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Sensitive Analysis of Epilepsy Drug, Phenobarbital, Based on Column Type Solid Phase Extraction and HPLC-DAD System

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ABSTRACT

An analytical approach has been developed for the sensitive determination of Phenobarbital, which is used as an antiepileptic drug molecule. The analysis of this active molecule, which has a very limited study even for its direct determination in the literature review, is mostly done with highly complex device systems such as LC-MS. In analysis with conventional HPLC systems, the limit of detection cannot be in most cases lower than 1 µg mL^{-1} With this study, a separation and preconcentration method based on solid phase extraction (SPE) was developed for trace phenobarbital molecules, so that even very low concentrations could be monitored. In the proposed method, the target molecules were enriched with column type SPE method, and then their analysis were carried out by with the HPLC-DAD system. As an SPE sorbent, a polymeric material, poly(ethylene glycol dimethacrylate-N-methacryloyl-L-tryptophan methyl ester) [poly(EGDMA-MATrp)], was used in extraction experiments. Experimental variables such as pH of medium, type and amount of desorption solvent, electrolyte effect have been studied and optimized step by step. The linear working range under the optimized conditions were determined in the range of 10.00-400.00 ng mL^{-1} with the limit of detection as 3.57 ng mL^{-1}. Quantitative results were obtained in recovery experiments with the help of model solutions including phenobarbital molecule.

Keywords: Phenobarbital, Solid phase extraction, HPLC, Analytical method development.

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Introduction

Phenobarbital (5-ethyl-5phenyl-1,3-diazinan-2,4,6-trione), as a barbiturate, is a hypnotic drug substance derived from barbituric acid. It is the oldest anticonvulsant drug still used today. Although it has hypnotic and sedative properties, it is not used for these effects today, benzodiazepines are preferred for this purpose[1]. Phenorbarbital, partial and is a general central nervous system depressant used in the control and treatment of generalized epilepsy disease [2–4]. Molecular formula of phenobarbital was given in Figure 1.

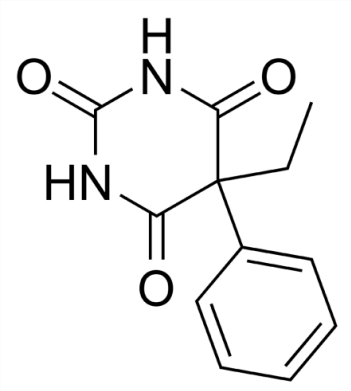

Figure 1. Chemical structure of phenobarbital

The degradation products of phenobarbital at high pHs are diethylmalonuric acid, diethylacetylurea, diethyl acetic acid, ammonia and carbonate, respectively. Barbituric acid derivatives are sufficiently acidic due to the nitrogens attached to the carbonyl group in their structures from two directions and the hydrogens in the -CH_2 group, and they form salts with alkalis. If the pH of the aqueous solutions of these salts falls below a certain value, the barbituric acid part precipitates in non-ionized form[5]. Injection solutions are prepared with organic solvents such as dimethylformamide, dimethylacetamide and propylene glycol to increase the solubility and stability of barbituric acid derivatives. Phenobarbital is a drug that stabilizes the work of the brain and nervous system and shows its function[6]. It is used in convulsions in grand mal epilepsy and other types of epilepsy , continuous treatment of eclampsia and spastic conditions, combined treatment of whooping cough, persistent insomnia, chorea minor, angina pectoris , hyperthyroidism and tensions in the climacterium[7,8].

The analysis of food, biological, pharmaceutical and environmental samples is the most important application area of solid phase extraction methods [9-11]. Especially in biological samples, SPE based methods are widely used

for the enrichment of organic molecules and trace heavy metals found mostly in water at µg/L and less [12–14]. Level of target molecules in biological samples is generally lower than detection limit of conventional analysis system. So, easy applicable and effective sample pre-treatment procedures are valuable for trace analysis of drug molecules. Use of new and useful adsorbents in extraction procedures presents new ways for this challenges.

The aim of the presented study is to develop a HPLC based determination method after a sample preparation procedure with a solid phase extraction method for the sensitive and selective determination of phenobarbital molecules that are present at trace levels in real samples and cannot be directly analyzed by classical determination devices.

Material Methods

Chemicals and Reagents

All reagents used during the experiments were of analytical purity and purchased from Sigma or Merck. All solutions used were prepared with ultrapure water with a resistivity of 18.2 M Ω obtained from ELGA Pure Lab Flex III device.

For pH 2.0-11.0 Britton Robinson (BR) buffer, a stock BR solution containing H_3BO_3, H_3PO_4 and CH_3COOH acids was prepared to contain 0.05 M each. 0.1 M NaOH was added dropwise by checking with a pH meter in the appropriate pH ranges according to the acidity constants and adjusted to the desired pH.

Phenobarbital stock solution; 50 mg of phenobarbital supplied by Sigma Aldrich was weighed and put into a volumetric flask and dissolved with some methyl alcohol to make up to 50 ml and transferred to a brown bottle and stored in the refrigerator at +4ºC.

Instrumentation

In all chromatographic measurements, Shimadzu (Prominence) HPLC (Kyoto, Japan) device was used. The HPLC device used has LC 20 AD quaternary pump, SPD-M20 A PDA detector, DGU-20A vacuum degasser and CTO-10 AS VP column oven equipment. All separations and determinations were performed on reverse phase C18 column (C18, 250 mm×4.6 mm, 5µm). Evaluation of chromatograms was done via LC Solution 2.0 software. The following devices were used during the optimization experiments; pH meter (pH-2005, JP Selecta , Barcelona, Spain), Vortex (Jeotech , Korea), Ultrasonic Water Bath (SK521 0HP), Shaker (WhyShake SHO-2D).

HPLC Determination Conditions for the Phenobarbital Analysis

Before proceeding to the SPE experiments, the parameters of direct determination were optimized for Phenobarbital molecule by HPLC-DAD. For this purpose, based on literature information, Luna Omega C-18 column was selected as the stationary phase and the ideal eluent composition was investigated to enable conditions for determination. Many experiments were conducted in isocratic and gradient elution modes with aqueous solutions containing buffers at different pHs of organic eluent phases such as methanol, ethanol and acetonitrile and different eluent phase compositions. Experiments were continued until good peaks were obtained for phenobarbital. As a result of the experiments, the most ideal mobile phase conditions were obtained with methanol and pH 3.0 phosphate buffer eluents. The ideal HPLC operating conditions obtained after optimization were given in Table 1. The chromatogram obtained for 3 different Phenobarbital concentrations under the optimized working conditions was also shown in Figure 2.

Table 1. HPLC operating conditions

Parameter Value	
HPLC Mode	**Isocratic**
Eluent	Methyl Alcohol: pH 3.00 Phosphate Buffer (70:30)
Eluent Flow Rate	1 mL/min
Run Time	12 min
Column	C18- Luna Omega (250 mm×4.6, 5.0 µm)
Column Temperature	30^o C
Injection Volume	10 µL

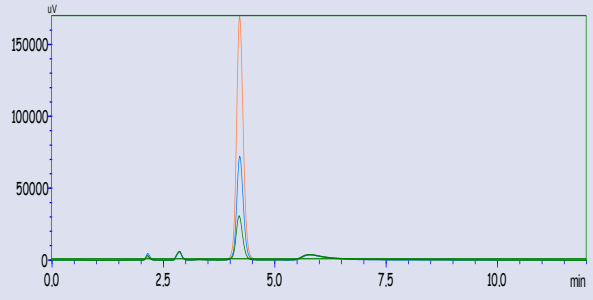

Figure 2. The standard chromatogram of Phenobarbital before SPE

The obtained calibration graph on the HPLC-DAD system with the help of standard solutions prepared at 5 different concentrations (2, 5, 10, 20 and 50 µg mL^{-1}) was given in Figure 3.

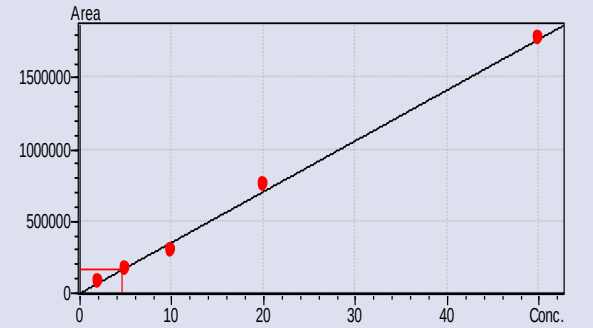

Figure 3. Calibration graph obtained for phenobarbital molecules before SPE (R^2: 0.9968)

As seen in Figure 4, the spectrum taken from the DAD detector of the HPLC device for phenobarbital molecules and the maximum absorption wavelength was determined as 199 nm for further studies. Under optimized HPLC conditions, Table 2 shows direct determination parameters before SPE for phenobarbital molecules.

Figure 4. UV-VIS Spectrum obtained in DAD Detector for Phenobarbital

Table 2. Direct analysis results by HPLC

Parameter	Value
Retention Time, min	4.25
Maximum absorption wavelength, λ	199 nm
Calibration Range	2.0-50.0 µg mL $^{-1}$
Limit of Detection, LOD	0.57 µg mL $^{-1}$
RSD % (for 5.0 µg mL $^{-1}$)	3.47
R^2	0.9968
Number of repetitions	3

Synthesis and Characterization of the Used Solid Phase Sorbent [poly(EGDMA-MATrp)]

Synthesis of the used polymeric sorbent was carried out by using N-methacryloyl-L-tryptophan methyl ester (MATrp) and (EGDMA) as a monomer and a cross-linker, respectively. The sorbent microbeads were synthesized by suspension polymerization technique. The details about synthesis and characterization of used adsorbent in SPE are available in our previous studies[15,16]. Briefly, a 200 mg of poly (vinyl alcohol) (PVA) was dissolved in 50 mL of the deionized water and then the dispersion phase was prepared by means of an organic phase including MATrp (4 mL), EGDMA (5 mL), toluene (10 mL) and PVA solution. After that, 100 mg of (AIBN) was transferred to the polymerization mixture. Polymerization was performed at 85 °C for 8 h with a 600 rpm stirring rate. The final product was washed with ethanol and water in order to remove unreacted chemicals and then dried at 50°C in a vacuum oven.

The Proposed Solid Phase Extraction Method

A solid phase extraction based separation and preconcentration method was developed for Phenobarbital molecules, which are found at trace levels in real samples and are difficult to determine due to the complex matrix of the sample. At the beginning of the experimental studies, preliminary experiments were carried out regarding all the parameters that will provide quantitative transition of the relevant substance to the rich phase. The main goal in solid phase extraction experiments is to create ideal experimental conditions that will provide the transition of analyte species to the solid phase with the highest possible efficiency.

200 mL of poly(EGDMA-MATrp) microbeads were weighed in a 1 mL empty SPE cartridge using an upper frit and a lower frit to prevent the leakage of adsorbent from the SPE cartridge. Prior to extraction, the cartridge packed with poly(EGDMA-MATrp) microbeads was preconditioned with 3 mL of methanol and then with 3 mL of water. The pH of samples was adjusted a value of 4.0 using Britton-Robinson Buffer solutions. The sample solution (50 mL) was passed through the cartridge at a flow rate of 0.75 mL min^{-1}. Then, 500 µL of ACN:MeOH (1:1) mixture was used to elute the analytes retained on the cartridge. The eluent solution was filtered through a 0.45 µm membrane, and 10 µL of the solution were injected into the HPLC-PDA system for analysis.

Results and Discussion

pH effect

The pH of the medium is a very important parameter, as it affects the interaction of the analyte with the solid phase and the reactions between species. Two milliliters (2 mL) of Britton-Robinson (BR) buffer in the range of pH 2–10 was added to all tubes containing phenobarbital, and their volumes were adjusted to 50 mL with ultrapure water. The prepared solutions were then filtered using an

SPE manifold, allowing the analyte molecules to attach to the solid phase, and subsequently analyzed using an HPLC-DAD system. The analytical responses obtained by this optimization were shown in Figure 5. It was observed that the optimum pH value for the enrichment process is 4.00. Consequently, it was understood that phenobarbital becomes positively charged in the acidic region due to its pKa value. According to the literature, the pKa value of phenobarbital is reported as 7.30 [17], which is consistent with our findings.

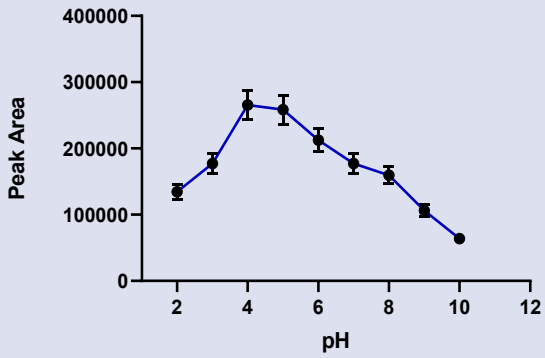

Figure 5. pH effect on the proposed SPE method.

Electrolyte Effect

To observe the effect of electrolyte concentration on the developed method, a series of experiments were performed using NaCl solution. Evaluating how the solid-phase extraction system is influenced by increased electrolyte concentration in the medium and determining whether there are changes in the analyte signals are important parameters for method stability. The ability of the method to perform even in the presence of high electrolyte concentrations is significant, as it confirms its reliability for real sample applications and demonstrates its potential to enhance signals by maintaining charge balance in the medium in certain cases. However, in some instances, analyte signals may be negatively affected as the electrolyte concentration increases. To assess these effects, increasing concentrations of NaCl were added to the model solutions containing analytes, and changes in the peak areas of phenobarbital were monitored. As shown in Figure 6, even at high electrolyte concentrations, no disruptive effect was observed on the analyte signals.

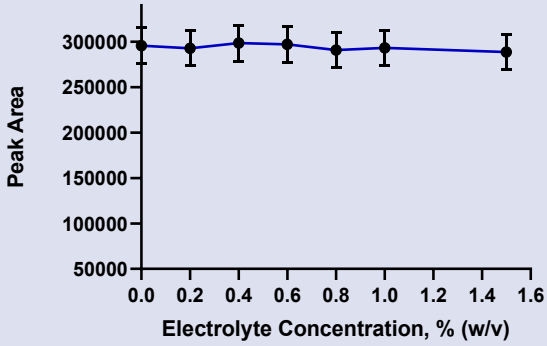

Figure 6. Effect of electrolyte concentration on the proposed SPE method.

Desorption Solvent Selection

After completing the filtration process with the SPE manifold, the next step was to select the most suitable solvent for separating the material bound to the solid phase. The selected solvent needed to completely dissolve the solid phase enriched with phenobarbital while ensuring it would not damage the HPLC system during analysis. When choosing the solvents for this purpose, several candidates were tested based on their compatibility with the HPLC eluent phase and their ability to quantitatively dissolve the SPE. For this process, 1 mL of various solvents, including methanol (MeOH), acetonitrile (ACN), isopropyl alcohol (IPA), ethanol (EtOH), ACN:MeOH (1:1), water, and n-hexane, were tested sequentially. The results obtained with these solvents after the enrichment process were presented in Figure 7.

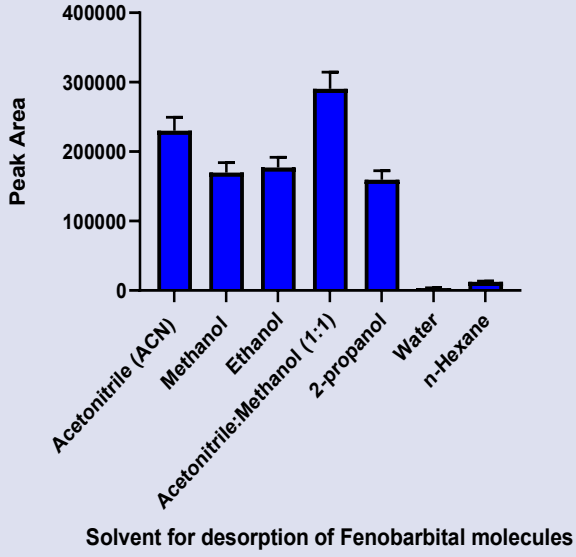

Figure 7. Eluents for desorption process.

As seen in Figure 7, the highest signals were obtained with Acetonitril:Methanol (1:1) solution. Therefore, in subsequent studies, the Phenobarbital-rich solid phase was dissolved with this solvent. Since the amount of solvent used to dissolve the phenobarbital-rich solid phase will directly affect the enrichment factor, it is important how much of the solvent volume will be taken. In order to obtain a high enrichment coefficient, the solvent volume should be at the smallest value. Because the enrichment coefficient decreases as the solvent volume increases. 200 µL of sample can be placed in HPLC micro vials. Volume optimization was performed by adding 250-2000 µL of solvent and, as expected and seen in Figure 8, the signals decrease as the solvent volume increases. In order to obtain the best signals, 1000 µL was determined as the appropriate volume, which is the volume in which the phenobarbital-rich solid phase can be dissolved and filtered, and this value was used in subsequent studies.

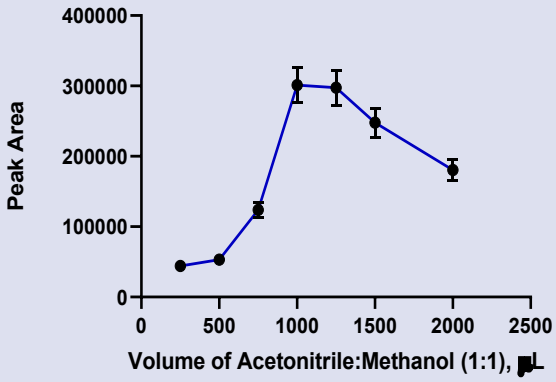

Figure 8. Optimization of eluent volume used for SPE.

Analytical Performance Parameters of the developed SPE-HPLC-DAD based Method

After determining the most suitable experimental conditions for solid phase extraction, enrichment experiments were applied to Phenobarbital solutions at different concentrations in order to determine the linear working range, and as a result, it was determined that the measured signals varied linearly in the range of 10.00-400.00 ng mL $^{-1}$. The calibration line obtained by applying the method we developed to these solutions is given in Figure 9. As can be seen, the signals increase proportionally with the concentration. All analytical parameters of the developed method were collectively presented in Table 3.

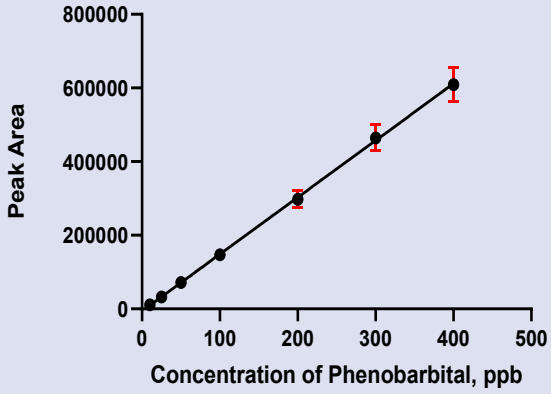

Figure 9. Linear Concentration Range of the developed method under optimum conditions.

Table 3. Analytical parameters of the proposed method

Parameter	Before SPE	After SPE
Linear range	2.00-50.00 µg mL $^{-1}$	10.00-400.00 ng mL $^{-1}$
Limit of detection(LOD) [a]	0.56 µg mL $^{-1}$	3.57 ng mL $^{-1}$
Limit of Quantification (LOQ) [b]	1.91 µg mL $^{-1}$	9.58 ng mL $^{-1}$
RSD (%) (for 250 ng mL $^{-1}$)	4.57	3.70
Calibration Sensitivity	1.12	69.44
Correlation Coefficient (R^2)	0.9968	0.9846
Enrichment Factor [c]	-	50
Enhancement Factor [d]	-	62

[a.,b] The selection limit was calculated using 3 times the standard deviation obtained from blank trial signals with at least 3 replicates, and the quantification limit was calculated using 10 times the standard deviation.
[c] Enrichment factor; It was calculated by taking the ratio of the initial aqueous phase volume (50 mL) to the volume obtained after enrichment (1.0 mL)
[d] Enhancement Factor; It was computed by taking the ratio of calibration sensitivity(slope) after and before SPE

Conclusion

Solid-phase extraction (SPE) is a method that has been extensively and increasingly used for the separation and enrichment of both organic and inorganic species, especially over the past twenty years. Hundreds of studies are published annually in this field, demonstrating its wide application for various species and sample types. In the last two decades, SPE has been effectively applied for the enrichment of organic species, followed by their determination using appropriate analytical methods.

The key factors contributing to the widespread application of SPE include its simplicity, environmental friendliness, low cost, and easy applicability in almost any laboratory. The primary goal of SPE experiments is to transfer target species from a relatively large volume of aqueous medium into a smaller volume of a phase immiscible with water—like classical liquid-liquid extraction—thereby enriching the analyte to concentrations detectable by analytical instruments.

Despite its advantages, several challenges may arise during the application of SPE. For example, variability in conditioning conditions before extraction, insufficient contact between the inhomogeneous solid-phase support material and the solution, and time-consuming procedures may hinder the process. Additionally, the retaining materials may sometimes lack sufficient selectivity for the analyte of interest. To minimize these drawbacks, optimization studies are carried out to improve method performance.

Like all enrichment methods, SPE aims to concentrate analytes that cannot be directly detected to levels measurable by analytical instruments. In this study, an enrichment and determination method was developed for this purpose. Direct determination of organic analytes, such as phenobarbital, is often challenging in many samples due to the complex matrix structure of real samples and the very low analyte concentrations. For such analytes, which are predominantly analyzed

chromatographically, the minimum concentration detectable using an HPLC system with an absorbance detector is typically no lower than 2 µg mL^{-1}.

To address this limitation, a cost-effective, accurate, precise, and easy-to-apply enrichment method was developed for use in any laboratory. Before initiating the enrichment process, direct determination conditions for phenobarbital were optimized. Following method optimization, analytical parameters were determined. Under optimized conditions, the linear working range for phenobarbital was found to be 10.00–400.00 ng mL^{-1}. The calculated limits of detection (LOD) and quantification (LOQ) were 3.57 ng mL^{-1} and 9.58 ng mL^{-1}, respectively.

Acknowledgment

This study is the graduation thesis of Seren Kilit Bülbül, who completed her degree at the Faculty of Pharmacy in 2020. The experimental data were collected and organized with the assistance of the other authors. Additionally, the experimental work for this study was conducted using project funds provided by the Scientific Research Projects Commission of Sivas Cumhuriyet University.

Conflict of interests

The authors declare that they have no conflicts of interest.

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