CHAPTER 3

Materials and Methods

3.1 Materials and Instruments

1. Acetonitrile HPLC grade	J.T.Baker USA.
2. Methanol HPLC grade (Analar®)	Lab Scan Co, Ltd. Thailand
3. Chloroform AR grade	Merck KgaA Germany
4. Tribasic sodium phosphate	E. Merck Germany
5. Standard carbamazepine powder	Sigma chemical Co. USA.
6. Standard propylparaben powder	
7. Disposable syringe (Nipro®)	Nissho Nipro Corporation Ltd.
8. Needle	Nissho Nipro Corporation Ltd.
9. Dispossible latex glove size S	
10. Cotton ball (Clinic®)	Thai gauze Co, Ltd.
11. Gauze	Thai gauze Co, Ltd.
12. Heparin 5,000 IU	Leo Ltd
13. Microcentrifuge tube 1.5 ml	Fabrik Fur Laborgerate Laboratory Equipment
14. Volumetric flask 50 ml	Glaswerk wertheim Western Germany
15. Volumetric flask 10 ml	Schott Duran Germany
16. Beaker 250 ml	Pyrex
17. Beaker 500 ml	Pyrex
18. Adjustable micropipet 100-1000 mcl	Glison France (Pipetman®)
19. Adjustable micropipet 1-100 mcl	Glison France (Pipetman®)
20. Vial for HPLC instrument	Hewlett Packard, USA.
21. Trip	Axygen Inc.
22. Filter	Schott Duran, Germany
23. Membrane filter.	Hewlett Packard, USA.

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3.2 Apparatus

1. HPLC (Hewlett Packard Serie 1100, Hewlett Packard, Germany)
2. Vertex Evaporator (HBI vortex-Evaporator®, Bucher Instrument)
3. Centrifuge (Sigma 302 K, Laboratory Centrifuges GmbH, W-Germany)
4. Sonicator (Sonorex RK 5105, Bandelin Electronic W-Germany)
5. Vaccuum pump (Gast, DOAP 104-BN, Bentor Harber, USA.)

3.3 HPLC System

Column :	Hypersil ODS C-18, 250*4.6 mm, particle size 5 mcm
Mobile phase :	water : actonitrile (50:50)
Flow rate :	1.5 ml / min
Injection volume :	20 mcl
Detector :	UV set at 280 nm
Running time :	6 min / injection

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3.4 Methods

3.4.1 Subjects

The subjects of this study are the patients who were taking CBZ for at least 1 month and were treated in Maharaj Nakorn Chiang Mai Hospital, Neurological Hospital and Salaklang Medical Clinic. The patients who did not have saliva were excluded. Every patient was clearly explained about the objectives of the study and willingly join with this study.

3.4.2 Sample collection techniques

Blood samples were collected about 5 ml by venipuncture and transferred into heparinized tubes. Then plasma samples were separated by centrifugation at 3,000 rpm for 10 minutes. After separation, the plasma samples were kept in a microcentrifuge tube. The saliva samples were collected simultaneously with the blood samples by insert a cotton ball between gum and buccal for 5 minutes. Then the cotton ball was transferd to 10 ml syringe and pressed to separate saliva from the cotton ball. The saliva samples were kept in a microcentrifuge tubes. The patients should wash their mouth and do not eat anything at least 1 hour before the saliva samples collection in order to prevent contamination. Both plasma and saliva were immediately frozen, until time for analysis, which would be performed within 7 days after sampling.

3.4.3 Extraction and assay procedure

The plasma and saliva samples were thawed to room temperature and then 250 mcl of the plasma or saliva sample were transferred into 1.5 ml microcentrifuge tube. 50 mcl of saturated tribasic sodium phosphate was added and vigorously vortexed about 1 minute. 700 mcl chloroform was added to each tube and vortexed 3 minutes. The samples were centrifuged at 7,000 rpm for 5 minutes and the aqueous phase was aspirated. Then 500 mcl of the organic phase (chloroform) was transferred into clean sample vial and evaporated to dryness under reduced pressure at 42°C. The residues were reconstituted with 200 mcl of 5 mcg/ml propylparaben (PP) for plasma sample and 200 mcl of 2.5 mcg/ml PP were used for saliva sample. Only 20 mcl of aliquot was injected into HPLC system.

3.4.4 Standard curve

The standard CBZ spiked plasmas were prepared with the following concentrations; 0, 0.5, 1.0, 2.0, 6.0 and 12.0 mcg/ml. And those of saliva were 0, 50, 100, 500, 1000 and 2000 ng/ml.

They were extracted and analyzed by the method as mentioned above. Peak area ratio of CBZ to PP of each concentration was calculated and plotted with concentration.

3.4.5 Accuracy and precision of the assay procedure

Three sets of standard CBZ spiked plasma and saliva were analyzed on the same day for three consecutive days. Then statistic analysis was used to investigate interday and intraday variation of analysis method. Percent recovery was also done to check the accuracy of analysis method too.

3.4.6 Stability of CBZ in frozen plasma and saliva

Standard CBZ spiked plasma and saliva with various concentrations were prepared and analyzed and then kept in refrigerator at $-40^{\circ}C$ for 7 days. After 7 days storage, the samples were analyzed again and compared their peak area ratio to those of the first day analysis.

3.4.7 Data Analysis

Linear regression analysis was used to investigate the correlation between plasma and saliva concentrations.

3.4.8 Application to patients

Blood and saliva collection procedure were the same as in volunteers. The saliva CBZ concentrations of the other 10 patients were substituted into the saliva-plasma concentration equation from volunteers to predict the plasma concentrations.

The predict plasma CBZ concentrations were compared to the actual plasma CBZ concentration of those patients and then calculated for percent accuracy.

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