Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **27** (4): 485-91 (2008)

Original Article

Received: April 30, 2007
Accepted: April 30, 2008

Micromethod for Quantification of Carbamazepine, Phenobartital and Phenytoin in Human Plasma by HPLC-UV Detection for Therapeutic Drug Monitoring Application

Cristina SANCHES [1], Karin V. LÓPEZ [1], Celia E. OMOSAKO [2], Maria A. BERTOLINE [2], Maria D. PEREIRA [2], & Silvia R.C.J. SANTOS [1]*

[1] *Department of Pharmacy, School of Pharmaceutical Sciences, University of Sao Paulo, Av. Professor Lineu Prestes, 580, Sao Paulo, SP, 05508-000, Brazil*

[2] *Instituto do Coração Hospital das Clinicas, Medical School, University of Sao Paulo, Av. Dr. Enéas de Carvalho Aguiar, 44 / CEP: 05403-000 - São Paulo - SP , Brazil*

SUMMARY. A simple, rapid, selective and sensitive analytical method by HPLC with UV detection was developed for the quantification of carbamazepine, phenobarbital and phenytoin in only 0.2 mL of plasma. A C18 column (150 x 3.9 mm, 4 micra) using a binary mobile phase consisting of water and acetonitrile (70:30, v/v) at a flow rate of 0.5 mL/min were proposed. Validation of the analytical method showed a good linearity (0.3 to 20.0 mg/L for CBZ, 0.9 to 60.0 mg/L for PB and 0.6 to 40.0 mg/L for PHT), high sensitivity (LOQ: 0.3, 0.9 and 0.6 mg/L respectively). The method was applied for drug monitoring of antiepileptic drugs (AED) in 27 patients with epilepsy under polytherapy.

RESUMEN. "Micrométodo para la Cuantificación de Carbamazepina, Fenobarbital y Fenitoina en Plasma Humano por CLAR- Detección UV para su Aplicación en Monitoreo Terapéutico". Se ha desarrollado un método analítico simple, rápido, selectivo y sensible por HPLC con detección ultravioleta para la cuantificación de carbamazepina, fenobarbital y fenitoína en sólo 0,2 mL de plasma. Se utilizó una columna C18 (150 x 3.9 cm, 4 micras) usando una fase móvil binaria constituida por agua y acetonitrilo (70:30 v/v) a una velocidad de flujo de 0,5 mL/min. La validación del método analítico mostró buena linearidad (0,3 a 20,0 mg/mL para CBZ, 0,9 a 60,0 mg/L para PB y 0,6 a 40,0 mg/L PHT), alta sensibilidad (LOQ: 0,3, 0,9 y 0,6 mg/L, respectivamente). El método fue aplicado para el monitoreo terapéutico de drogas en 27 pacientes epilépticos sometidos a politerapia.

INTRODUCTION

A single drug therapy for all patients is the desirable goal in the treatment of the epilepsy, but many practitioners know that around 50% of their patients require two drugs or more, which, in combination, may produce toxicity. Nevertheless, the compliance is a major problem related to patients who require long term treatment for the seizure disorder. Relationship between blood concentration and therapeutic effect have been established for antiepileptic drugs, for which can be evaluated through use of therapeutic drug monitoring, that is recommended for the clinical management of epileptic patients receiving carbama-zepine (CBZ), phenobarbital (PB) and phenytoin (PHT) [1,2].

Nevertheless, polytherapy may be necessary and pharmacokinetic interactions can occur, with significant changes in drug level, and unexpected toxicity [3,4]. It is established that the interaction between antiepileptic drugs is clinically significant and that these interactions depends on their metabolic enzymes. Dose administered and plasma level are sometimes unpredictable, since blood concentration of antiepileptic drugs (AED) may be affected by age, sex, variable absorption, autoinduction and heteroinduction [5].

Analytical methods reported for the determination of these drugs describe immunoassays, chromatographic approaches and techniques based upon capillary electrophoresis [2,3,5,7,9-12,14,15]. If a high percentage of patients will be on two drugs or more for the control of seizure, it is well know that which, in combination, may produce sedative and behavioural toxicity [16-19]. For the routine of therapeutic drug monitoring

KEY WORDS: Antiepileptic drugs, Analytical method , HPLC-UV, TDM application, Validation.
PALABRAS CLAVE: Drogas antiepilepticas, HPLC-UV, Método analítico, TDM aplicación, Validación.

* Author to whom correspondence should be addressed. *E-mail:* pharther@usp.br

the assay must be rapid, simple, selective and low cost. High-performance liquid chromatography (HPLC-UV) presents efficiency, rapid purification of biological matrices and relative low cost; also this technique has the potential for the simultaneous multi-drug measurements [7,8]. Simultaneous multiple drug analysis is the most rapid and cost-effective approach for drug monitoring in plasma of epileptic patients under treatment with several drugs [3].

In the present study, an isocratic reversed-phase HPLC-UV method is reported for measurements of CBZ, PB and PHT in human plasma of 27 epileptic patients under polytherapy with these AED.

MATERIALS AND METHODS

Clean-up of plasma samples

All solvents and reagents for the clean up and chromatographic analysis HPLC grade were purchased from Merck (Darmstadt, Germany) and drug standards carbamazepine (CBZ), phenytoin (PHT), phenobarbital (PB) and methyl-phenyl-phenytoin (structural analog of phenytoin) were purchased from Sigma (St. Louis, USA). Methyl-phenyl-phenytoin was applied as external standard for CBZ plasma measurements or internal standard for simultaneous analysis of PHT and PB in plasma.

For the determination of plasma levels of AED, two liquid-liquid sequential extractions was performed with 2 mL methylene dichloride. On the first extraction, for CBZ measurements, 0.2 mL of serum was added to a glass extraction tube (11 x 1.5 cm), alkalinised with 0.02 mL of 1.25 M sodium hydroxide and extracted with methylene dichloride, vortex mixed for 1 min and the phases separated in a centrifuge 3000 g at 4 °C for 40 min. The organic layer was immersed in a nitrogen bath and the extract was transferred to the conical tubes containing 0.02 mL/assay (ES) (methanolic solution 40 mg/L), evaporated in the water bath at 40 °C to dryness under a gentle stream of nitrogen. Aliquots of alkaline plasma aqueous phase (0.2 mL supernatant of the first extraction) were transferred to extraction tubes containing dried IS (0.02 mL/assay), acidified with the same volume of 0.3M NaH_2PO_4 and then extracted with 2 mL methylene dichloride in vortex mixer for 1 minute. The tube from the acidic extraction, was centrifuged at 3000 g for 40 min, immersed in a liquid nitrogen bath, the extract was transferred to the conical tubes, the solvent evaporated to dryness under a gentle stream of nitrogen at 40 °C.

Chromatography

In parallel, the same procedure was utilised daily for the preparation of the calibration curve, internal controls (high, medium and low) and unknown samples. Both residues from acidic and alkaline extractions were dissolved with 0.2 mL of mobile phase, transferred to microvials on the rack of an autosampler and volumes of 0.01 mL were automatically injected into a SIL 10AV, Shimadzu (Kyoto, Japan) liquid chromatograph connected to a CBM 101 controller, using a software LC10. A LC-10 pump was connected to a C18 Nova Pak column (150 x 3.9 mm, 4 micra, Waters Assoc., Milford, USA) protected by a C18 Nova Pak inserts (4 x 4 mm, Waters Assoc., Milford, USA). For the chromatographic separation, a binary mobile phase consisting of water and acetonitrile (70:30, v/v) was pumped at a flow rate of 0.5 mL/min. The peaks were monitored with an ultraviolet detector (Shimadzu SDP 10 AV) at 210 nm. Areas of the peaks were integrated using the Class VP software from Shimadzu and an integrator CR6A (Shimadzu) and peak area ratios were plotted against drug plasma concentrations to obtain the day calibration curve.

Validation of the analytical method

The working solution containing a mixture of three AED was prepared before use from the stock solution of AED (10 mg/mL) by dilution with methanol. The calibration curve was prepared from spiked drug-free human plasma (blank) by adding of the working solution containing a mixture of CBZ, PB and PHT, and stored at -20º until assay. Standard solution series concentrations of CBZ were 20.0, 15.5, 10.0, 5.0, 2.5, 1.25, 0.62, 0.31 and 0.15 mg/L; concentrations of PB were 60.0, 45.0, 30.0, 15.0, 7.5, 3.75, 1.87, 0.94 and 0.47 mg/L; and of PHT were 40.0, 30.0, 20.0, 10.0, 5.0, 2.5, 1.25, 0.62 and 0.31 mg/L. The internal standard (IS), methyl-phenyl-phenytoin (1 mg/mL, methanolic stock solution, 40 mg/L, working solution) was prepared in methanol and stored at -20 °C.

The nominal value of CBZ, DPH and PB in plasma was plotted as a function of the peak area ratio obtained for each drug and its internal standard, and the linear correlation coefficient ($r^2 > 0.99$) and its respective equation $Y = AX + B$ were obtained. At least five calibrators were considered for the construction of the daily calibration curve.

Internal quality controls (for CBZ were 8.0, 4.0 and 0.8 mg/L; for PB were 24.0, 12.0 and 2.4

mg/L; and for PHT were 16.0, 8.0 and 1.6 mg/L) were prepared in duplicate and analysed during each analytical run to guarantee the acceptance of day calibration curve. A daily run was accepted when at least four of the six controls showed a deviation lower than 15% (high, medium and low quality control) compared to their respective nominal value. At least one control of each concentration must be within the acceptable deviation. Once accepted the calibration curve was used for CBZ, PHT and PB measurements in plasma samples from the patients.

The linearity of the method was determined in triplicate using ten CBZ, PHT and PB concentrations. The limits of detection (LOD) and of quantification (LOQ) were determined based on the analysis of 10 replicates. The limit of quantification was defined as the lowest plasma concentration of the calibration daily curve quantified with acceptable precision and accuracy. The limit of detection was defined as 0.5 times of the limit of quantification. In addition, the limit of quantification has a ratio of 5:1. Absolute recovery, reported as percentage, was determined using three drug concentrations, for CBZ were: 8.0, 4.0 and 0.8 mg/L; for PB were 24.0, 12.0 and 2.4 mg/L; and for PHT were 16.0, 8.0 and 1.6 mg/L, in five replicates. The accuracy of the method was evaluated using three concentrations (low, medium and high) six replicates, and the systematic error of the assay was determined based on the percentage of inaccuracy; for accuracy and precision the mean value should be within 15% of the actual value, except at LOQ, where it should not deviate by more than 20% .

Intraday accuracy or intraday precision were evaluated based on replicate analysis of three concentrations (n = 6) in the same day, while for the determination of inter-day precision analysis (duplicates) of three concentrations for each drug (8.0, 4.0 and 0.8 mg/L for CBZ, 24.0, 12.0 and 2.4 mg/L for PB, and 16.0, 8.0 and 1.6 mg/L for PHT) were carried out on three consecutive days (n = 18).

The stability of CBZ, PB and PHT in the plasma and in the organic extracts before the chromatography was also investigated by the concentrations of all the stability samples compared to the mean of back-calculated values for the standards and the appropriate concentrations for the first day of stability testing. Probability, $p > 0.05$ value was adopted as an acceptance criterion for all concentrations studied. Analysis consisted of the evaluation of stability after three freeze-thaw cycles of the drug in biological matrices; samples of three AED concentrations (8.0, 4.0 and 0.8 mg/L for CBZ; 24.0, 12.0 and 2.4 mg/L for PB and 16.0, 8.0 and 1.6 mg/L for PHT) were analysed in triplicate.

In addition, the short-term stability of the drug was determined for the time and conditions under which the analyses were performed. For this study, three samples of each AED (8.0, 4.0 and 0.8 mg/L for CBZ, 24.0, 12.0 and 2.4 mg/L for PB, and 16.0, 8.0 and 1.6 mg/L for PHT) were required.

The robustness of the method was determined, after analysis of 6 replicates, based on the use of two different analytical C18 columns and small variations in the proportion of constituents and little changes on the flow rate of the mobile phase. The results were expressed as a function of the coefficient of variation. An influence of variation in the room temperature was excluded since the temperature of the laboratory was monitored daily and kept at 20 ± 2 °C.

Therapeutic Drug Monitoring - Patients Specimens

The clinical protocol was approved by the Ethical Committee of the Hospital and of the School of Pharmaceutical Sciences / University of Sao Paulo. Therapeutic drug monitoring was applied to 27 epileptic patients adults, without renal, hepatic, cardiac or endocrine dysfunctions under long term polytherapy with the antiepileptic drugs (CBZ, PB, PHT). Patients were informed by the physician in details about the procedures as drug administration, blood collection for therapeutic drug monitoring before the next clinical evaluation. In the morning, before drug administration, in the rest room of ambulatory, venous blood samples were withdrawn directly to tubes (Vacutainer: BD, Becton Dickinson and Company, MG, Brazil) containing sodium EDTA. After centrifugation (3000 g for 30 min), the plasma was stored in a freezer at -20 °C until assay. The samples were analysed using the above described HPLC method. For therapeutic drug monitoring, 6 calibrators, internal controls (replicates, n = 2) and samples from patients were analysed daily.

RESULTS

Validation of analytical method

The method described in the present study was validated based on confidence limits for the measurement of CBZ, PHT and PB in plasma (Table 1).

The specificity of the analytical method for

Confidence limits	CBZ	PB	PHT
Linearity	0.3 to 20.0 mg/L	0.9 to 60.0 mg/L	0.6 to 40.0 mg/L
Linear correlation, r^2	0.999	0.991	0.992
Linear equation	Y= 0.29x + 0.02	Y = 0.06x +0.13	Y = 0.07x + 0.09
Absolute recovery	98.5%	95.4%	98.4%
Sensitivity			
LOD	0.1 mg/L	0.4 mg/L	0.3 mg/L
LOQ	0.3 mg/L	0.9 mg/L	0.6 mg/L

Table 1. Linearity, recovery and sensitivity of the analytical method. LOQ: limit of quantification. LOD: limit of detection.

CBZ, PHT and PB in biological matrices was evaluated by the injection of extracts of normal (n = 2), lipemic (n = 2) and hemolysed (n = 2) plasma blanks into the chromatographic system. Endogenous components eluted from the chromatographic column did not interfered with the analysis under the conditions described above, once endogenous peaks on the chromatogram were eluted at the first minutes of run. Peaks eluted from the chromatographic column were illustrated in the Figure 1. Detection at 210 nm, guarantees the sensitivity and the total time required for each chromatographic run was 10 min. Once the selectivity of chromatographic analysis is maintained after the quite simple clean up of plasma samples in alkaline or acidic medium, ten minutes for each run is reasonable to quantify CBZ, PHT, PB in plasma samples at a relative low cost.

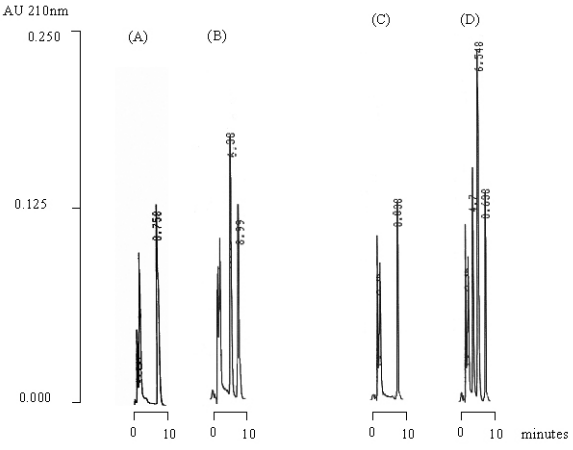

Figure 1. Chromatographic profile of AED in plasma extracts. Chromatograms of alkaline extraction/Run 1 (A) Blank plasma extract. (B) Standard plasma extract. Chromatograms of acidic extraction/Run 2 (C) Blank plasma extract. (D) Standard plasma extract. Peaks (**Run 1**): CBZ (1mg/L 6.4 min), IS: 8.9 min; (**Run 2**): PB (3 mg/L 4.7 min), PHT (6 mg/L 6.5 min) and methyl phenyl phenytoin: 8.8 min.

Peak areas were integrated for CBZ, PHT, PB and the peak area ratio to methyl-phenyl-phenytoin was required for the construction of the daily calibration curve considered for the determination of CBZ, PHT, PB in plasma samples obtained from the patients monitored. In addition, the acceptation of the daily calibration curve is based on three internal controls (low, medium and high concentrations) analysed in parallel. Absolute recovery, reported as mean percentage, was determined using three drug concentrations, for CBZ were 98.5% (0.8-8.0 mg/L, for PB were 95.4% (2.4-24 mg/L), and for PHT were 96.7% (1.6-16 mg/L), Table 1.

The present procedure showed high sensitivity (LOQ: 0.3, 0.9 and 0.6 mg/L and LOD: 0.15, 0.45 and 0.3 mg/L for CBZ, PB and PHT respectively), good linearity between 0.3 a 20 mg/L for CBZ, 0.9 a 60 mg/L for PB and 0.6 a 40 mg/L for PHT with a linear correlation coefficient of 0.999, 0.991 and 0.992 respectively as illustrated in Figure 2 and Table 1. Good recovery and acceptable accuracy and precision were obtained; in addition the stability of drugs in the biological matrices (Table 1 and 2). The robustness of the method for CBZ, PB and for PHT measurements was determined based on some small variation in the chromatographic system using two analytical C18 columns (1.35%), 1 to 2% acetonitrile in the mobile phase (0.11%) and 0.05 mL/min changes on the flow rate of mobile phase (8.7%).

Three plasma concentrations (low, medium and high) were used for each assay. In addition when different analytical C18 columns were compared, data obtained expressed also by the Coefficient of variation were: -0.28% to 3.4% for CBZ, -6.0% *versus* 7.6% (CV%) for PB, -5.5% to 4.2% for PHT. Variations of 1-2% of acetonitrile in the original mobile phase (water:acetonitrile, 7:3, v/v) showed coefficients of variation equivalent to 4.8% to 3.4% for CBZ, 0.77% to 7.6% for PB, and -4.9% to 4.2% for PHT. In addition,

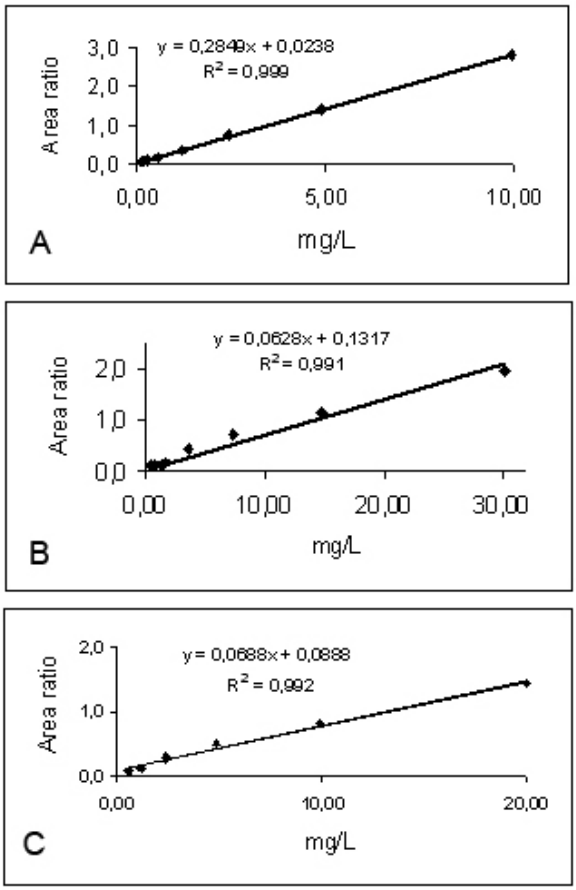

Figure 2. Calibration day curve for AEDs measurements: **A**) Carbamazepine **B**) Phenobarbital **C**) Phenytoin. Average of duplicates.

when little changes on the flow rate of the mobile phase were tested, coefficients of variation ranged from 3.4 to 12.1% (CBZ), 7.6 to 10.2% (PB) and 4.2 to 14.9% (PHT).

The stability of CBZ, PB and PHT in the plasma and in the organic extracts before the chromatography were also investigated by the concentrations of all the stability samples compared to the mean of back-calculated values for the standards and the appropriate concentrations for the first day of stability testing; probability, p value adopted as an acceptance criterion for all concentrations studied are showed in Table 2. Analysis of the short-term stability of CBZ, PB and PHT, time and condition of assay didn't show any reduction on plasma concentrations of AED drugs in the organic extract after chromatographic analysis within a period of 24 hours (vials on a tray). Additionally, good stability for CBZ, PB and PHT in the biological matrices was showed after three consecutive freeze-thaw cycles since no significant changes were observed, $p > 0.05$, Table 2.

Therapeutic monitoring Application

Analytical method validated was applied to routine analysis of 27 patients under polytherapy for therapeutic drug monitoring of CBZ, PB and PHT. Analysis was performed using the daily plasma calibration standards, three different plasma internal controls (low, medium and high concentrations, duplicates) and the samples from patients.

Plasma concentration of drugs (AED) for patients were expressed as mean value (IC95%) for CBZ 3.5 (2.1-4.9) mg/L, PB 31.3 (21.8-40.6) mg/L and PHT 26.8 (16.8-35.7) mg/L and presented on Table 3 and in Figure 3.

Considering the therapeutic range for carbamazepine (4-12 mg/L), plasma levels obtained from patients under polytherapy were within the range only for 29% (8/27) patients. It is important to point out that the drug plasma concentrations were lower than 4 mg/L for 67% (18/27) patients. Only one patient (4%:1/27) showed plasma level higher than 12 mg/L.

Considering the therapeutic range for phenobarbital (10-30 mg/L), trough plasma levels obtained from patients under polytherapy were within the range for only 30% (8/27) patients,

	CBZ (mg/L)			PB (mg/L)			PHT (mg/L)		
	8.0	**4.0**	**0.8**	**24.0**	**12.0**	**2.4**	**16.0**	**8.0**	**1.6**
Precision (%)									
Within-day	7.4	0.3	5.6	3.8	0.1	7.9	0.1	0.1	13.7
Inter-day	10.9	5.7	5.2	2.9	11.5	6.3	11.7	0.1	11.5
Accuracy (systematic error)									
(%)	7.2	1.9	10.5	1.9	7.3	4.4	7.6	5.8	3.7
Stability*									
Freezer-thaw	0.0717	0.6372	0.2238	0.2053	0.6421	0.1147	0.3582	0.3652	0.6547
Autosampler/24h	0.3436	0.1328	0.7888	0.8882	0.8315	0.5504	0.4359	0.1588	0.5091

Table 2. Precision, accuracy and stability studies from the validation of analytical method. *Test t Student, statistical significance $p<0.05$, probability of obtained data from each period investigated based on the day curve against the nominal value; $p>0.05$, not significant difference.

Patients, n=27	CBZ	PB	PHT
Mean (mg/L)	3.5	31.3	26.2
SEM (mg/L)	0.8	5.1	5.1
CI95% (mg/L)	2.1-4.9	21.8-40.6	16.8-35.7
Therapeutic range (mg/L)	4 -12	10-30	10-20

Table 3. Plasma concentration of AED in 27 adults patients under long term polytherapy, data expressed as mean and SEM, CI 95%. CBZ: carbamazepine, PHT: phenytoin and PB: phenobarbital. SEM: Standard error of mean, CI95%: confidence interval 95%.

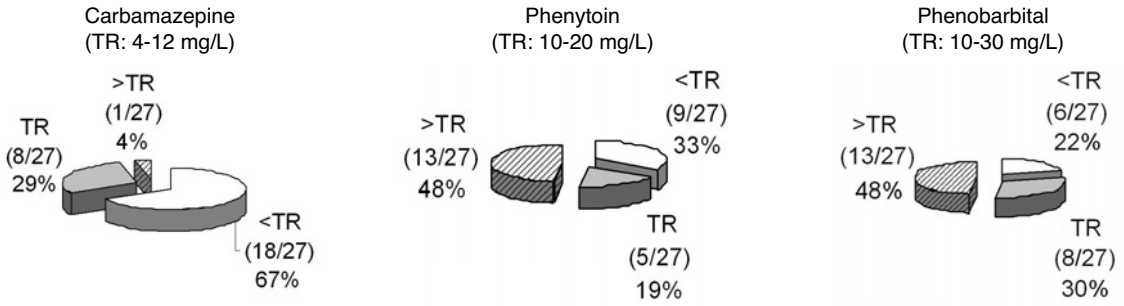

Figure 3. Plasma concentrations of AED, expressed as percentage of patients under polytherapy with carbamazepine, phenytoin and phenobarbital n=27: data obtained within the therapeutic range (TR), plasma concentrations lower than TR (value < TR), and plasma concentrations higher than TR (value > TR).

while 70% of drug levels from patients were out of therapeutic window as follows: plasma concentrations were higher than 30 mg/L for 48% (13/27) patients and 22% (6/27) patients showed subtherapeutic levels lower than 10 mg/L for phenobarbital. When phenytoin was considered (10-20 mg/L), only 19% (5/27) patients were in the range, while for 81% (22/27) of patients, the plasma levels were out of the therapeutic range as follows 48% (13/27) higher than 20 mg/L and 33% (9/27) lower than 10 mg/L.

DISCUSSION

Various methods for the determination of AED in plasma using HPLC-UV (high-performance liquid chromatography with ultraviolet detection) have been described previously [2,3,5,7,9-12,14,15]. The volume of the biological sample required for the quantification of a drug is a decisive factor for the choice of an analytical method. However, most methods reported previously have employed a volume of 0.5 - 1.0 mL of plasma or whole blood to determine CBZ, PHT and PB by HPLC-UV.

The method described in the present study requires only 0.2 mL of biological sample for the analysis of CBZ, PHT and PB in plasma which consists of a relative rapid, simple and low-cost analytical procedure, using a purification by liquid-liquid extraction. Furthermore, the method showed absolute recovery higher than 95%, good linearity, precision, accuracy and also high sensitivity (LOQ: 0.3, 0.9 and 0.6 mg/L and LOD: 0.15, 0.45 and 0.3 mg/L for CBZ, PB and PHT, respectively). It should be emphasised that robustness was not investigated in the procedures previously published.

For the preparation of biological matrices several methods were reported. A liquid-liquid extraction was preferred [7,9,12,14,15] instead of the precipitation of plasma protein for CBZ, PHT and PB measurements [2,3,5,10,11]. The main advantage of the liquid-liquid extraction is the higher selectivity as a consequence of the reduction of endogenous substances in the organic extract increasing also the sensitivity [7]. Furthermore, the direct injection of plasma on an automated chromatographic system reported without an internal standard compromises the recovery and precision of the assay [8]. Consequently, for the routine laboratory the automatic preparation of biological samples isn't cost effectiveness for therapeutic drug monitoring.

Chromatographic systems to determine CBZ, PHT and PB in plasma reported earlier describe very complex mobile phases at high flow rate (1.0 to 2.8 mL/min) [2,3,5,7,9-12,14,15]. Considering the large number of components in the mobile phase, the main disadvantages of data published previously are the reduction of the column life-

time, reproducibility impaired, and a low robustness of the method, besides the additional cost. The main advantage of the method described in the present study is related to the simplicity of chromatographic system consisting of reversed phase column C18 (Nova Pak), a binary mobile phase consisting of water and acetonitrile (70:30, v/v, 0.5 mL/min) at low flow rate, guaranteeing high selectivity and sensitivity, good accuracy, precision and also symmetry for the peaks of CBZ, PHT and PB and the internal standard. The analytical run lasts 10 min each, permitting a large number of assays for therapeutic drug monitoring purpose.

Data obtained in the present study from patients under long term polytherapy showed that therapeutic drug monitoring of these AED are cost effective since 70-80% of them presented drug plasma levels out of therapeutic windows.

CONCLUSION

The analytical method showed robustness, good linearity, sensitivity, accuracy and precision and good stability for measurements of CBZ, PB and PHT in plasma for routine assay. The analytical method to determine in small volumes of plasma these more commonly prescribed antiepileptic drugs was applied in 27 patients. Based on data obtained, therapeutic monitoring is cost effective and always required for dose adjustment for patients under long term therapy.

Acknowledgments. To Brazilian foundations (CNPq and FAPESP) for the financial support. To Mr. Edgar, M. Machado Jr, for the technical support in the Laboratory of Pharmacology and Therapeutics.

REFERENCES

1. Warner, A., M. Privitera & D. Bates (1998) *Clin. Chem.* **44**: 1085-95.
2. Patil, K.M. & S.L. Bodhankar (2005) *J. Pharmaceut. Biomed. Anal.* **39**:181-6.
3. Liu H., M. Delgado, L.J. Forman, C.M. Eggers & J.L. Montoya (1993) *J. Chromatogr. B* **616**: 105-15.
4. Manoj Babu, M.K.M. (2004) *J. Pharmaceut. Biomed. Anal.* **34**: 315-24.
5. Liu H., M. Delgado, S.T. Iannaccone, L.J. Forman & C. Eggers (1993) *Ther. Drug Monit.* **15**: 317-27.
6. Thormann W., R. Theurillat, M. Wind & R. Kuldvee (2001) *J. Chromatogr. A* **924**: 429-37.
7. Matar, K.M., P.J. Nicholls, A. Tecle, A.S. Bawazir & M.I. Al-Hassan (1999) *Therapeutic Ther. Drug Monit.* **21**: 559-66.
8. Kouno, Y., C. Ishikura, M. Homma & K. Oka (1993) *J. Chromatogr. B* **622**: 47-52.
9. Romanyshyn, L.A, J.K. Wichmann, N. Kucharczyk, R.C. Shumaker, D. Ward & R.D. Sofia (1994) *Ther. Drug Monit.* **16**: 90-9.
10. Ramachadran, S., S. Underhill & S.R. Jones (1994) *Ther. Drug Monit.* **16**: 75-82.
11. Ou, C. & C.L. Rognerud (1984) *Clin. Chem.* **30**: 1667-70.
12. Szabo, G.K., R.J. Pylilo, R.J. Perchalski & T.R. Browne (1990) *J. Chromatogr.* **535**: 271-7.
13. Babu, M.K.M. (2004) *J. Pharmaceut. Biomed. Anal.* **34:** 315-324.
14. Lensmeyer, G.L., B.E. Gidal & D.A. Wieb (1997) *Ther. Drug Monit.* **19**: 292-300.
15. Remmel, R.P., S.A. Miller & N.M. Graves (1990) *Ther. Drug Monit.* **12**: 90-6.
16. Eadie, M.J. (1998) *Brit. J. Clin. Pharmacol.* **46**: 185-93.
17. Richens, A. (1995) *Seizure* **4**: 211-214.
18. Stephen, L.J. & M.J. Brodie (2002) *Seizure* **11**: 349-51.
19. Kwan, P. & M.J. Brodie (2002) *Seizure* **11**: 77-84.