQ was defined as the lowest concentration on the calibration curve.

#### 2.5.2 Accuracy and precision

The intraday accuracy and precision were evaluated by carrying out six independent assays of each QC (LLOQ, low, medium, and high), on the same day under the same experimental conditions. The interday accuracy and precision was examined by carrying out the assays on three different days. The accuracy was calculated as relative error (RE) in percentage (measured concentration-nominal concentration)/nominal concentration x 100), and the precision was expressed as the relative standard deviation (RSD, %) at each level.

To meet the acceptance criteria of accuracy and precision, the mean concentration and RSD should be ± 20 % of nominal value for the LLOQ and ± 15 % for the other QC samples.

#### 2.5.3 Extraction recovery

The extraction recovery was determined by comparing the analyte responses in saliva spiked before extraction with those spiked after extraction, at low, medium, and high QC levels. Recovery need not be 100 %, but the results should be consistent and reproducible.

#### 2.5.4 Carryover

Carryover was evaluated by injecting blank samples after injections of the highest calibration standard concentration (5,5 µg/mL). To meet the acceptance criteria, carryover should not exceed 20 % of LLOQ.

#### 2.5.5 Selectivity and specificity

The selectivity was evaluated by analyzing blank saliva samples from six different volunteers, and specificity was evaluated by analyzing potential concomitant medication and CBZ metabolite, in order to ensured that no interfering peaks were present at the respective retention times of CBZ, and the IS. In addition, the internal standard was assessed to avoid interference with the CBZ peak. If interfering compounds are present, their response should be less than 20 % of the CBZ at the LLOQ, and less than 5 % of the IS response.

#### 2.5.6 Stability

The stability of stock solutions of CBZ was evaluated at room temperature (23 ± 2°C), 4 ± 2°C, and -20 ± 2 °C, for 1, 7 and 14 days, respectively. The stock solution was diluted to 4,5 µg/mL in ACN / water (35:65 v/v) prior to analysis. The peak area relation of CBZ to the IS of stored samples was compared with that of fresh stock solutions. The stability of QC was evaluated at low and high concentrations in triplicate at -20 ± 2 °C for 30 days.

Samples were analyzed after being thawed for 1 h at room temperature when they were stored at -20 °C, or after several minutes when they were stored at 4 °C. Freshly-prepared IS was added to each sample before analysis. The stability was determined by comparing the concentration at each time with the initial concentration. To meet the acceptance criteria the results should be within ± 15 % of the nominal concentration.

#### 2.5.7 Robustness

Although robustness is not requested in the FDA and EMA guidelines, it was decided to carry out this study to determine the factors that may affect the method. The robustness was tested by modifying the flow rate in ± 0,2 units, the composition of the organic phase in the mobile phase in 2 %, and the pH of aqueous phase of mobile phase in ± 0,2 units. Replicate injections (n=3) of stock solutions diluted to 4,5 µg/mL in ACN / water (35:65 v/v) were performed. The resolution, retention time and peak tailing factor were calculated as comparison parameter.

## 3. RESULTS AND DISCUSSION

### 3.1 Optimization of chromatographic conditions

Studies were performed for the optimization of the chromatographic conditions with the aim of achieving efficient separation and good resolution between CBZ and endogenous compounds within a short run time. Different columns, including C8, C18, and various compositions of the mobile phase were evaluated. MeOH and ACN were tested for use as the organic phase, different proportions of ACN (30-60 %) with water or water with mobile phase additives (TEA, phosphoric and formic acid) at 0,8 and 1,0 mL/min flow rate, were tested to provide adequate retention, resolution and peak shapes, in a short run time. In addition, different compounds were evaluated to find a suitable IS, and, ultimately, bromazepam was found to be the most appropriate for the present purpose.

C18 column and ACN as organic solvent, instead of C8 and MeOH gave the best results. With TEA and phosphoric acid, the peak shapes and resolution between CBZ and SI were better. Finally, optimal chromatographic conditions were achieved using 35 % ACN and 65 % water (with TEA 0,05 %, pH adjusted to 7,3 with phosphoric acid) at 1mL/min with a Purospher ® STAR RP-18 column (125 mm x 4 mm, 5 µm). Under these conditions, total run time was 5,0 min, enabling high-throughput sample processing. Retention time of CBZ was 4,3 min, and retention time of SI was 3,2 min. A chromatogram of the standard solution obtained by optimized HPLC method is presented in Figure 2B.

System suitability parameters were found to be within the suitable ranges: Resolution > 2,0 between all peaks, peak tailing factor 1,1 and 1,2, and theoretical plates 2300 and 2200 for CBZ and SI respectively.

### 3.2 Sample preparation

The samples were centrifuged at 2500 rpm for 5 min. And the supernatant was used, without requiring any special treatment. With this treatment a chromatogram without interference from endogenous compounds was obtained (results show in selectivity study), and the recovery did not differ from the 100 % (results show in extraction recovery study). The sample treatment of the present work is simpler, faster, and cheaper than others previously published, which are more complex and time consuming, such as liquid extraction [6, 11], protein precipitation with ACN [7, 21], solid phase extraction [18], and dried saliva spots [22].

### 3.3 Method validation

#### 3.3.1 Linearity and sensitivity

A linear correlation was obtained between peak areas relation of CBZ to IS versus the CBZ concentrations over the range of 0,5 -5,5 µg/mL. All three calibration curves showed good linearity with $r^2 > 0,9980$. The typical equation of the calibration curve was y= 0,4900 x – 0,0235. All calibration standards meet acceptance criteria. According to statistical analysis by ANOVA, the curves were linear with $p < 0,005$.

The LLOQ was established at 0,5 µg/mL, and considering the therapeutic range in saliva, it was found that the LLOQ is below the lower therapeutic level (1,2 µg/mL), therefore, is adequate for quantitative determination of CBZ in saliva.

#### 3.3.2 Accuracy and precision

The results of accuracy and precision studies are shown in Table 1. The RE and RSD for intraday and for interday accuracy and precision were within ± 15 % of the nominal value, indicating that the method was accurate and precise.

**Table 1.** Intraday and interday accuracy and precision of QC samples for CBZ

| Nominal concentration (µg/mL) | Intraday[a] | | | Interday[b] | | |
|---|---|---|---|---|---|---|
| | Measured concentration (µg/mL) | RE (%) | RSD (%) | Measured concentration (µg/mL) | RE (%) | RSD (%) |
| 0,5 | 0,48 ± 0,01 | -3,33 | 2,93 | 0,49 ± 0,03 | -2,67 | 5,81 |
| 1,5 | 1,51 ± 0,01 | 0,69 | 0,67 | 1,51 ± 0,00 | 0,72 | 0,29 |
| 2,5 | 2,52 ± 0,05 | 0,68 | 2,14 | 2,50 ± 0,00 | 0,20 | 0,17 |
| 4,5 | 4,53 ± 0,04 | 0,67 | 0,83 | 4,48 ± 0,42 | -0,35 | 9,33 |

[a]Analyzed on the same day (n = 6).
[b]Analyzed on three different days (n = 18)

#### 3.3.3 Extraction recovery

The results of extraction recovery study are shown in Table 2. According to the t-test (n = 15, $\alpha = 0,05$) the recovery obtained did not differ from the real value (100%), which proved that CBZ was successfully recovered, in addition, the RSD was less than 2,2 %, indicating that the recovery was reproducible.

**Table 2.** Extraction recoveries of CBZ in saliva

| Nominal concentration (µg/mL) | Extraction recovery (%) | |
|---|---|---|
| | Mean ± SD[a] | RSD (%) |
| 1,5 | 100,69 ± 0,67 | 0,67 |
| 2,5 | 100,68 ± 2,16 | 2,14 |
| 4,5 | 100,67 ± 0,83 | 0,83 |

[a]n = 5

#### 3.3.4 Carryover

Following the injection of the highest calibration standard, no interfering peaks of the CBZ and the IS were observed, indicating that carryover does not occur.

#### 3.3.5 Selectivity and specificity

The analysis of blank saliva did not show interference from endogenous compounds with CBZ and IS retention times, demonstrating that the method is selective. The analysis of potentially co-prescribed drugs, such as phenytoin and phenobarbital, and carbamazepine-10,11-epoxide metabolite, showed no interference with the CBZ or IS peak, therefore the method is specific. The retention times were 2,5, 4,8 and 2,4 minutes for phenobarbital phenytoin and 10,11-epoxide metabolite, respectively. Phenytoin is separated from the CBZ with a resolution of 1,2. Also, CBZ and IS peaks were well separated, with a resolution value up to 2, demonstrating no interference between them. Figure 2 presents the typical chromatograms of blank saliva sample (Figure 2A), and blank sample spiked with CBZ at 3,5 µg/mL plus IS at 1,5 µg/mL (Figure 2B).

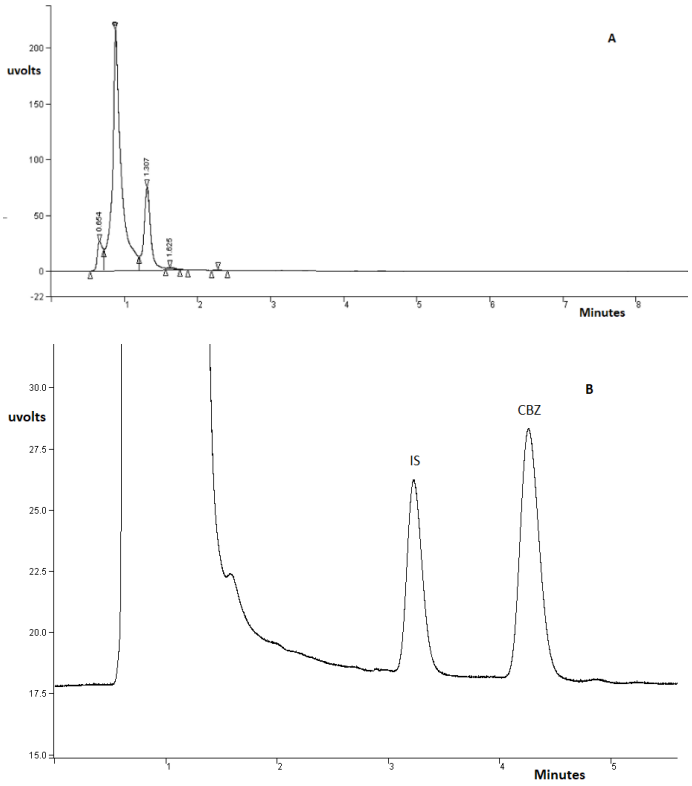


**Figure 2.** Chromatogram of of blank saliva sample (A), and blank sample spiked with CBZ at 3,5 µg/mL plus IS at 1,5 µg/mL (B). Column, Purospher ® STAR C18; mobile phase, water (TEA 0,05 %, pH 7,3) and ACN, 65:35 v/v; detection, at 230 nm; flow rate, 1,0 mL /min.

#### 3.3.6 Stability

As shown in Table 3, the results for the stock solutions of CBZ confirm that all the solutions were stable under different storage conditions, also, QC samples were stable at low and high levels at -20 ± 2 °C for 30 days, as there the original value remained almost unchanged, and are within ± 15 %, therefore, samples can be stored under these conditions prior to analysis. These results are in accordance with a stability study of CBZ in saliva, in which CBZ remained stable at different storage conditions [6].

**Table 3.** Stability of CBZ under different storage conditions

| Temperature (°C) | Days | | Stability (%) |
|---|---|---|---|
| Stock solutions | | | |
| 23 ± 2 | 1 | | 100,5 ± 0,5 |
| 4 ± 2 | 7 | | 99,9 ± 0,4 |
| -20 ± 2 | 14 | | 98,7 ± 0,8 |
| QC | | | |
| | | Level | |
| -20 ± 2 | 30 | Low | 97,5 ± 0,2 |
| | | High | 96,9 ± 0,9 |

#### 3.3.7 Robustness

With pH 7,1 and 33 % ACN in mobile phase an increase in the retention time of CBZ and IS peaks was observed but this does not affect the separation or the peaks shape. With pH 7,5, flow rate 1,2 mL/min, and 37 % ACN in mobile phase, no relevant changes were observed in the studied parameters; therefore, the method is robust for such fluctuations. In the case of flow rate 0,8 mL/min an increase of > 3 minutes in the retention time of CBZ (RT = 9,9 min) and IS (RT = 6,7 min) and an increase of peak tailing factor of SI (T = 1,4) was observed, but peaks separation was adequate (Rs > 2), therefore, the flow rate must be properly adjusted.

## 4. CONCLUSIONS

A simple and rapid HPLC method was developed and fully validated to quantify CBZ in saliva samples. The proposed method was properly validated according to the FDA and EMA guidelines. The present method showed excellent performance, presenting advantages such as simple sample collection and treatment, simple HPLC-UV technique, and short chromatographic run time, enabling high-throughput sample processing. The method is suitable to routine analysis of CBZ in patients in treatment of epilepsy, allowing results to be obtained quickly, which reduces the time needed for therapeutic intervention.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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