

## Development and Validation of an HPLC Method for the Determination of Carbamazepine in Human Plasma

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**Abstract:** A simple method for the determination of carbamazepine using high performance liquid chromatography (HPLC) with ultraviolet absorbance detection (UV) was developed. The method involves two steps (protein precipitation and liquid-liquid extraction). Diclofenac sodium was used as the internal standard (is). The separation was carried out using an analytical Thermo C<sub>8</sub> (250 x 4.6 mm), 5 µm column with mobile phase consisted of acetonitrile: isopropyl alcohol: phosphate buffer pH: 3 (36:15:49). The flow rate was 1.2 mL/min. The eluent was monitored at 220 nm with a sensitivity setting at 0.05 absorbance units. Linear detection response was obtained for concentrations ranging from 0.1 to 8.0 µg mL<sup>-1</sup>. The limit of quantification (LOQ) was 0.1 µg mL<sup>-1</sup>. The method was validated successfully for the determination of carbamazepine and, the proposed HPLC method is simple, rapid and highly sensitive, and it could be reliable for pharmacokinetic studies in humans.

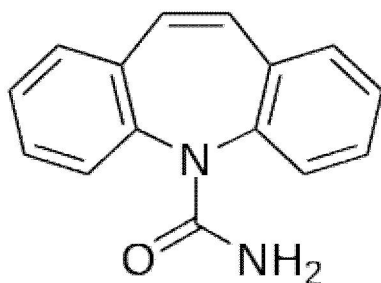
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**Key words:** carbamazepine. HPLC. Diclofenac, Plasma

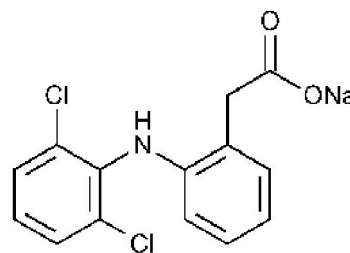
### 1. Introduction

Carbamazepine (CBZ) (Fig. 1) is widely used as the first line treatment for partial and generalized tonic-clonic seizures (Beghi et al., 2002) CBZ also has other indications, including bipolar disorder, trigeminal neuralgia and other neuropathic pain syndromes (Wyllie 2001, Trimble 2002). Clinical effects of CBZ bear a relatively close relation to serum drug concentrations in epileptic patients (Tergau et al., 2003). Carbamazepine is considered safe when used appropriately (Franceschi et al., 1988;

Matlow, and Koren, 2012). Carbamazepine overdose most commonly leads to prolonged coma associated with wide complex tachycardia (Askenazi et al., 2004; Furlanut et al., 2006). Massive overdose with controlled-release carbamazepine resulting in delayed peak serum concentrations and life-threatening toxicity (Matlow, and Koren, 2012). The aim of this study was to establish a simple HPLC-UV method for determination of carbamazepine in human plasma in order to facilitate pharmacokinetic and toxicokinetic studies.



Carbamazepine



Diclofenac sodium

**Fig. 1:** Chemical structures of carbamazepine and diclofenac sodium (IS)

### 2. Experimental

#### Chemicals and Reagents

Carbamazepine was supplied by Medizen pharmaceutical industries Co, Egypt; diclofenac sodium was supplied by Adwia Co., Egypt. Isopropanol was supplied by Panreac, Spain,

potassium dihydrogen phosphate was supplied Adwic, Egypt. Dichloromethane (LAB-SCAN, Ireland). Human plasma obtained from VACSERA blood bank. Deionized water was obtained from a Milli-Q water purification system (Millipore, France).

### Instrumentation

The HPLC system (Waters, New York, USA) equipped with pump controlled by Waters 610 controller, Waters 717 autosampler injector, Waters 486 variable wavelength UV detector. For the data acquisition and integration Waters millennium software operated by Pentium III (450 MHz) processor (Compaq, UK) was used.

### Chromatographic conditions

The mobile phase consisted of acetonitrile: isopropyl alcohol: phosphate buffer pH: 3 (36:15:49). The flow rate was 1.2 ml/min. The eluent was monitored at 220 nm with a sensitivity setting at 0.05 absorbance units. The separation was performed using an analytical Thermo C<sub>8</sub> (250 x 4.6 mm), 5 µm particle size column.

### Method validation

The method validation assays were carried out according to the Food and Drug Administration (FDA) bioanalytical method validation guidance (FDA, 2001).

### Standard Solution of carbamazepine and diclofenac

Stock solutions of carbamazepine and diclofenac sodium (I.S), were prepared separately in methanol at concentration of 100.00 µg/ml each and stored at 4 °C. Evaluations of the assay were performed by seven point calibration curves made by serial dilution of the stock solution of the drug at the nominal concentration range 0.1-8.00 µg/ml of carbamazepine in mobile phase as well as in human plasma. Slope and intercept of the calibration lines were determined.

### Calibration Curve

Standard calibration curves were prepared by spiking 1 ml of plasma by different concentration of carbamazepine to yield concentrations of 0.1, 0.2, 0.5, 1.0, 2.0, 4.0 and 8.0 µg/ml. To 1 ml plasma in screw capped glass tubes, 25 µl of internal standard (diclofenac, 500 µg/ml) was added and samples were shaken by vortex for 5s, 2 ml of acetonitrile was added and centrifuged for 5 min at 3000 rpm. 2.0 ml of the supernatant was transferred to another glass tube, 5 ml of dichloromethane was added, and the mixture was shaken by vortex for 5s and centrifuged for 5 min. Four ml of lower organic layer transferred to glass tube and evaporated under nitrogen and reconstituted in 300 µl of mobile phase and 200 µl were injected. The mobile phase consisted of acetonitrile: isopropyl alcohol : phosphate buffer pH: 3 (36:15:49).

### Selectivity

Selectivity was evaluated by extracting drug-free plasma samples from a pool of human plasma. The absence of interfering peaks at the same retention time of carbamazepine or diclofenac was considered as evidence for selectivity.

### Accuracy, precision and recovery

The intra-day precision of the assay was assessed by calculating the coefficients of variation (CV %) of samples in six replicates on the same day. The interday precision was determined through the analysis of the samples on three consecutive days. Accuracy was determined by comparing the calculated concentrations to known concentrations with calibration curves. The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard.

### Linearity

Calibration curves were prepared by determining the best-fit of peak area ratios (peak area of analyte/ peak area of internal standard) versus concentration, and fitted to the linearity equation  $Y = bC + a$  and the regression coefficient was calculated. The linearity of the calibration curve (18 replicates for each concentration) for carbamazepine was assessed in the range 0.1- 8.0 µg/ml in plasma samples.

### Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

LLOD is a parameter that provides the lowest concentration of analyte in a sample that can be detected, but not quantified, under the stated experimental conditions. LLOQ is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions. LLOD and LLOQ were determined as the concentrations with a signal-to-noise ratio of 3:1 and 10:1, respectively, by comparing test results from samples with known concentrations of analyte to blank samples. Each concentration standard should meet the following acceptable criteria: a precision of 20% and accuracy of 80-120%. The LLOD and LLOQ were 0.05 µg /ml and 0.1 µg/ml, respectively, for carbamazepine in 18 standard calibration curves.

### Stability

The stability of carbamazepine in plasma was studied under a variety of storage and handling conditions using a set of plasma samples containing carbamazepine. Short-term stability indicated reliable

stability behavior under the experimental conditions of the regular runs. It was assessed by analyzing six aliquots each of low, medium and high concentration samples that were thawed at room temperature and kept at this temperature for 6 h. Post-preparation stability was measured by re-analyzing the extracted low, medium and high concentration plasma samples kept under the auto-sampler conditions for 24 h. Freeze-thaw stability (at  $-80^{\circ}\text{C}$  in plasma) was checked through three freeze-and-thaw cycles. Six aliquots at each of the low, medium and high concentrations were stored at  $-80^{\circ}\text{C}$  for 24 hour and thawed at room temperature. When completely thawed, the samples were refrozen for 24 h under the same conditions. The freeze-thaw cycles were repeated three times, and then the samples analyzed on the third cycle. The long-term stability was determined by analyzing six aliquots of each of the low, medium and high concentrations stored at  $-80^{\circ}\text{C}$  for 6 weeks. The precision and accuracy for the stability samples must be within  $\pm 15\%$  of their nominal concentrations.

### 3. Results and Discussion

There are several published methods for determination of carbamazepine in biological fluid (Yoshida et al., 2006); Fortuna et al., 2011; Mandrioli et al., 2001; Subramanian et al., 2008). Some of these methods require high retention times, or using chiral column for chromatographic analysis (Fortuna et al., 2011). Other characterized with more complicated extraction procedures such as solid-phase extraction

were also reported (Franceschi and Furlanut 2005; Loureiro et al., 2011). Solid-phase extraction procedures are requiring solid-phase extraction cartridges, increasing the cost of the analysis. Recent study reported the determination of carbamazepine using LC-MS-MS detection (Bhatt et al., 2011), but mass spectrometers are expensive and not readily available. The present study describes a simple, sensitive, accurate and reproducible HPLC method for the determination of carbamazepine in human plasma. This method has several advantages over the previously reported methods. Sample preparation is simpler. The very low quantification limit obtained with a UV detector in the present work makes this method particularly useful for pharmacokinetic studies and allowed us to avoid using fluorimetric detection, which require more expensive equipment.

Under the chromatographic conditions described, carbamazepine and diclofenac sodium peaks well resolved. Fig. 2 showed typical chromatograms of spiked samples. The retention times of carbamazepine and the internal standard was approximately 11.4 min and 4.1 min, respectively. No endogenous compounds appear to interfere with their peaks. The baseline was relatively free from drift. Seven concentrations defined the calibration curves. A calibration curve was obtained by plotting the peak- area ratio against the concentration of carbamazepine in plasma. The linearity of the calibration curves was verified from 0.1- 8  $\mu\text{g/ml}$  and the corresponding regression equation was,  $r = 0.998$  (Fig. 3). The lower limit of detection was 0.1  $\mu\text{g/ml}$ .

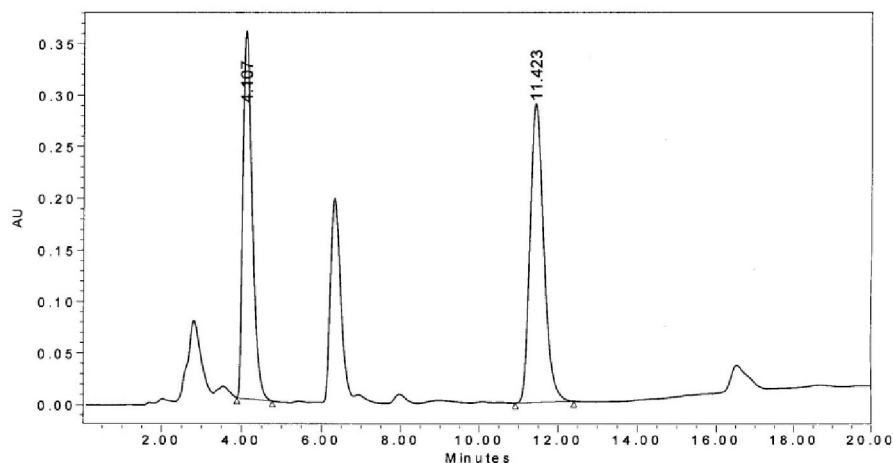


Fig. 2: HPLC Chromatograms showing separation of diclofenac (internal standard) (4.1) and carbamazepine (11.42 min) extracted from plasma spiked with 0.1  $\mu\text{g/ml}$  Carbamazepine and internal standard.

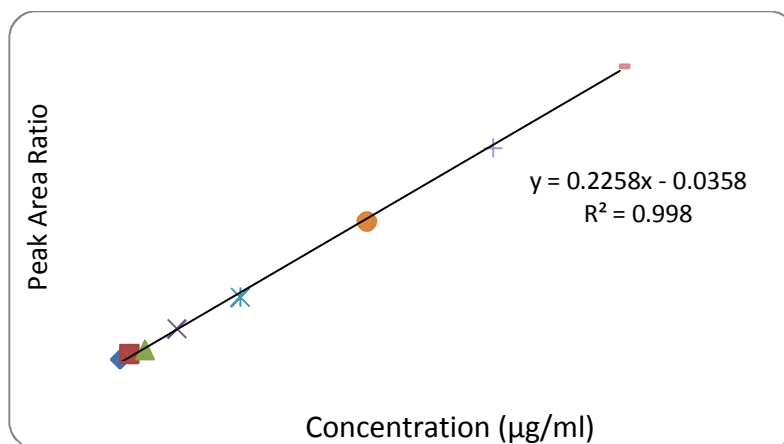


Figure 3. Standard calibration curve of carbamazepine in plasma

Intra-day accuracy and the precision were determined with six determinations per concentration in one day (Table 1). Inter-day accuracy and precision calculated from the results of the assay of six calibration curves in different three days (Table 2). Intra and inter-day accuracy ranged from 85.0% - 95.0% and 85.20% – 100.0 % respectively while precisions were less than 15% (Tables 1-2). The average recovery was 87.00%.

The method described here provides a simple, reliable and reproducible HPLC assay of carbamazepine in human plasma. This method could be reliable for pharmacokinetic and toxicokinetic studies in humans.

**Table 1:** Precision and accuracy of standard calibration curves of carbamazepine (Intra-day) in human plasma. (n=6).

| Theoretical<br>Conc.(µg/ml) | Conc. Found (µg/ml) |      |      | Mean ± SD    | CV %  | Accuracy (%) |
|-----------------------------|---------------------|------|------|--------------|-------|--------------|
|                             | 1                   | 2    | 3    |              |       |              |
| 0.10                        | 0.09                | 0.08 | 0.09 | 0.09 ± 0.006 | 6.66  | 86.7         |
| 0.20                        | 0.2                 | 0.19 | 0.21 | 0.20 ± 0.010 | 5.00  | 100.0        |
| 0.50                        | 0.39                | 0.48 | 0.41 | 0.43 ± 0.047 | 11.08 | 85.3         |
| 1.00                        | 0.9                 | 0.8  | 0.9  | 0.87 ± 0.058 | 6.66  | 86.7         |
| 2.00                        | 0.18                | 0.17 | 0.16 | 0.17 ± 0.010 | 5.88  | 8.5          |
| 4.00                        | 3.8                 | 3.7  | 3.5  | 3.67 ± 0.153 | 4.17  | 91.7         |
| 8.00                        | 7.9                 | 7.1  | 7.2  | 7.40 ± 0.436 | 5.89  | 92.5         |

**Table 2:** Precision and Accuracy of Standard Calibration Curves of Carbamazepine (Inter-day) in human plasma (n=18).

| Theoretical<br>Conc.(µg/ml) | Concentration founds (µg/ml) |       |       |       |       |       | Mean ± SD    | CV%   | Accuracy (%) |
|-----------------------------|------------------------------|-------|-------|-------|-------|-------|--------------|-------|--------------|
|                             | 1                            | 2     | 3     | 4     | 5     | 6     |              |       |              |
| 0.10                        | 0.085                        | 0.081 | 0.082 | 0.098 | 0.099 | 0.078 | 0.09 ± 0.01  | 9.06  | 87.17        |
| 0.20                        | 0.182                        | 0.16  | 0.15  | 0.18  | 0.21  | 0.18  | 0.18 ± 0.02  | 9.86  | 88.42        |
| 0.50                        | 0.490                        | 0.44  | 0.43  | 0.39  | 0.42  | 0.38  | 0.43 ± 0.04  | 7.87  | 85.00        |
| 1.00                        | 0.800                        | 0.85  | 0.79  | 0.9   | 0.87  | 0.97  | 0.86 ± 0.066 | 12.02 | 86.3         |
| 2.00                        | 1.820                        | 1.20  | 1.80  | 1.90  | 1.79  | 1.78  | 1.71 ± 0.26  | 12.78 | 85.71        |
| 4.00                        | 3.300                        | 3.90  | 3.80  | 4.10  | 3.90  | 3.80  | 3.80 ± 0.27  | 6.71  | 95.00        |
| 8.00                        | 6.300                        | 6.90  | 7.10  | 7.90  | 6.90  | 7.20  | 7.05 ± 0.52  | 6.51  | 88.13        |

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