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Research Article

BIO-ANALYTICAL METHOD DEVELOPMENT AND ITS VALIDATION FOR ESTIMATION OF PHENOBARBITAL IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY COUPLED WITH TANDEM MASS

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ABSTRACT

A high throughput and sensitive liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the estimation of Phenobarbital in human plasma. Phenobarbital was extracted from human plasma using solid-phase extraction technique using Phenytoin Sodium as internal standard. A Beta basic 8 column provided chromatographic separation of analyses followed by detection with mass spectrometry. The mass transition ion-pair was followed as *m/z* 251.4 → 98.0for Phenobarbital. The method involves a simple multiplexing, rapid solid-phase extraction, simple isocratic chromatography conditions and mass spectrometric detection which enable detection at sub-nanogram levels. The proposed method has been validated for a linear range of 5.5 – 10.3 ng/mL with correlation coefficient≥0.99 79. The precision and accuracy were within 10% for intra-HPLC runs and inter-HPLC runs. The overall recoveries for Phenobarbital were 99.89%. Total MS run time was 4 min. The developed method was applied for the determination of pharmacokinetic parameters of Phenobarbital following a single oral administration of a 30 mg Phenobarbital tablet in human plasma.

Keywords: LC/MS/MS, Phenobarbital, HPLC, Validation, Human Plasma.

INTRODUCTION

Phenobarbital (PBT) chemically 5-ethyl-5-phenylpyrimidine-2,4,6(1H,3H,5H)-trione[1] belongs to anticonvulsant drugs it also has sedative and hypnotic properties[2] (Figure. 1) but, as with other barbiturates, has been superseded by the benzodiazepines for these indications[3]. Phenytoin Sodium an antiepileptic drug was used as an internal standard. The mass transition ion-pair for Phenobarbital (PBT) *m/z* 251.4 →98.0. Different methods have been reported in the literature for monitoring plasma levels of PBT[4], and in dosage forms also[5]. Some other techniques used in individual analysis of PBT from plasma include HPLC with Mass Spectroscopy, UV-Spectroscopy [6,7,8,9], but these are not sensitive. Since there is no specific method was available in literature for the quantification of Phenobarbitol in human plasma using LC/MS/MS system, the study was done by different literature survey[9,10,11,12,13,14,15] the aim of the study was the development and validation of simple, sensitive, rapid and specific method.

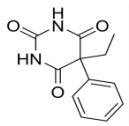


Fig. 1: Chemical structure of Phenobarbital

MATERIALS AND METHODS

Chemicals used

Acetonitrile (Merck), Methanol (Merck), Millipore water were used in method development.

Instrumentation

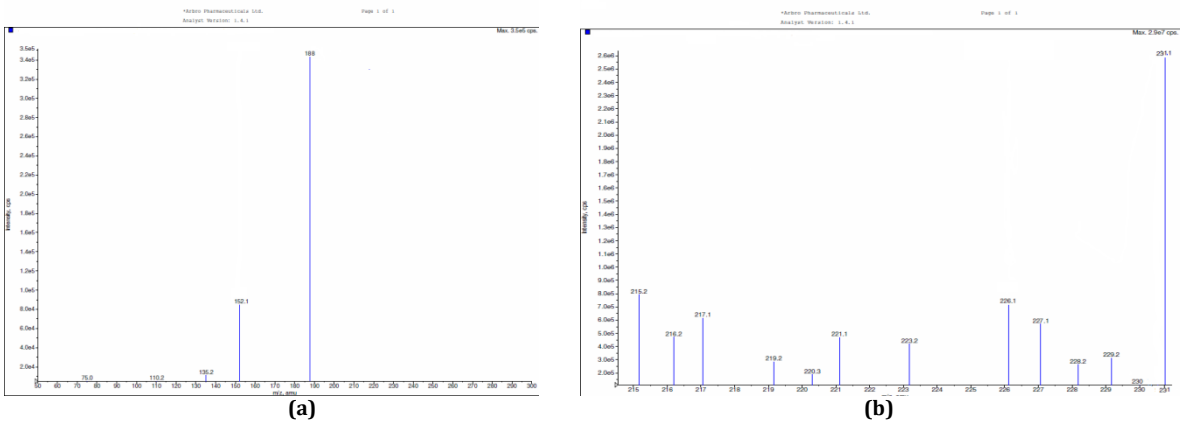
API 3000 LC/MS/MS was used.

Mass Parameters

The mass spectrometer was operated in the positive ion mode. The developed mass and LC parameters for the estimation of PBT and Phenytoin Sodium (PTS) as internal standard are given below. The compound dependent parameters for Phenobarbitol was 55eV, Focussing potential was 160eV, Entrance potential 10eV, Collision energy CE 30 psi. For Phenytoin Sodium potential was 50eV, Focusing potential was 190 eV, Entrance potential 10 eV, Collision energy CE 100 V. Source dependent parameters for the method are CUR -15 psi, TEMP -550°c, ISV-5500 V, CAD-18psi.

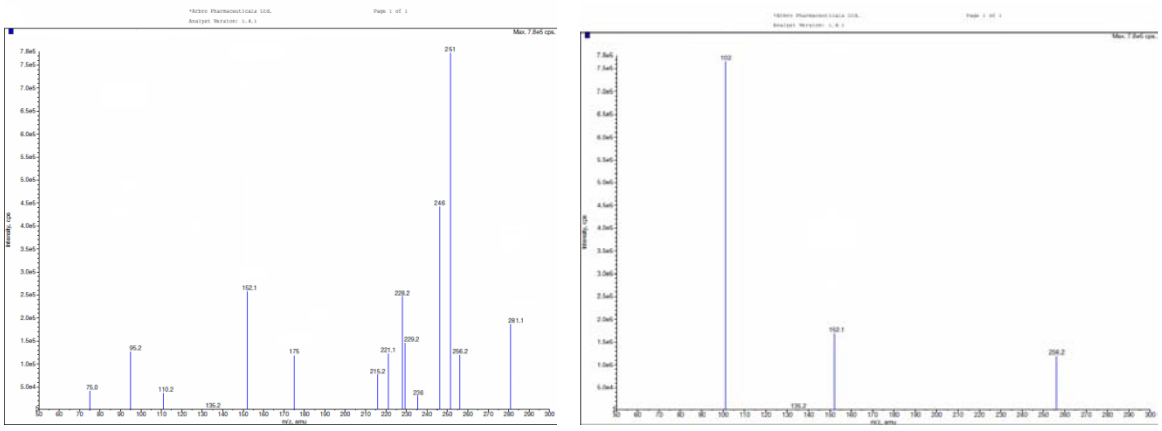
Multi Reaction Monitoring (MRM)

The mass transition ion-pair has been followed as *m/z* 251.4 → 98.0 for PBT and *m/z* 423.2→91.0 for PTS.



(a) **(b)**

Fig. 2: Mass spectrum of Phenobarbital (a) Parent ion (b) Daughter ion



(a) **(b)**

Fig. 3: Mass spectrum of Phenytoin sod. (a) Parent ion (b) Daughter ion

From the chromatogram above the retention time (Rt) for Phenytoin Sodium was found to be 0.87 min and Phenobarbitol 0.69 min.

LC parameters

Column: Hypurity advanced 50×4.6 mm, 5 µm (thermo)
Mobile phase: Acetonitrile:methanol (50:50)
Run time: 4.0 min.
Injection volume: 5 µL
Flow Rate: 600 µL /min.
Column oven temperature: 40°c

Table 1: Gradient System

Time	Flow	Mobile Phase A %	Mobile Phase B%
0	600µl/min	95	5
2.5	600µl/min	95	5
3	600µl/min	30	70
4	600µl/min	30	70

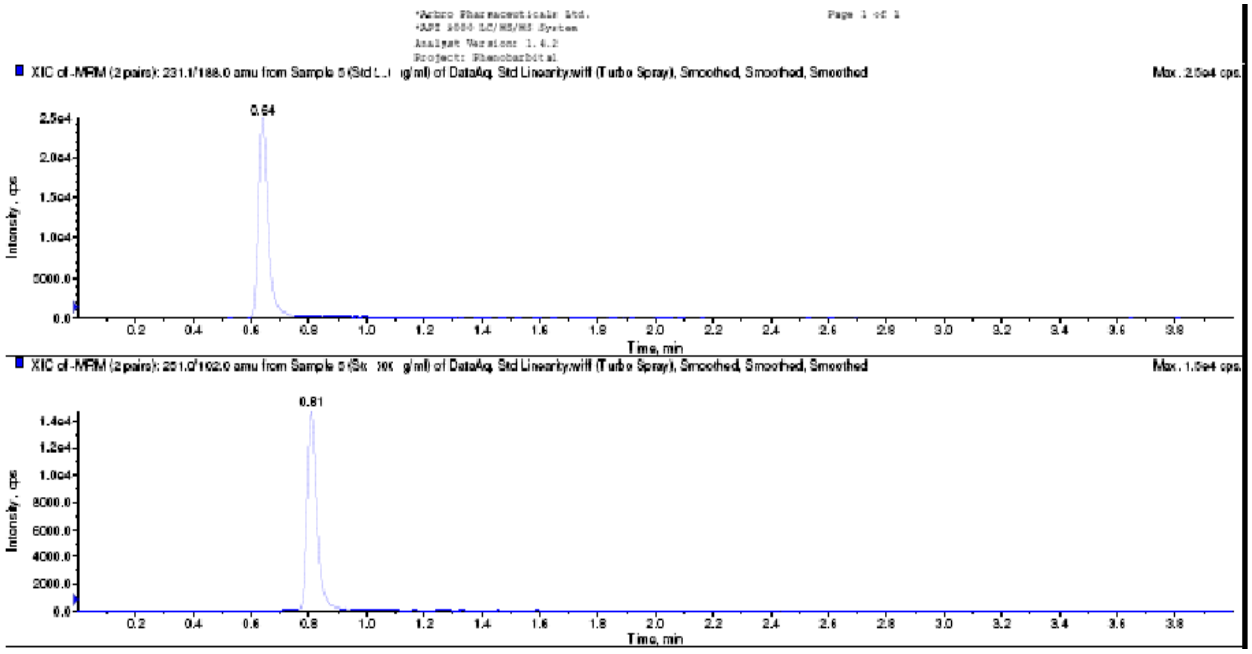


Fig. 4: Chromatogram for Optimization of Mobile Phase (Water: Acetonitrile) [(a) Phenobarbital, (b) Phenytoin Sodium (ISTD).

Preparation of calibration curve standards and quality controls for estimation of Phenobarbitol

To 96µl of plasma 4µL of standard solution (SS) of Phenobarbitol was added and vortexed. The spiked plasma samples were pooled and 100µL was taken from it. To it acetonitrile containing IS (Phentoin Sodium 200 ng/ml) was added, vortexed and kept for centrifugation which rotates at 13000 rpm at 5°c. The supernatant was taken and transferred to plates for analysis. In similar way remaining calibration standards and quality controls were prepared

to get calibration curve standards with arrange of 5.5% to 10.3% ng/ml.

System suitability

Six replicates of Extracted standard 9 (ULOQ) sample was injected Results are presented in Table 2.

Table 2: System suitability analysis

Replicate	Analyte Area/IS area
1	7.905695
2	8.096845
3	7.956168
4	8.743318
5	8.384934
6	8.468316
Mean	**8.259213**
SD	**0.327738**
% CV	**3.968151**

Acceptance Criteria(s)

The % CV of the drug/IS area ratio of last five injections should not be more than 5.0

Conclusion: System is suitable for determination of Phenobarbital from human blood plasma.

Method validation

Objective

The objective of the work is to validate specific LC/MS/MS method for the determination of PBT in human plasma for the study. Injector carries over effect for analyte and is Carry over test was performed in the following sequence.

MP → STD9 → MP → MP → STD1 → Plasma Blank.

No significant injector carries over was observed for PBT and internal standard. Results are presented in table 3.

Blank matrix screening (Specificity)

During validation, blank plasma samples from 4 different lots were processed according to the extraction procedure and evaluate the interference at the retention times of analytes and internal standard. The 3 free interference lots were selected from the 4 lots. Presented in table 4.

Extraction recovery

The percentage recovery of PBT and PTS was determined by comparing the mean peak area of PBT in extracted LQC, MQC, HQC samples with freshly prepared un-extracted LQC, MQC, HQC samples. The percentage recoveries were found to be above 75%.

Table 3: Assessment of Injector carry over effect for Analyte PBT and IS (PTS)

Sample Identification	Analyte Response	Internal standard Response	Carry Over observed with Analyte	Carry Over observed with IS
MP	0	0	Nil	Nil
S9	673905	779645	Nil	Nil
MP	0	0	Nil	Nil
MP	0	0	Nil	Nil
S1	15562	875943	Nil	Nil
Plasma Blank	0	0	Nil	Nil

Result: Injector carry over effect for Analyte (PBT) and IS was passed

Acceptance criteria

The carry over must be less than 20% response of analyte (S1) and less than 5% response of internal standard.

Table 4: Screening of Different batches of blank matrix (HUMAN PLASMA) Screening (SPECIFICITY)

S. No.	Response of signal (Height) (S)	Response of noise (Height) (N)	Signal to Noise ratio S/N
1	1770.000	12.300	143.90
2	1755.000	15.000	117.00
3	1542.000	11.000	140.18
4	1743.000	11.250	154.93
5	1698.000	13.010	130.51
6	1648.000	10.500	156.95
7	1769.000	12.550	140.96
8	1779.000	10.990	161.87
9	1691.000	13.840	122.18
10	1780.000	13.170	135.16
Mean	-	-	**140.37**

Acceptence criteria: Signal (LLOQ)/ noise ratio NLT 5:1

Analysis of subject samples preparation (Extraction procedure)

A 100µL of subject plasma sample was mixed with 300µL of internal standard working solution (200ng/ml of PBT) vortexed for two minutes and kept in centrifuge for 5 minutes at 5°C which rotates at a speed of 13000 rpm. After that the supernatant layer was collected and analyzed in LC/MS/MS system. The chromatograms of mobile phase , Blank plasma, Internal standard, Calibration standards, subject samples and table representing time points vs. plasma drug concentrations are shown in illustrations.

RESULTS AND DISCUSSION

The goal of present work was to develop and validate a simple, rapid, sensitive method for extraction and quantification of PBT, was suitable for pharmacokinetic studies. To achieve the goal different options was evaluated to optimize sample extraction, detection parameters and chromatography. Different columns (X bridge C_{18},C_8, Phenomenex C_{18},C_8 but polar RP column was found to be better with minimum tailing and good retention. Coming to the mobile phase the combination of methanol and Acetonitrile has good eluting capacity and is cheaper. When acetonitrile is used the analytes are not at all retaining. A flow rate of 600 µL /min was optimized for good retention and the column oven temperature was kept at 40°c. The LOD, LOQ values are also low (5.01ng/ml) indicating that method is very sensitive. The injection carry over, specificity, system suitability was in limits. While coming to extraction procedure precipitation was found to be better than Liquid-liquid extraction with good recoveries.

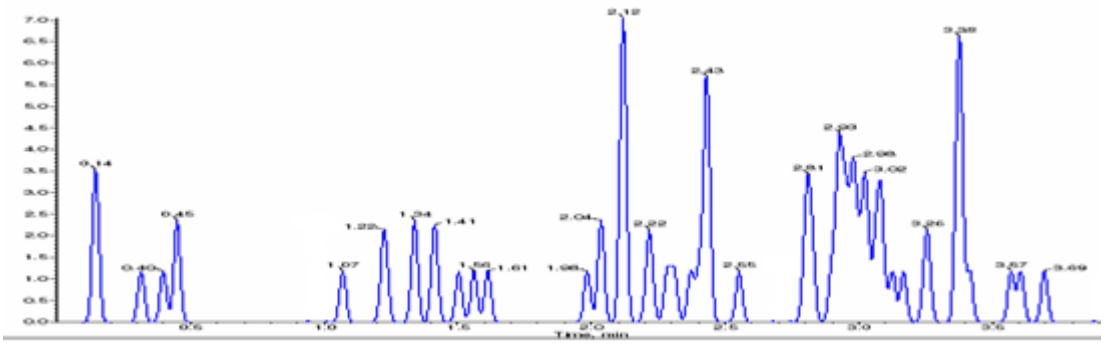


Fig. 5: Mobile Phase Chromatogram

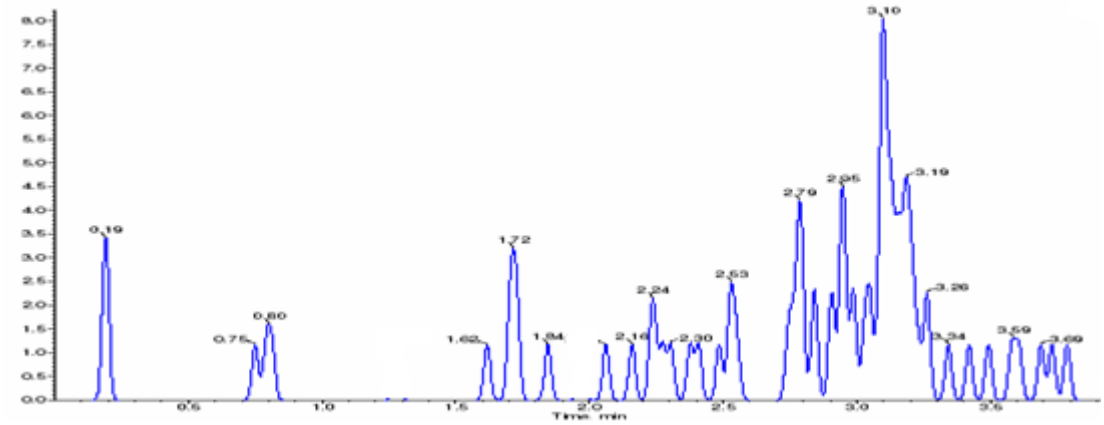


Fig. 6: Blank plasma chromatogram of losartan calibration curve

CONCLUSION

A simple, specific, rapid and sensitive analytical method was developed for the determination of PBT in human plasma. Most of the analytical methods reported, for quantization of PBT individually from human plasma, require laborious extraction procedure like liquid–liquid extraction, long run time and high quantification limit. The method was provided excellent specificity and linearity. The other major advantage of this method is the short run time of 4 min which allows the quantization of over 300 plasma samples per day. The recoveries in the method were also good.

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