A diagnostic intoxication tool for clozapine and phenytoin using hybrid micelle liquid chromatography

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\textbf{ABSTRACT}
Micellar liquid chromatography was employed for the quantification and separation of the antidepressant drug clozapine (CLZ) and antiepileptic agent phenytoin (PHY). This mixture was separated using C8 column operating at 40°C using a mobile phase consisting of 0.075 M sodium dodecyl sulphate (SDS), 10% acetonitrile, 0.3% triethylamine (TEA), adjusted to pH 2.5 by 2 M ortho-phosphoric acid and applying a simple gradient program starting with a flow rate of 0.8 mL/min for the first 10 min of analysis applying 220 nm as detection wavelength, followed by raising the flow rate to 1.5 mL/min till end of analysis, adopting UV detection wavelength of 250 nm. The proposed method was used to quantify the concentration of both (CLZ) and (PHY) in rat whole blood whether spiked or \textit{in vivo} suggesting its utility as a diagnostic tool for intoxication with either drugs in emergency rooms.

\textbf{ARTICLE HISTORY}
Received 10 November 2020
Revised 29 December 2020
Accepted 31 January 2021

\textbf{KEYWORDS}
Clozapine; phenytoin; micellar liquid chromatography; intoxication

\section{1. Introduction}
Clozapine (CLZ) is one of the examples of efficient antipsychotic agents which makes it a candidate for the treatment of resistant schizophrenia reducing patients attempts for suicide [1], being more effective than other agent of the same pharmacological class [2]. Chemically, it is nominated (8-chloro-11-(4-methylpiperazin-1-yl)-5H-benzo [b,e][1,4]-diazepine [3] (Figure 1A). On the other hand, the etiology of epilepsy was divided into three groups: genetic, structural change and related to unknown cause. There are two main classes of seizures: focal and generalized one [4]. Phenytoin is an important medication for the management of different types of seizure disorders especially, simple focal disorder [5].

Phenytoin is widely distributed and highly effective against psychomotor activity, possessing a narrow therapeutic index, consequently any small change in its dose will be highly significant in its plasma concentration and hence in its bioavailability [6]. It is chemically configured as sodium 4-oxo-5,5-diphenyl-4,5-dihydro 1H-imidazol-2-olate [3] (Figure 1B).

There are many reasons of clozapine or phenytoin toxicity including: excessive drugs ingestion, misunderstanding of the prescription order or prolonged treatment. Some research articles proved that the intoxication of CLZ or PHY results in symptoms that mimic stroke [7,8], which misleads the emergency medical team for diagnosis and treatment of the patient, leading eventually to death.

Several liquid chromatographic methods were reported for the determination of clozapine [9–16] and phenytoin [17–21] in different biological fluids applying HPLC as an analysis tool. Most of the reported methods utilized tedious and time-consuming extraction steps [9–12,17–21], some of them applied specific extraction procedures to yield high recovery percentages as solid phase extraction [13] or liquid–liquid extraction [14–16], while others required a specified stationary phase designed for their determination [10], which might not be available in some laboratories. In all the published articles, high ratios of organic solvents were required for successful separation of the concerned analytes, which constitute a significant health hazard on both the analysts and the environment.

Micellar liquid chromatography is one of the separation techniques applying reversed phase mode, where it consists of an aqueous phase containing a surfactant over its critical micelle concentration CMC [22]. Organic modifiers are usually added to create the well-known hybrid micelle mobile phases [22], where the presence of micelles promotes the solubility of organic solvents and inhibits their evaporation, hence provides a safe environment for analysts. By increasing the surfactant concentration over its CMC, increase in the number of micelles is expected, while surfactant monomers number within the solution remains constant [22]. A fixed number of monomers are adsorbed onto the stationary phase surface, which...
Contribute to the retention behaviour in micelle-based mobile phases [22]. The adsorbed amount of monomers depends mainly on the type of the reversed phase column [22]. Introduction of organic solvents to micellar mobile phases sweeps away monomers from the stationary phase [22], hence they contribute to improving separation efficiency and reduce analysis time. Retention mechanism in micellar liquid chromatography could be attributed to three equilibria, namely, partition between micelles and aqueous solvent phase, partition between aqueous solvent phase and stationary phase, and partition between micelles and stationary phase [22].

Hybrid micelle liquid chromatography (HMLC) was selected as an analysis tool owing to its unique features proposing a green analytical method, where it mainly depends on applying aqueous surfactant solution which proved to be safe on the skin [23], besides, lower consumption of organic solvents is offered. Clean waste is generated from HMLC, moreover, recycling of the mobile phases is attainable [23]. Another attractive advantage of HMLC is that it does not require any extraction steps for biological samples, since micelles have a strong solubilizing ability to proteins, thus direct injection of physiological fluids is possible which allows analysis of large number of samples [23]. Eventually, compatibility of HMLC with reversed phase instruments and columns is well established [23].

The authors were encouraged to perform this study to serve as a diagnostic analytical tool for intoxication with either CLZ or PHY aiming to separate and quantify these two pharmaceutical agents in their pure form and further apply the proposed method to determine the studied drugs in rat whole blood samples both in vitro and in vivo. Further application of the proposed method to biological fluids requires conducting full validation procedures according to an approved protocol, to acquire the validity for such application. This fact motivated the authors to follow ICH guidelines [24] to validate both the analytical and bioanalytical methods.

2. EXPERIMENTAL

2.1. Materials and reagents

- Clozapine (ClZ of 99.7% purity, Batch # 031016) is kindly supplied by (Pharco Pharmaceutical, Alexandria, Egypt).
- Clozapex R⃝ tablets (Batch # MT5690818) containing 100 mg clozapine/tablet, product of (Multi-Apex Pharma, Cairo, Egypt) was purchased from local company.
- Phenytoin sodium (PHY) of purity is 99.8%, kindly provided by (Memphis Pharmaceuticals and Chemical Industries Co. S.A.E, Cairo, Egypt).
- Phenygin R⃝ ampoules containing phenytoin sodium 250 mg/5 mL for I.V. injection, (Batch # A 981302) and Phenygin R⃝ capsules containing 100 mg phenytoin sodium/capsule (Batch # C 983707), a product of (El-Nile company for pharmaceutical and chemical industries, Cairo, Egypt) were obtained from local pharmacies.
- Acetonitrile (99.9% purity, a product of Fisher scientific, UK) HPLC grade.
- Sodium dodecyl sulphate (SDS, ≥ 98% purity), methanol, triethylamine (TEA) and 1-propanol (all of 99.9% purity) (products of Sigma Aldrich, USA) HPLC grade.
- Orthophosphoric acid (of 85% purity) (El Naser Company, Cairo, Egypt).

2.2. Optimal condition of chromatographic separation

Two main apparatus were used during method development: Shimadzu SPD-20A, a product of Kyoto, Japan. The instrument is supplied with an injection loop (20 µL)
loop, UV/VIS detector, in addition to column oven (CTO-20A) and degasser unit (DGU-207) and pH meter, Jenway, UK.

Separation was carried out on a Shimadzu Shim-Pack VP-C8 column (250 mm × 4.6 mm), which was sustained at 40°C during the analysis time. The mobile phase is composed of 0.075 M SDS, 10% acetonitrile containing 0.3% TEA, having final pH of 2.5 modified by 2M orthophosphoric acid. A gradient program was used for controlling the flow rate and detection wavelength where the mobile phase was pumped at 0.8 mL/min during the first 10 min with UV detection at 220 nm, then increased to reach 1.5 mL/min till the end of analysis, adopting UV detection at 250 nm during this period of analysis time.

2.3. Stock solution and calibration graphs
Stock solutions were prepared using methanol as diluting solvent in all steps of the proposed method. The solutions were stable when kept in the refrigerator for 1 week. The stock solutions were prepared as 4.0 mg/mL for clozapine and 1.0 mg/mL for phenytoin owing to the dissimilarity in the sensitivities of the studied drugs. The final obtained working concentration covered the corresponding linearity ranges of 40.0–800.0 µg/mL and 20.0–300.0 µg/mL for clozapine and phenytoin respectively. The solutions were then injected in triplicates using the described chromatographic conditions.

The resultant peak areas were plotted versus the final concentration in µg/mL in order to obtain the final calibration curve for each individual drug, after that least square regression equations were calculated. Mixture of CLZ and PHY was measured by transferring the accurate calculated volumes of the drugs (10.0 mL volumetric flask) and diluting to the mark with methanol. Further dilution with same solvent was carried out to reach final linearity range of (50.0–200.0 µg/mL). The procedures described under “stock solution and calibration graph” were then applied.

2.5. Analysis of Phenytin® ampoules
A volume of 2 mL from the ampoule was transferred to 100.0 mL volumetric flask, and complete to the mark with methanol. Further dilution with same solvent was carried out to reach final linearity range of (50.0–200.0 µg/mL). The procedures described under “stock solution and calibration graph” were then applied.

2.6. Analysis of spiked drugs in rat blood samples
Control rat blood (1.0 mL) was transferred to 10.0 mL volumetric flask, then 3.0 mL of 0.075M SDS were added, after which, vortex mixing for 5 min was performed. Spiking with different volumes of the studied drugs was carried out to reach final concentration ranges of 60.0–400.0 µg/mL and 30.0–100.0 µg/mL for clozapine and phenytoin respectively. Then the solutions were completed to the mark with SDS, injected in triplicates and analysed using the described chromatographic conditions. Calibration plot for each pharmaceutical agent was constructed followed by the deduction of the least square regression equation as previously mentioned to determine the concentration of each analyte in rat blood samples.

2.7. In vivo analysis of clozapine and phenytoin
Stock solutions of each drug having concentrations of 1.0 mg/mL for PHy and 0.66 mg/mL for CLZ were prepared in order to attain their toxic dose in blood as reported previously in the literature [7,8]. The pharmaceuticals were dissolved in a mixture of propylene glycol: 0.9% sodium chloride (40: 60, v/v). Such solvent system was chosen under guidance of a previous report [25], so as to reduce irritation caused by organic solvents. Authors followed the instruction offered by the local authorities of the Institutional Animal Care and Use Committee (IACUC) at faculty of Pharmacy, Delta University for Science and Technology, Egypt and commit that every effort to minimize the suffering of animals during experiments is guaranteed. The manuscript carries Ethical committee approval number # FPDU 16/2020.

This promised study was performed on four rats, having average weight of 150–200 g. The experimental animals were maintained under controlled temperature and ventilation in cages, keeping one animal as a control; the other three rats were revived the toxic doses of CLZ and PHY. All rats were slaughtered after 2.5 h where blood samples of the three rats were collected and stored at −80°C till used for analysis. For in-vivo analysis, 1 mL blood sample was transfer to 10.0 mL volumetric flask, then the steps mentioned under “Analysis of spiked drugs in rat blood samples” were followed to deduce concentration of each of CLZ and PHY in blood samples.
3. Results and discussion

3.1. Method optimization

The hybrid micelle liquid chromatographic method was optimized intensively by studying the effects of pH of the mobile phase, SDS concentration and organic modifiers (type and ratio) on the separation performance of the studied drugs. Moreover, a gradient program was used in this study by controlling the flow rate and detection wavelength with time to obtain a better resolution and higher sensitivity for both drugs.

pH was studied over the range of (2.5–7), at pH values higher than 3, clozapine (CLZ) eluted with solvent front, while retention time of phenytoin was slightly affected by pH change. This behaviour of the studied drugs towards pH effect could be attributed to their pKa values (3.7 and 7.35 for CLZ and 8.3 for PHY) [26], keeping in mind that their corresponding log \( P \) values are 3.4 and 2.5 [26]. By referring to Figure 2(A), it is obvious that CLZ carries two positive charges at pH values lower than 3. By elevating the pH of the mobile phase, a subsequent decrease in the cationic charge carried by CLZ takes place, to reach a single charge at pH 6. Since CLZ is highly lipophilic as indicated from its log \( P \) value [26], it is expected to undergo mass transfer [22], which transports the drug from the micelles where it binds with electrostatic attraction, to the stationary phase where SDS monomers are adsorbed. At pH values higher than 3, weak binding between CLZ (carrying one positive charge) and SDS monomers is expected, so it fails to be retained on the column and elutes with the solvent front. Below pH 3, more attraction between CLZ and the surfactant monomers takes place, which enhances retention of CLZ on the stationary phase for a reasonable time, resulting in its resolution from the solvent front. Meanwhile, PHY is expected to be neutral all over the studied pH range (Figure 2B), and thus its retention time is slightly affected by pH changes. It elution follows the nonbinding behaviour [22], where it is expected to bind to the hydrophobic core of SDS micelles, owing to its lipophilic nature; log \( P \) 2.5 [26], followed by mass transfer in a way similar to CLZ, except that it is transported to the stationary phase to bind by Vander Waals forces to the lipophilic tail of SDS monomers, and to the alkyl chain of the column, which in turn elongates its retention on the column, causing it to elute later after CLZ. It is worth to notice that mass transfer exhibited by both drugs is enhanced by column heating [23].

The concentration of SDS was studied from 0.05 to 0.18 M SDS. At a molar strength of 0.05, the surfactant did not have sufficient strength to separate CLZ

Figure 2. Charges carried by CLZ (A) and PHY (B) at different pH values as documented by the software: Chemicalize – Instant Cheminformatics Solutions, https://chemicalize.com/app/calculation/clozapine.
from the solvent front, while PHY eluted after 27 min. 0.075 M SDS succeeded to separate CLZ from the solvent front and eluted PHY after 21.7 min. On the other hand at concentrations higher than 0.075 M SDS, clozapine eluted with solvent front, while phenytoin retention time decreased gradually. Consequently, 0.075M SDS was sorted out as an optimal concentration for the separation of both drugs.

The addition of organic modifier to micelle based mobile phase would enhance column efficiency and reduce analysis time. Various types of organic modifier such as methanol, 1-propanol and acetonitrile were studied in this method. Upon using (10%) of organic modifier the retention behaviour of both drugs was different. 1-propanol and methanol eluted CLZ with the solvent front, while phenytoin eluted after 15 min. Upon utilizing acetonitrile as an organic modifier, it was possible to separate CLZ from the solvent front after 8 min, where it was efficiently separated from PHY which appeared after 21.7 min. Different ratios of acetonitrile were also studied. The optimal concentration of acetonitrile was 10%. Lower ratios failed to separate clozapine from phenytoin. While higher percentages of acetonitrile resulted in elution of CLZ with solvent front.

The combination of triethyl amine with acetonitrile has a synergistic effect on separation efficiency parameters [23] than using organic solvents alone, which was reported by El-Shaheny et al. in their review article. Besides, TEA is also utilized for pH adjustment of the mobile phase.

The mobile phase flow rate has a vital effect on chromatographic peak shape and retention time. To compromise between peaks shapes and retention times of both drugs, a simple gradient program was applied using 0.8 mL/min. as a flow rate during the first 10 min of analysis, followed by increasing the rate to reach 1.5 mL/min till the end of analysis.

The choice of UV detection wavelength was challenging, where different wavelength settings (220–280 nm) were investigated for both drugs to reach maximum sensitivity parameters. To achieve this goal, the gradient program of flow rate was accompanied with the adjustment of UV detector to be set at 220 nm for the first 10 min, then changed to be 250 nm till the end of analysis, as these two particular wavelengths resulted in maximum sensitivities for CLZ and PHY respectively. By referring to the absorption spectra of the studied drugs (Figure 3), it could be easily deduced that the selected wavelengths for analysis represent $\lambda_{\text{max}}$ of the concerned analytes.

Eventually, column oven was also investigated over temperature settings from 20°C to 40°C, where 40°C gave optimum baseline separation and was applied through the whole analysis. It is worth to mention that keeping the column at a thermostatically fixed temperature enhances the repeatability of the proposed method. A summary of separation parameters obtained at different mobile phase composition is demonstrated in Table 1.

As a result, CLZ and PHY were separated using a mobile phase composed of 0.075 M SDS, 10% acetonitrile, 0.3% TEA, adjusted to pH 2.5 with o-H$_3$PO$_4$, keeping the column temperature at 40°C, and applying a

![Figure 3. Absorption spectra of CLZ (A) and PHY (B) in methanol.](image)

| Table 1. Separation efficiency parameters of the proposed method. |
|----------------------|------------|------------|------------|
| **pH** | CLZ | PHY | CLZ | PHY | PHY/CLZ |
| 2.5 | 7.673 | 22.722 | 0.343 | 2.373 | 6.913 |
| 3 | 3.326 | 20.591 | 1.015 | 8.703 | 8.577 |
| 4 | 4.526 | 12.124 | 2.616 | 9.341 | 3.571 |
| 5 | 13.793 | 24.596 | 1.818 | 9.249 | 7.619 |
| 6 | 14.153 | 30.444 | 1.818 | 27.238 | 9.128 |
| 7 | 4.657 | 25.514 | 0.933 | 9.523 | 10.83 |

| **SDS concentration** | CLZ | PHY | CLZ | PHY | PHY/CLZ |
|----------------------|------------|------------|------------|
| 0.05 M | 4.747 | 28.377 | 0.48 | 6.813 | 14.189 |
| 0.075 M | 7.575 | 22.267 | 0.373 | 2.442 | 6.547 |
| 0.15 M | 6.275 | 21.217 | 0.824 | 6.372 | 7.736 |
| 0.18 M | 3.03 | 20.844 | 0.55 | 4.772 | 8.675 |

| **Type of organic modifier** | CLZ | PHY | CLZ | PHY | PHY/CLZ |
|----------------------|------------|------------|------------|
| 1-Propanol | 2.776 | 19.75 | 0.572 | 3.584 | 10.843 |
| Methanol | 9.491 | 18.64 | 2.691 | 3.897 | 6.345 |
| Acetonitrile | 7.673 | 22.722 | 0.343 | 2.373 | 6.913 |

| **Ratio of organic modifier** | CLZ | PHY | CLZ | PHY | PHY/CLZ |
|----------------------|------------|------------|------------|
| 5% | 3.215 | 27.535 | 0.957 | 14.945 | 15.612 |
| 7% | 9.011 | 27.101 | 2.294 | 13.699 | 5.972 |
| 10% | 7.673 | 22.722 | 0.343 | 2.373 | 6.913 |
| 12% | 11.55 | 27.382 | 1.389 | 10.016 | 7.164 |
| 15% | 5.154 | 31.186 | 1.185 | 10.435 | 8.809 |

| **Flow rate** | CLZ | PHY | CLZ | PHY | PHY/CLZ |
|----------------------|------------|------------|------------|
| 0.8 mL/min. | 7.687 | 21.94 | 1.917 | 12.759 | 6.656 |
| 1 mL/min. | 10.819 | 26.083 | 1.778 | 12.645 | 7.871 |
| 1.2 mL/min. | 4.481 | 24.846 | 0.926 | 8.219 | 8.871 |

| **Oven temperature** | CLZ | PHY | CLZ | PHY | PHY/CLZ |
|----------------------|------------|------------|------------|
| 20°C | 14.153 | 26.123 | 1.818 | 16.598 | 9.128 |
| 30°C | 14.946 | 27.288 | 1.824 | 15.143 | 8.301 |
| 35°C | 13.793 | 26.598 | 1.818 | 13.849 | 7.619 |
| 40°C | 7.673 | 22.722 | 0.343 | 2.373 | 6.913 |
A presentative chromatogram of the studied drugs applying the described chromatogram conditions could be illustrated in Figure 4.

3.2. Validation parameters

Validation parameters were determined under guidance of ICH [24], including linearity and range, detection limit DL and quantitation limit QL, trueness, precision and specificity.

The proposed method showed linearity over the concentration ranges of 40.0–800.0 µg/mL and 20.0–300.0 µg/mL for clozapine and phenytoin respectively. The calibration graphs were obtained by plotting the peak area versus the final concentration of each drug, from which the following least squares linear regression equations were obtained:

\[ Y = 26.15X + 45510 \] (for CLZ) and \[ Y = 31697X - 201064 \] (for PHY), where \( Y \) is the peak area and \( X \) is the concentration of the drug (µg/mL). With corresponding correlation coefficient \( r \) of 0.9996 and 0.999945, which proves acceptable linearity of the proposed method. \( S_{ar} \), the standard deviation of the intercept was found to be 389.94 and 37741.69 for CLZ and PHY respectively, while their corresponding \( S_{b} \) values, standard deviation of the slope were 0.72 and 235.31.

Detection limit defined regarding to ICH as the lowest possible concentration of the sample at which the method can detected but not quantified. While quantitation limit is described as the lowest concentration of the analyte that can be quantified by the reliable way. Both values were determined, where DL values for CLZ and PHY were 49.18 and 3.96 µg/mL respectively, with corresponding QL values of 149.0 and 12.02 µg/mL.

The trueness was investigated by calculating different concentrations of pharmaceutical drugs within their linearity ranges, where the final outcome was compared statistically with the comparison methods [16,21]. Small values of \( t \) and \( F \) tests were obtained emphasizing on the trueness of the proposed method (Table 2). Concerning precision, analysis of three different concentrations of each drug within their linearity ranges whether on the same day or on three successive days (intraday and interday) was performed. Small values of standard deviation SD were recorded indicating the high precision of proposed method (Table 3). Specificity was
assured by the absence of any interfering peaks at retention time of CLZ and PHY which may arise from dosage forms excipients, impurities, degradation products or biological matrix.

3.3. Applications

3.3.1. Application of proposed method to dosage form

The proposed method was applied to assay CLZ and PHY in their dosage forms. The obtained results were statistically compared with those of reference methods [16,21]. The calculated \( t \) and \( F \) values [27] were lower than the tabulated ones, emphasizing on the accordance between the suggested and reference methods. The obtained data are abridged in Table 4. The selectivity of the proposed method was illustrated from dosage form analysis, where the inactive ingredients showed no peaks at the retention time of either drugs applying the described chromatographic conditions (Figure 5).

3.3.2. Analysis of CLZ and PHY in spiked blood sample

The trueness and precision of the proposed method allow its application to biological fluids. Spiked blood sample experiments were performed in order to construct calibration curves and deduce least square regression equations of the studied pharmaceuticals in the presence of biological fluid, as regression equations of the analytes in their pure form do not reflect their accurate concentrations in whole blood – whether in vitro or in vivo – owing to quantification in a different matrix.

Bioanalytical validation procedures in spiked whole blood experiments were carried out according to reported methods [28,29]. Trueness, as reflected from the obtained percentage recoveries, was judged through spiking the investigated drugs in rat blood and analysing the samples to deduce the concentration of each analyte using the obtained least square regression equation. Table 5 summarizes the obtained data and shows the trueness of the bio-assay. Meanwhile, precision was also assessed via inter-day and intra-day experiments, where the obtained SD values illustrate the precision of the bioanalytical method (Table 5). A presentative chromatogram for spiked rat blood experiment is demonstrated in Figure 6.
3.3.3. In vivo analysis
Further application of the proposed method to in vivo experiment was attained by injecting experimental rats with the reported toxic doses of CLZ and PHY (0.66 and 1 mg/mL) respectively [7,8], so as to mimic the stroke syndrome accompanied with their toxicity. The drugs were injected in three rats to take the average response, where the mean concentration of CLZ and PHY were 61.73 and 25.0 µg/mL respectively. A representative chromatogram of in-vivo experiment is illustrated in

Figure 5. Presentative chromatogram of the extracts of (A) clozapex® tablets (800.0 µg/mL), (B) phenytin® ampoule (200.0 µg/mL) and (C) phenytin® cap (60.0 µg/mL).
Figure 6. Representative chromatogram of rat blood spiked with (A) 200.0 μg/mL CLZ and (B) 100.0 μg/mL PHY.

Figure 7. It is worth to mention that both Figures 6 and 7 reflect the specificity of the bioanalytical technique, where various components in biological matrix showed no interfering peaks at retention times of the studied analytes.

4. Conclusion

Hybrid micellar liquid chromatography was applied for the quantitation and separation of clozapine and phenytoin. Chromatographic parameters were studied to reach the optimal condition for separation. This work was validated according to ICH guidelines. Furthermore, the developed method was applied to assay the studied drugs in rat blood samples (in vivo and in vitro) without any extraction procedure. The validation of the bioanalytical method was also performed to assess its...
trueness and precision. The obtained results suggest the application of the described method as a diagnostic tool for CLZ and PHY both qualitatively and quantitatively in emergency rooms to assist physicians in identifying patients suffering from stroke-like syndrome owing to intoxication with the studied drugs. Such application may help to rescue lives from misdiagnosis with stroke.

Disclosure statement
No potential conflict of interest was reported by the author(s).

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