

## DEVELOPMENT AND VALIDATION OF AN UPLC METHOD FOR THE QUANTIFICATION OF CARBAMAZEPINE IN INTESTINAL SAC SAMPLES

Adukondalu devandla<sup>1,2</sup>, Madhusudan Rao Yamsani<sup>1,2</sup>

<sup>1</sup>University College of Pharmaceutical Sciences, Kakatiya University, Warangal. A.P., India

<sup>2</sup>Vaagdevi College of Pharmacy, Ramnagar, Hanamkonda, Warangal, A.P., India

\*Corresponding Author Email: [devandlaadukondalu@gmail.com](mailto:devandlaadukondalu@gmail.com)

### ABSTRACT

An isocratic simple rapid assay has been developed and validated for the determination of carbamazepine (CBZ) in both solution form and intestinal sac samples. The assay was performed using a Analytical C18 (100 mm · 4.6 mm i.d) with a mobile phase consisting of methanol and water (50:50), the flow rate was 1 ml/min and UV detection at 285 nm. The method was found to be specific for CBZ, no interfering peaks were observed with an overall analytical run time of 5 min. Accuracy reported as % recovery were found to be 95.83–103.34% and 93.18–104.49% for inter-day and intra-day accuracies, respectively. Inter-day precision (reproducibility) was found to be 0.22–1.19 SD, while intra-day precision (repeatability) was found to be 0.14–2.12 SD for the samples studied. The calibration curve was found to be linear with the equation  $y=13474x+16969$ , with a correlation coefficient of 0.997 ( $R^2$ ) over a concentration range of 0.5–40 lg/ml. The limit of quantitation was the lowest concentration. The method is simple and rapid and does not require any preliminary treatment of the sample. The method was fully validated. The method was successfully applied for the intestinal sac samples to study the drug transport.

### KEY WORDS

Carbamazepine; Validation; Intestinal sac samples; HPLC method

### 1. INTRODUCTION

Carbamazepine (CBZ), 5-H-dibenze[b,f]azepine-5-carboxamide (Fig. 1), is a tricyclic lipophilic compound that is a first choice antiepileptic drug for the treatment of simple and complex partial seizures (Duche and Loiseau, 1995). It is almost completely metabolized in the body and only small traces are excreted unchanged in urine (Goodman et al., 2001). Therapeutic concentrations have been reported to be 6–12 µg/ml, although considerable variations may arise (Goodman et al., 2001). The use of an established therapeutic range for CBZ concentration is limited by the presence of carbamazepine-10, 11-epoxide (CBZ-E), its active metabolite that significantly contributes to the efficacy and toxicity and also

possesses pharmacological activity as an anticonvulsant (He et al., 1992), though it reaches lower concentrations than CBZ.

Various methods have been reported in the literature for the determination of CBZ, in particular those using chromatography (Bhatti et al., 1998; Dasgupta et al., 1999; Ashy et al., 1986; Raggi et al., 2000; Franceschi and Furlanut, 2005; Chelberg et al., 1988; Hartley et al., 1986; Oh et al., 2006; Yoshida et al., 2006; Elizabeth et al., 2007; Budakova et al., 2008; Hemenway et al., 2010). High-performance liquid chromatography (HPLC) and fluorescence polarization immunoassay (FPIA) are usually employed as routine techniques for the determination of this and other

anticonvulsants (Sanchez et al., 1999). Other techniques such as micellar electrokinetic capillary chromatography (MECC) (Lancas et al., 2003) and chemiluminescence (Lee et al., 2003) were used. Very recently, two approaches were presented in the literature. One of them use spectrophotometry and multivariate calibration (Rezaei et al., 2005) for the simultaneous determination of CBZ and phenytoin. The other method exploits the unusual fluorescence

of CBZ on a nylon membrane (Escandar et al., 2004). Simultaneous determinations of CBZ and its metabolites in biological fluids and drug products (Burke and Thenot, 1985; Owen et al., 2001) have been published, including, solid-liquid extraction (Wad, 1984), liquid-liquid extraction (Rouan et al., 1992), column-switching (Juergens, 1984), deproteinization (Liu et al., 1993), spectrofluorimetry method (Huang et al., 2002), gas-liquid chromatography (Chen and Bashi, 1991), FT-Raman spectroscopy (Auer et al., 2003), planar chromatography (Mennickent et al., 2009), stir bar-sorptive extraction and high-performance liquid chromatography-UV detection (SBSE/HPLC-UV) (Queiroz et al., 2008) and high-performance thin layer chromatography (HPTLC) (Patel et al., 2011). Also, LC-mass spectrometry methods (Breton et al., 2005; Van Rooyen et al., 2002; Miao and Metcalfe, 2003; Zhu et al., 2005) have been reported for the detection of CBZ and its metabolites in aquatic environments and in plasma. Only one article that focused on forced degradation of CBZ used acid, base, oxidation, heat and photolytic conditions. Much degradation was not observed in CBZ samples under stress conditions like acid hydrolysis photolysis and thermal exposure. However, mild degradation was observed during alkaline hydrolysis and significant degradation was observed when the drug was exposed to

oxidation by hydrogen peroxide (Srinivasa and Belorkar, 2010). There is no article concerning complete analysis and validation of CBZ in intestinal sac samples, So, the aim of this study was to establish a method based on UPLC-UV that is capable of analyzing CBZ in both solution and intestinal sac samples in order to facilitate transport studies of CBZ.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Carbamazepine was kindly supplied by Dr Reddy's laboratories, Hyderabad, Andhra pradesh. Methanol (HPLC grade) was purchased from E. Merck private limited, Mumbai. Water for HPLC was prepared in laboratory. All other chemicals and solvents were of analytical reagent grade.

### 2.2. Equipment and chromatographic conditions

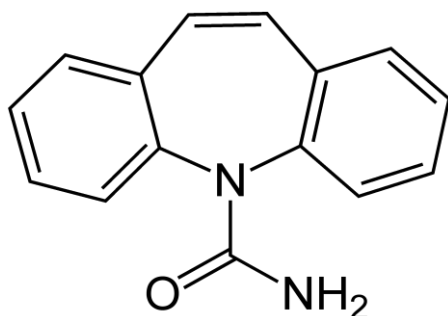
The study employed a ultra performance liquid chromatography (Schimadzu isocratic controller, Koyoto, Japan) equipped with a LC20AT Absorbance PDA detector and an 20µl capacity loop containing sampling system. The mobile phase consisted of methanol and water (50:50), and the flow rate was 1 ml/min. Separation was achieved using a 100 mm · 4.6 mm (i.d.) C18, Analytical, reversed phase column with an average particle size of 10 lm, and the column was kept at ambient temperature. The column effluent was monitored at 285 nm and the chromatographic data analysis was performed with the LC Solutions software (Schimadzu, Koyoto, Japan).

### 2.3. Stocks solutions and standards

Stock solutions of CBZ were prepared in triplicate by dissolving 50.0 mg CBZ in 50 ml methanol, resulting in a solution containing 1 mg/ml. This solution was diluted 20-fold by methanol to give working solution (50µg/ml). Working solutions of CBZ (50µg/ml), were prepared by dilution of the stock solutions in methanol. Calibration curves were constructed in methanol by preparing a

series of concentrations of the drug (3.125, 6.25, 12.5, 25, and 50 µg/ml). Calibration curve was also constructed in intestinal sac samples. These involved replicate analysis of samples spiked with varying concentrations of CBZ (3.125, 6.25, 12.5, 25, and 50 µg/ml)

**Figure 1 Chemical structure of carbamazepine.**



#### 2.4. Treatment of intestinal sac samples

The working solutions (100 µl) were separately transferred to clean dry centrifugation tubes. Intestinal sac sample (500 µl) was added to each tube. These were vortex mixed for 2 min before adding 400 µl of methanol. These were vortex mixed before centrifugation at 5000 rpm for 5 min. The supernatant was separated and loaded into HPLC vials before injecting 50 µl into the HPLC system.

#### 2.5. Method validation

The calibration curve of CBZ is a plot of the peak area of the drug and the drug concentration (C). This gives the following equation: Peak area = Slope · C + Intercept. The slope and the intercept are determined from the determined peak area and the nominal concentration of the drug. The unknown CBZ concentrations are determined from this equation. The precision of the method based on intraday variability was determined by replicate analysis of the calibration standards in

the same day. The reproducibility was taken as the inter-day variability and was determined by replicate analysis of the calibration standards in different days with one replicate being analyzed each day. The standard deviation (SD) to the mean was calculated and recovery expressed as percentage. The accuracy of the method was determined by comparing practical amounts recovered from the control samples with actual values present in the samples (theoretical values). The selectivity of the method was determined by examining the interference from the endogenous materials in intestinal sac samples or from the degradation products of the drug. The limit of quantification LOQ was taken as the lowest concentration that can be accurately (relative error <2 for biological samples and <2 for the in vitro samples) and precisely determined (SD <2 for biological samples and <2 for the in vitro samples). The LOD for CBZ was found to be 0.25 µg/ml and this result is in agreement with data reported in Elizabeth et al. (2007). The drug is stable in liquid preparation and the assay was not validated as stability indicated (Srinivasa and Belorkar, 2010).

#### 2.6. In vitro transport study

The transport of carbamazepine across rat intestine (duodenum, jejunum, ileum and colon) was studied by using in vitro non-everted sac methods (8). The rats were separated into groups of 3 sacrificed, isolated the intestinal segments isolated and then the sacs were prepared. The drug solution was placed in sac and kept in dulbeccos buffer. Samples were collected at pre-set time points by replacing with fresh buffer and their drug contents were estimated using validated HPLC method and the results represented in Table 3.

**Table3. Cumulative amount ( $\mu\text{g}$ ) of carbamazepine (Mean $\pm$ S.D) transported from different parts of albino wistar rat small intestine (n=3) after 120 min.**

Part of intestine	Cumulative amount of drug transported
Duodenum	12.4 $\pm$ 0.14
Jejunum	13.3 $\pm$ 0.66
Ileum	9.97 $\pm$ 0.77
Colon	9.57 $\pm$ 0.48

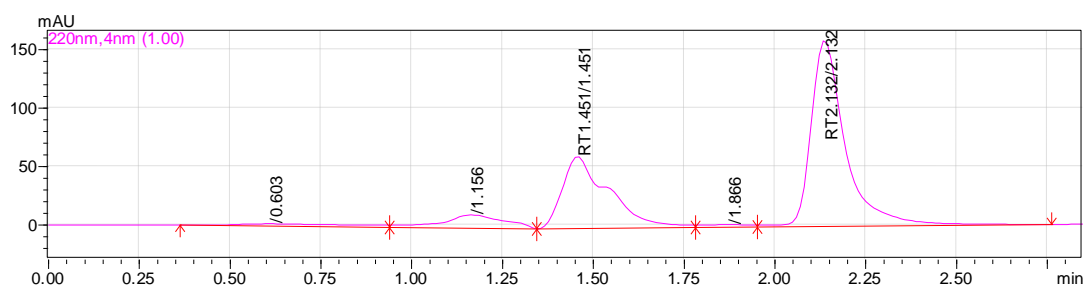
### 3. RESULTS AND DISCUSSION

#### 3.1. HPLC assay of CBZ

HPLC with UV detection was chosen as a simple, fast, and effective separation method for the determination of CBZ (Bhatti et al., 1998; Dasgupta et al., 1999; Ashy et al., 1986; Raggi et al., 2000; Franceschi and Furlanut, 2005; Chelberg et al., 1988; Hartley et al., 1986; Oh et al., 2006; Yoshida et al., 2006; Elizabeth et al., 2007; Budakova et al., 2008; Hemenway et al., 2010). In extensive preliminary experiments, a series of aqueous mobile phases with different pH values

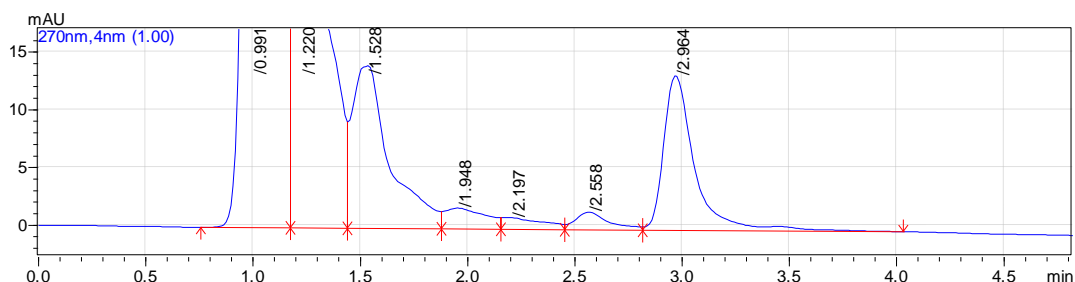
in combination with different organic modifiers were tested. Best results were obtained when using methanol and water (50:50) and adjusting the pH of the solution to 5, allowing adequate separation of the drug and the internal standard using a C18 Analytical column at a flow-rate of 1.0 ml/min. In addition, depending on the weak tissue protein binding of drug, methanol was used for protein precipitation (1:1) in order to obtain satisfactory values for recovery of CBZ. The selected chromatographic conditions provided optimum resolution of CBZ.

**Figure 2 Representative chromatograms of carbamazepine showing 0.5  $\mu\text{g}/\text{ml}$  as lower concentration (left) and 40  $\mu\text{g}/\text{ml}$  as higher concentration (right) carbamazepine.**



Using the chromatographic conditions described above, the selectivity was further evidenced by the ability of the assay to separate the drug and the internal standard from plasma samples without interference from any endogenous material with a good separation and resolution of their peaks. This is clearly indicated from Fig. 3.

Figure 3 shows representative chromatograms of low and high concentrations of CBZ in intestinal sac samples.



### 3.2. Method validation

The linearity of the assay method was evaluated by seven-point standard curves; with concentration range selection of (3.125–50 µg/ml) based on drug concentration. These standards

were analyzed in replicates of the range mentioned above. Standard curves were constructed over a 6-week period to determine the variability of the slopes and intercepts. The results show little day-to-day variability in the slopes and intercepts. The mean ( $\pm$ standard deviation; SD) regression equation for replicated calibration curves constructed on different days was determined using the least-squares linear regression analysis method. Standard calibration

curves reflected good linearity of the assay in the concentration range of 3.125–50 µg/ml. The linear regression of the calibration curve produced an equation of ( $y=13474+16969$ ), with a correlation coefficient of 0.997 ( $R^2$ ). The inter-day and intraday accuracies were expressed as the closeness to the true value and are calculated as the percent recovery related to the nominal values. Tables 1 and 2 present the percentage of drug recovered relative to the nominal values.

Table1. Inter day validation parameters (n=3)

Added con(µg/ml)	Recovered con(µg/ml)	S.D	% Recovery
0.00	0.00	0.00	0.00
3.12	3.16	0.22	103.34
6.25	6.36	0.75	101.77
12.50	12.15	0.81	95.18
25.00	24.22	0.69	97.19
50.00	49.16	1.19	95.83

**Table2. Intra-day validation parameters (n=3)**

Added con(µg/ml)	Recovered con(µg/ml)	S.D	% Recovery
0	0	0	0
3.13	3.09	0.14	97.96
6.25	6.14	0.71	95.36
12.50	11.91	0.71	93.18
25.00	25.22	0.71	104.49
50.00	52.16	2.12	101.47

The recovered values were close to the true value suggesting the accuracy of the assay. The % recoveries of CBZ were ranged from 95.18 to 103.34% and 93.18% to 104.47% for interday and intraday, respectively. These high values of the % drug recovered reflect the accuracy of the assay method. The interday and intraday precisions were measured as the relative standard deviation (SD) expressed as percentage over the concentration range of CBZ during the course of validation. This is presented in Tables 1 and 2 for the interday and intraday precisions. The results indicated an acceptable precision for all concentrations assayed for both intraday and inter-day samples. The SD of CBZ ranged from 0.22 to 1.19 and 0.14 to 2.12 for both inter-day and intraday precisions, respectively. The low values of RSD% reflect the precision of the assay method. The LOQ for this method was found to be 0.5µg/ml. This indicated from the precision and accuracy of such concentration. Both accuracy and precision values throughout the concentration range (0.5–40 µg/ml) were acceptable (ICH Guideline Q2 (R1), 2005). The specificity of the method with regard to CBZ was ascertained by the absence of any co-eluted or chromatographic interference peaks (Figs. 2 and 3).

#### 4. CONCLUSION

The HPLC method developed in this article is rapid, sensitive, and specific. The accuracy and

precision of the method are within the acceptable range (ICH Guideline Q2 (R1), 2005).

The simplicity of technique and the high sensitivity make this technique particularly attractive for the quantification of CBZ in both solution and intestinal sac samples. The method can also be readily adapted to routine quality control analysis.

#### 5. REFERENCES

- Ashy, A.R., El Sayed, Y.M., Islam, S.I., 1986. Comparison of fluorescence polarization immunoassay and high performance liquid chromatography for the quantitative determination of phenytoin, phenobarbitone and carbamazepine in serum. *J. Pharm. Pharmacol.* 38, 572–577.
- Auer, M.E., Griesser, U.J., Sawatzki, J., 2003. Qualitative and quantitative study of polymorphic forms in drug formulations by near infrared FT-Raman spectroscopy. *J. Mol. Struct.* 661–662 (1–3), 307–317.
- Bhatti, M.M., Hanson, G.D., Schultz, L., 1998. Simultaneous determination of phenytoin, carbamazepine, and 10,11-carbamazepine epoxide in human plasma by high-performance liquid chromatography with ultraviolet detection. *J. Pharm. Biomed. Anal.* 16, 1233–1240.
- Breton, H., Cociglio, M., Bressolle, F., Peyriere, H., Blayac, J.P., Hillaire-Buys, D., 2005. Liquid chromatography–electrospray mass spectrometry determination of carbamazepine, oxcarbazepine and eight of their metabolites in human plasma. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* 828, 80–90.
- Budakova, L., Brozmanova, H., Grundmann, M., Fischer, J., 2008. Simultaneous determination of antiepileptic drugs and their two active metabolites by HPLC. *J. Sep. Sci.* 31, 1–8.
- Burke, J.T., Thenot, J.P., 1985. Determination of antiepileptic drugs. *J. Chromatogr.* 340, 199–241.



- Chelberg, R.D., Gunawan, S., Treiman, D.M., 1988. Simultaneous high-performance liquid-chromatographic determination of carbamazepine and its principal metabolites in human plasma and urine. *Ther. Drug Monit.* 10, 188–193.
- Chen, K., Bashi, H.K., 1991. Comparative analysis of antiepileptic drugs by gas chromatography using capillary or packed columns and by fluorescence polarization immunoassay. *J. Anal. Toxicol.* 15 (2), 82–85.
- Dasgupta, A., Vega, A.E., Wells, A., Datta, P., 1999. Effect of heating human sera at a temperature necessary to deactivate human immunodeficiency virus on measurement of free phenytoin, free valproic acid, and free carbamazepine concentrations. *Ther. Drug Monit.* 21, 421–425.
- Duche, P., Loiseau, B., 1995. In: Levy, R.H., Mattson, R.H., Meldrum, B.S., Penry, J.K., Dreyfuss, F.E. (Eds.), *Antiepileptic Drugs*, fourth ed. Raven, New York, p. 555.
- Elizabeth, G.-S., Darla, R.L., Mohamed, A.V., Matthew, D.K., 2007. Simultaneous determination of lamotrigine, zonisamide, and carbamazepine in human plasma by high-performance liquid chromatography. *Biomed. Chromatogr.* 21 (3), 225–228.
- Escandar, G.M., Gonzá lez Go´ mez, D., Espinosa Mansilla, A., Mun˜ oz de la Pen˜ a, A., Goicoechea, H.C., 2004. Determination of carbamazepine in serum and pharmaceutical preparations using immobilization on a nylon support and fluorescent detection. *Anal. Chim. Acta* 506, 161–170.
- Franceschi, L., Furlanut, M., 2005. A simple method to monitor plasma concentration of oxcarbamazepine, carbamazepine, their main metabolites and lamotrigine in epileptic patients. *Pharmacol. Res.* 51, 297–302.
- Goodman, A.G., Hardman, J.G., Limbrid, L.E., 2001. Goodman and Gilman's the Pharmacological Basis of Therapeutics, 10th ed. McGraw Hill, New York.
- Hartley, R., Lucock, M., Cookman, J.R., Becker, M., Forsythe, W.I., 1986. High-performance liquid-chromatographic determination of carbamazepine and carbamazepine-10,11-epoxide in plasma and saliva following solidphase sample extraction. *J. Chromatogr.* 380, 347–356.
- He, J., Shibukawa, A., Nakagawa, T., 1992. Direct injection analysis of carbamazepine and its active 10,11-epoxide metabolite in plasma by use of a semipermeable surface (SPS) silica column in LC. *J. Pharm. Biomed. Anal.* 10 (4), 289–294.
- Hemenway, J.N., Jarho, P., Henri, J.T., Nair, S.K., Velde, D.V., Georg, G.I., Stella, V.J., 2010. Preparation and physicochemical characterization of a novel water-soluble prodrug of carbamazepine. *J. Pharm. Sci.* 99 (4), 1810–1825.
- Huang, C., He, Q., Chen, H., 2002. Flow injection photochemical spectrofluorimetry for the determination of carbamazepine in pharmaceutical preparations. *J. Pharm. Biomed. Anal.* 30(1), 59–65.
- ICH Guideline Q2 (R1), 2005. Validation of Analytical Procedures: Text and Methodology.
- Juergens, U., 1984. Routine determination of eight common antiepileptic drugs and metabolites by high-performance liquid chromatography using a column-switching system for direct injection of serum samples. *J. Chromatogr.* 310, 97–106.
- Lancas, F.M., Sozza, M.A., Queiroz, M.E.C., 2003. Simultaneous plasma lamotrigine analysis with carbamazepine, carbamazepine-10,11-epoxide, primidone, phenytoin, phenobarbital, and PEMA by micellar electrokinetic capillary chromatography (MECC). *J. Anal. Toxicol.* 27, 304–308.
- Lee, S.H., Li, M., Suh, J.K., 2003. Determination of carbamazepine by chemiluminescence detection using chemically prepared tris(2,2Vbipyridine) ruthenium(III) as oxidant. *Anal. Sci.* 19, 903–906.
- Liu, H., Delgado, M., Forman, L.J., Eggers, C.M., Montoya, J.L., 1993. Simultaneous determination of carbamazepine, phenytoin, phenobarbital, primidone and their principal metabolites by high-performance liquid chromatography with photodiode-array detection. *J. Chromatogr.* 616, 105–115.
- Mennickent, S., Fierro, R., Vega, M., de Diego, M., Godoy, C.G., 2009. Instrumental planar chromatographic method for determination of carbamazepine in human serum. *J. Sep. Sci.* 32 (9), 1454–1458.
- Miao, X.-S., Metcalfe, C.D., 2003. Determination of carbamazepine and its metabolites in aqueous samples using liquid chromatography–electrospray tandem mass spectrometry. *Anal. Chem.* 75, 3731–3738.
- Oh, E.K., Ban, E., Woo, J.S., Kim, C.-K., 2006. Analysis of carbamazepine and its active metabolite, carbamazepine-10,11-epoxide, in human plasma using high-performance liquid chromatography. *Anal. Bioanal. Chem.* 386, 1931–1936.
- Owen, A., Tettey, J.N., Morgan, P., Pirmonhamed, M., Park, B.K., 2001. LC determination of carbamazepine in murine brain. *J. Pharm. Biomed. Anal.* 26 (4), 573–577.
- Patel, R.B., Patel, M.R., Bhatt, K.K., Patel, B.G., 2011. Development and validation of HPTLC method for estimation of carbamazepine in formulations and its in vitro release study. *Chromatogr. Res. Int.*, 8pp..
- Queiroz, R.H.C., Bertucci, C., Malfara´ , W.R., Dreossi, S.A.C., Chaves, A.R., Vale´ rio, D.A.R., Queiroz, M.E.C., 2008. Quantification of carbamazepine, carbamazepine-10,11-epoxide, phenytoin and phenobarbital in plasma samples

- by stir bar-sorptive extraction and liquid chromatography. *J. Pharm. Biomed. Anal.* 48, 428–434.
- Raggi, M.A., Casamenti, G., Mandrioli, R., Sabbioni, C., Volterra, V., 2000. A rapid LC method for the identification and determination of CNS drugs in pharmaceutical formulations. *J. Pharm. Biomed. Anal.* 23, 161–167.
- Rezaei, Z., Hemmateenejad, B., Khabnadideh, S., Gorgin, M., 2005. Simultaneous spectrophotometric determination of carbamazepine and phenytoin in serum by PLS regression and comparison with HPLC. *Talanta* 65, 21–28.
- Rouan, M.C., Campestrini, J., Le Clanche, V., Lecaillon, J.B., Godbillon, J., 1992. Automated microanalysis of carbamazepine and its epoxide and trans-diol metabolites in plasma by column liquid chromatography. *J. Chromatogr.* 573 (1), 65–68.
- Sanchez, A., Garcia, R., Abadin, J.A., Duran, J.A., 1999. Determination of free serum carbamazepine by protein precipitation with sulfosalicylic acid. *Pharm. Pharmacol. Commun.* 5, 435–438.
- Srinivasa, R.K., Belorkar, N., 2010. Development and validation of a specific stability indicating liquid chromatographic method for carbamazepine in bulk and pharmaceutical dosage forms. *J. Adv. Pharm. Res.* 1, 36–47.
- Van Rooyen, G.F., Badenhorst, D., Swart, K.J., Hundt, H.K., Scanes, T., Hundt, A.F., 2002. Determination of carbamazepine and carbamazepine-10,11-epoxide in human plasma by tandem liquid chromatography–mass spectrometry with electrospray ionisation. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* 769 (1), 1–7.
- Wad, N.J., 1984. Simultaneous determination of eleven antiepileptic compounds in serum by high-performance liquid chromatography. *J. Chromatogr.* 305, 127–133.
- Yoshida, T., Imai, K., Motohashi, S., Hamano, S., Sato, M., 2006. Simultaneous determination of zonisamide, carbamazepine, carbamazepine-10,11-epoxide in infant serum by high-performance liquid chromatography. *J. Pharm. Biomed. Anal.* 41, 1386–1390.
- Zhu, Y., Chiang, H., Wulster-Radcliffe, M., Hilt, R., Wong, P., Kissinger, C.B., Kissinger, P.T., 2005. Liquid chromatography/ tandem mass spectrometry for the determination of carbamazepine and its main metabolite in rat plasma utilizing an automated blood sampling system. *J. Pharm. Biomed. Anal.* 38 (1), 119–125.



**\*Corresponding Author:**

[devandlaadukondalu@gmail.com](mailto:devandlaadukondalu@gmail.com)