SIMULTANEOUS LIQUID CHROMATOGRAPHIC ANALYSIS OF
PHENOBARBITAL, PHENYTOIN AND CARBAMAZEPINE IN HUMAN SERUM
– AN APPLICATION TO THERAPEUTIC DRUG MONITORING.

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Summary

A rapid, sensitive and specific reverse phase-HPLC method with UV detection was developed and validated for the simultaneous estimation of three antiepileptic drugs (AEDs) phenobarbital (PB), phenytoin (PHT) and carbamazepine (CBZ) in human serum. Chromatography separation was carried out using Phenomenex, Luna C18 (250 x 4.6 mm, 5µ) as a stationary phase and the mobile phase consisted of 25mM disodium phosphate buffer (pH 7.0) and acetonitrile (68:32, v/v) with isocratic flow rate of 1 ml/ min. The assay was linear over the concentration range of 0.5 - 50µg/ml for all the three drugs. The method was convenient for estimation of phenobarbital (PB), phenytoin (PHT) and carbamazepine (CBZ) with absolute recoveries of 97.65±1.47, 98.13±0.56 and 99.67± 3.37 respectively. The method requires 11 minutes for analysis with good peak resolution and the LLOQ was 0.5µg/ml. The method was validated in terms of linearity, accuracy, precision, recovery, selectivity, and stability. The developed method was simple, convenient, and suitable for the analysis of AEDs in human serum. The method has been successfully applied for the therapeutic drug monitoring (TDM) of AEDs in epileptic patients.

Key Words: Phenobarbital, Phenytoin, Carbamazepine, RP-HPLC, Serum, Simultaneous determination.

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Introduction

Monitoring serum levels of antiepileptic drugs is important for optimizing drug therapy since there is a distinct relationship between serum levels and therapeutic effects, which has been clearly established for several drugs (1). Routine monitoring of serum levels is especially important since drug bioavailability as well as the drug absorption, metabolism and excretion, varies widely from patient to patient. Serum or plasma concentration monitoring is widely used for the clinical management of epileptic patients receiving phenytoin (PHT), phenobarbital (PB) and carbamazepine (CBZ) (Fig 1). An increase in the Phenyltoin dose usually gives an unpredictable increase in the serum level, thus monitoring is necessary to assure that optimal levels are maintained (2). Phenyltoin serum levels with in a range of 10-20 µg/ml gives satisfactory seizure control in most patients (3-4) and hence it is used as a first line agent in the treatment and prevention of tonic-clonic seizures control and other forms of epilepsy (5) Because of its narrow therapeutic range and saturation kinetics, Phenyltoin is one of the most suitable drugs for therapeutic drug monitoring (6) Carbamazepine has been shown to be as effective as Phenyltoin or Phenobarbital in the grand mal and complex partial seizures (7) Several clinical studies indicate that effective serum level of Phenyltoin, Phenobarbital and Carbamazepine range between 10-20 µg/ml, 15-40 µg/ml and 4-12 µg/ml respectively (8, 9).

The comprehensive therapeutic monitoring of anticonvulsant therapy is based on the availability of suitable method for measuring various combinations of these drugs. Several reversed phase liquid chromatographic methods have been reported in the literature (10-13) for the simultaneous estimation of commonly administered antiepileptic drugs. Earlier reported methods suffer from various shortcomings like longer analyzing time, tedious extraction procedure, complicated mobile phase and maintaining column temperature etc. These problems increase the analysis time, cumbersome extraction procedure and consume lot of reagents (10-17).

In the present study, the isocratic reversed phase HPLC–UV method for the simultaneous estimation of phenobarbital, phenytoin and carbamazepine in human serum was developed on C18 column with a total analytical time of less than 11 min. The developed method was applied for monitoring drug levels in epileptic patients. The study was carried out after obtaining approval from Institutional ethical committee of J.S.S College of Pharmacy and Government Head Quarters Hospital, Ootacamund, India.

Experimental

Chemicals and reagents
Reference standards of phenytoin and carbamazepine were received as a gift samples from Sun Pharmaceuticals (Mumbai, India) and phenobarbital from Sigma Chemicals, USA. All the solvents used were of HPLC grade (Ranbaxy Chemicals) and Milli-Q water (Millipore, India) was used to prepare the mobile phase. All other chemicals and reagents were of analytical grade.
Apparatus and chromatographic conditions
The liquid chromatographic system consisted of a Shimadzu LC-10AT pump, SIL-10A manual injector and SPD-10A UV-VIS absorbance detector (Shimadzu, Japan) integrated via Class VP Chromatography Data System Version 4.2 computer software. The chromatographic analysis was performed using a Luna Phenomenex C18 RP analytical column (250 mm x 4.6 mm), 5µ (Phenomenex). A Guard-Pak precolumn module (Phenomenex) containing an ODS cartridge insert was used. The sample was introduced through a Rheodyne injector valve with a 20 µL sample loop using 25 mM of disodium phosphate buffer (pH 7.0) and acetonitrile (68:32 v/v) as mobile phase with an isocratic flow rate of 1ml/min. The mobile phase was filtered through a 0.22 µm membrane filter, degassed in an ultrasonic bath and the eluate was monitored at 254 nm for all the three drugs.

Standard and working solutions
The Standard solutions of PB, PN, and CBZ containing 1000 µg/ml were prepared by dissolving in methanol. From the primary stocks the secondary stock solutions were prepared to get 5, 10, 50, 100, 200, 400 and 500 µg/ml of working solutions. All solutions were stored at 4° C until use.

Extraction procedure
Blood samples (10 ml) of volunteers were collected in evacuated glass tubes, was centrifuged at 11000 rpm for 5 minutes and the serum was separated and stored at –20°C until analysis. The 500 µL of human serum was taken in the 2ml of eppendorf centrifuge tubes and 500µl of deprotenizing agent (acetonitrile) was added and vortexed for 1 min and centrifuged at 11,000 rpm for 5 min. The 20 µL of clear supernatant was injected into the column.

Results and Discussion
The proposed HPLC method required only fewer reagents, less time and easier extraction procedure. The easy sample preparation and the use of HPLC technique make it an ideal quality control tool for regular pharmaceutical analysis and the therapeutic drug monitoring of epileptic patients.

Method development and optimization
The variation in the mobile phase leads to considerable changes in the chromatographic parameters, like peak symmetry and retention time. However, the ratio 25 mM of disodium phosphate buffer (pH 7.0) and acetonitrile 68:32 (v/v) yielded best results. The retention time of Phenobarbital, Phenytoin and Carbamazepine were 4.57, 8.08 and 9.56 minutes respectively (Fig.2). The resolution was found to be more than 2 with shorter run time of 11 minutes. The method was found to be selective and there were no interferences from the endogenous matrix. All three peaks were separated clearly with good symmetry. Chromatogram of blank human serum is presented in fig. 3.
Fig. 1 Chemical Structure of Phenobarbital, Phenytoin and Carbamazepine.
Fig. 2 Chromatogram of serum sample (PB, PN, CBZ)

Fig. 3 Chromatogram of Blank serum
Validation of The Methods

Selectivity
The selectivity of the assay methodology was established using a minimum of six independent human sources. There were no interferences from the endogenous material at the retention time of all the three drugs.

Linearity and sensitivity
The calibration curve was plotted between the peak area and concentration. The calibration curve was constructed with working concentrations of 0.5, 1, 5, 10, 20, 40 and 50 µg/ml for all the three drugs. Correlation coefficients for Phenobarbital, Phenytoin and Carbamazepine were 0.9979, 0.9918, and 0.9946 respectively.

Precision and accuracy
Intra-day and inter-day accuracy and precision were determined by duplicate analysis of six sets of samples spiked with four different concentrations for all three drugs at low, medium, high quality control samples (5, 20, 50µg/ml) including LLOQ (0.5 µg/ml) within a day or on 6 consecutive days. For acceptance criteria for intra and inter-day accuracy should be within 85–115% of nominal concentration and coefficient of variation (%CV) values should be <15% over the calibration range, except at the LLOQ, where accuracy should be between 80 - 120% and %CV should not be more than <20% of the nominal concentration.

The intra day precision (%CV) ranged from 2.89 to 7.95, 2.33 to 4.54 and 4.28 to 10.63 for Phenobarbital, Phenytoin and Carbamazepine respectively. The inter day precision (%CV) ranged from 2.00 to 8.91, 7.51 to 12.18 and 5.57 to 11.58 for Phenobarbital, Phenytoin and Carbamazepine respectively. The intra day accuracy ranged from 97.17 to 107.03, 93.97 to 101.07 and 99.67 to 105.93 for Phenobarbital, Phenytoin and Carbamazepine respectively, where as the inter day accuracy ranged from 99.78 to 110.95, 97.23 to 108.43 and 97.21 to 104.70 for Phenobarbital, Phenytoin and Carbamazepine respectively. The results are showed in the table no 1.

Sensitivity:
The lower limit of quantification (LLOQ) for all the three analytes found to be 0.5µg/ml. The developed method was able to quantify all three drugs with acceptable precision and accuracy.

Stability
The antiepileptic drugs were stable in serum when stored at −20°C for up to four weeks. Stability of the extracted drug samples from serum with AED concentrations covering the low, medium and higher ranges of the calibration curve were tested. The extracted drug samples (n = 4) were stored at room temperature (25°C) for 24 h prior to analysis. The results show that Phenobarbital, Phenytoin and Carbamazepine were stable and did not show any significant variation with time.
Table 1: Intra Day and Inter Day Precision and Accuracy of Phenobarbitone,

<table>
<thead>
<tr>
<th>Concentration added (µg/ml)</th>
<th>Intra day (n = 6)</th>
<th>Inter day (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured</td>
<td>% CV</td>
</tr>
<tr>
<td></td>
<td>concentration</td>
<td>(%)</td>
</tr>
<tr>
<td></td>
<td>(mean ± SD)</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.49 ± 0.03</td>
<td>5.66</td>
</tr>
<tr>
<td>5</td>
<td>5.13 ± 0.19</td>
<td>3.70</td>
</tr>
<tr>
<td>20</td>
<td>20.18 ± 0.58</td>
<td>2.89</td>
</tr>
<tr>
<td>50</td>
<td>53.51 ± 4.26</td>
<td>7.95</td>
</tr>
<tr>
<td>Phenytoin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.47 ± 0.02</td>
<td>4.54</td>
</tr>
<tr>
<td>5</td>
<td>4.96 ± 0.12</td>
<td>2.33</td>
</tr>
<tr>
<td>20</td>
<td>19.63 ± 0.86</td>
<td>4.39</td>
</tr>
<tr>
<td>50</td>
<td>50.53 ± 1.55</td>
<td>3.06</td>
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<tr>
<td>Carbamazepine</td>
<td></td>
<td></td>
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<tr>
<td>0.5</td>
<td>0.51 ± 0.05</td>
<td>10.63</td>
</tr>
<tr>
<td>5</td>
<td>5.22 ± 0.22</td>
<td>4.28</td>
</tr>
<tr>
<td>20</td>
<td>21.19 ± 2.06</td>
<td>9.75</td>
</tr>
<tr>
<td>50</td>
<td>49.83 ± 4.16</td>
<td>8.34</td>
</tr>
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</table>
Absolute and relative recovery

To calculate the absolute and relative recovery, four different concentration (0.5, 5, 20, 50µg/ml) of Phenobarbital, Phenytoin and Carbamazepine were spiked with drug free human serum samples (n=6). Absolute recovery was calculated by comparing the resultant peak areas of spiked samples with the peak areas of pure samples in methanol at the same concentrations. Similarly, the relative recovery was calculated by comparing the concentrations of the spiked samples with the actual added concentration (Table 2). The absolute recoveries of Phenobarbital, Phenytoin and Carbamazepine ranged from 97.65±1.47, 98.13±0.56 and 99.67± 3.37 respectively and relative recovery ranged from 98.37±4.85, 99.58±2.14 and 101.25±1.48 respectively.

Table 2: Absolute and Relative Recovery of Phenobarbital, Phenytoin and Carbamazepine in Human Serum.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Concentration added (µg/ml)</th>
<th>Absolute recovery (% mean ± SD, n=6)</th>
<th>Relative recovery (% mean ± SD, n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbital</td>
<td>0.5</td>
<td>97.88 ± 1.73</td>
<td>99.91 ± 1.45</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>98.01 ± 1.09</td>
<td>98.37 ± 4.85</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>97.65 ± 1.47</td>
<td>99.84 ± 2.05</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>100.09 ± 1.49</td>
<td>101.28 ± 1.52</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>0.5</td>
<td>98.13 ± 0.56</td>
<td>99.75 ± 2.06</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>99.53 ± 2.63</td>
<td>100.42 ± 1.83</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>99.06 ± 2.32</td>
<td>100.08 ± 1.97</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>99.22 ± 2.66</td>
<td>99.58 ± 2.14</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>0.5</td>
<td>99.84 ± 0.59</td>
<td>101.25 ± 1.48</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>99.67 ± 3.37</td>
<td>102.10 ± 1.83</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>101.13 ± 1.65</td>
<td>101.31 ± 1.78</td>
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<tr>
<td></td>
<td>50</td>
<td>100.06 ± 0.88</td>
<td>101.50 ± 1.19</td>
</tr>
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</table>

System Suitability

System suitability of the method was evaluated by analyzing the peak symmetry of Phenobarbital, Phenytoin and Carbamazepine (symmetry factor), theoretical plates of the column, resolution between the peaks of Phenobarbital, Phenytoin and Carbamazepine, mass distribution ratio (capacity factor) and relative retention.

Advantages of the developed method:

The developed method is found to be specific, sensitive, accurate and precise. The method is economical in terms of shorter run time, simple and single step extraction (deproteinisation) procedure as compared to previously reported methods for simultaneous estimation of Phenobarbital, Phenytoin and Carbamazepine in human serum by using time consuming liquid-liquid and solid phase extraction procedures. The low limit of quantification, small sample volume and short chromatographic time makes it suitable for routine use.
The method enables simultaneous determination of Phenobarbital, Phenytoin and Carbamazepine in human serum because of good separation and resolution of the chromatographic peaks. The obtained results are in a good agreement with the limits of validation parameters. The accuracy and precision of the results are confirmed by the statistical parameters. Reliability, simplicity, economical nature and good recovery of this method make it advantageous for simultaneous determination in a single chromatographic run.

Conclusion
The developed HPLC method was simple with easier extraction steps and economical. The method was successfully applied for the therapeutic drug monitoring of epileptic patients who were undergoing treatment with different antiepileptic drugs (Phenobarbital, Phenytoin and Carbamazepine).

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