OFAT versus DOE as two optimization protocols for the chromatographic analysis of some OTC pharmaceuticals carrying negative cardiovascular effects and administered by pregnant and breast-feeding females: Application to dose dependent effect

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ABSTRACT

A simple HPLC technique has been utilized for rapid and sensitive quantitative analysis of two mixtures of drugs that are used during pregnancy and lactation. Drugs of the first mixture are used to manage gastrointestinal tract illness that are common during early stages of pregnancy, while pharmaceutical agents of the second mixture are administered over the counter as galactagogues or to overcome postpartum depression. Mixture I includes famotidine (FMT), ranitidine (RNT), nizatidine (NZT), and pantoprazole (PNT), which were separated on a C18 column using a mobile phase composed of methanol: 0.02 M sodium dihydrogen phosphate (60:40, v/v) of pH 6.9, adopting UV detection at 240 nm at a flow rate of 1 mL/min. Mixture II on the other hand, consists of domperidone (DOM), metoclopramide (MET), and sulpiride (SUL). These drugs were eluted using the same column and flow rate as those in mixture I, using a mobile phase consisting of acetonitrile: 0.075 M sodium dihydrogen phosphate (30:70, v/v) of pH 6 adopting a detection wavelength 270 nm. Two optimization protocols were utilized to optimize the chromatographic separation conditions, namely one factor at a time (OFAT) and design of experiments (DOE) where face centered cube response surface experimental design was chosen for this investigation. Comparison of the results obtained from both protocols reveals the accordance between them. Full validation procedure under guidance of United States Pharmacopoeia (USP) was applied to the proposed methods which enabled their application to separate the drugs of both mixtures in spiked rat whole blood samples and in vivo analysis of rat heart blood.

KEYWORDS

pregnancy, lactation, D₂ antagonist, H₂ antagonist, proton pump inhibitor

INTRODUCTION

Pregnancy and lactation are two important stages in females’ maternal lives. During these stages, some health conditions may be encountered leading to the use of some pharmaceutical products whether prescribed by a specialized physician, or taken over the counter.
Upper gastrointestinal tract illness like heartburn and acid reflux, are common during early stages of pregnancy. Histamine H₂ blockers and proton pump inhibitors are most frequently used to manage nausea and vomiting symptoms associated with pregnancy [1, 2].

Examples of H₂ blockers used are: famotidine (FMT); [1-amino-3-[(diminomethylene) amino]-4-thiazolyl]methyl|thio|propyldiene|sulfamide [3] (Table 1), ranitidine (RNT); N-[2-[[5-[(dimethylamino)methyl]-2-furanyl]methyl|thio|ethyl]-N-methyl-2-nitro-1,1-ethenediamine [3] (Table 1), and nizatidine (NZT); N-[2-[[2-[(dimethylamino)methyl]-4-thiazolyl]methyl|thio|ethyl]-N-methyl 1-2-nitro-1,1-ethenediamine [3] (Table 1). While pantoprazole (PNT); 5-(difluoromethoxy)-2-[[3,4-dimethoxy-2-pyridyl]methyl|sulfinyl]benzimidazole [3] (Table 1), was selected as an example of proton pump inhibitor.

The world health organization (WHO) encourages breast feeding owing to its remarkable advantages for both the neonates and the mothers. Protection of infants from many diseases such as respiratory and urinary tract infections, allergies and diabetes is well known [4]. Maternal health benefits that are clearly recognized include: decrease in post partum bleeding and low risk of breast cancer [4]. As a consequence, many post partum mothers that suffer from low milk production resort to the use of galactagogues. Domperidone (DOM); [2H-benzimidazol-2-one,5-chloro-1-[1-[3-(2,3-dihydro-2-oxo-1H-benzimidazol-1-yl)propyl]-4-piperidinyl]-1,3-dihydro-,(Z)-2-butenedioate [5] (Table 1), and metoclopramide (MET); 4-amino-5-chloro-N-[2-

| Drug | Chemical structure | LogP [29] | Pka [29] |
|------|-------------------|----------|---------|
| FMT  | ![Chemical structure](image1) | -3.1     | 7.61 & 7.75 |
| RNT  | ![Chemical structure](image2) | 0.98     | 8.4     |
| NZT  | ![Chemical structure](image3) | 0.77     | 7.31    |
| PNT  | ![Chemical structure](image4) | 2.18     | 9.15 & 3.55 |
| DOM  | ![Chemical structure](image5) | 3.9      | 7.9     |
| MET  | ![Chemical structure](image6) | 2.6      | 9.27    |
| SUL  | ![Chemical structure](image7) | 0.6      | 8.9     |
(diethylamino)ethyl)-o-anisamide [3] (Table 1), are dopamine D_2 receptor antagonist drugs used mainly for treatment of many gastrointestinal problems such as gastric paresis, gastro esophageal reflux preventing undesirable symptoms like nausea and vomiting [6]. In spite that remarkable increase in serum prolactin concentration (hyperprolactinemia) is one of the side effects of the two drugs [6], maternal mothers take advantage of the exploitation of this drawback, using these two medications to increase milk production [7–14].

Another health problem that may be frequently encountered after childbirth is post partum depression, which is a result of combination of many factors, the most pronounced of them, is the sharp drop in estrogen and progesterone blood concentration levels [6]. Sulpiride (SUL); of them, is the sharp drop in estrogen and progesterone blood concentration levels [6]. Sulpiride (SUL); another D_2 receptor antagonist classified as atypical antipsychotic, is usually used to overcome post partum depression, in addition to its action as a galactagogue [6]. Utility of these agents as galactagogues is questionable, and was not thoroughly evaluated in previous studies.

Although limited findings about adverse effects of these three categories (D_2 and H_2 antagonists and proton pump inhibitors), on both mothers and neonates are reported, their negative cardiovascular effects were the subject of many articles during the last few years [15–23]. From the most pronounced clinically reported effects mentioned in the literature are: ventricular arrhythmia, significant fall in stroke volume and cardiac output, blockage of pre-synaptic auto receptors, remarkable delay in cardiac repolarization associated with QT interval prolongation. Another important fact which attracts attention of many researchers is the presence of a distinct relationship between the administered dose of drugs in mixture II, and their negative cardiovascular effect which was proved in many clinical research studies [24–27].

Owing to these facts, the present research was dedicated to separate and quantify some drugs used during pregnancy; (FMT, RNT, NZT, and PNT), and lactation; (DOM, MET, and SUL), using two HPLC methods. In spite that the studied drugs are not expected to be co administered or co formulated (especially for mixture I), this research paper includes detailed investigation concerning their separation efficiency and quantification owing to their clinical impact which was previously discussed, which in turn is expected to offer help to analysts caring to analyze any of the studied drugs in both mixture, particularly in vivo. For this reason both OFAT (one factor at a time), and DOE were both applied to conclude optimum separation conditions. Face centered cube response surface experimental design was chosen to carry out the optimization experiments studying the effect of six factors; mobile phase pH, type and ratio of organic solvent, molar strength of the buffer, flow rate, and detection wavelength. By comparing the separation efficiency parameters obtained by the two optimization tools, the obtained results were found to be in accordance.

Full validation procedure was carried out for each method allowing their application to quantify the studied pharmaceutical compounds in pure form. The proposed methods were further extended to determine the concentration of the studied drugs both in spiked whole rat blood samples and in the heart blood of rats after administration of the specified clinical doses. A study of the dose dependent cardiac concentrations of drugs in mixture II was also performed. Simple extraction procedure was followed which adds another advantage to the method. It is worth to mention that analytical separation and quantification of these two pharmaceutical combinations was not a subject of any research study in the literature.

**EXPERIMENTAL**

**Instrumentation**

Separation was conducted on Azura® analytical HPLC equipped with P6.1 L pump, UVD 2.1 L detector, DG 2.1 S degasser, product of Knauer, Germany.

All computations were carried out using version 6.5 “Matlab for WindowsTM”, Mathworks Inc., 2002 (USA).

pH meter, Jenway, UK.

**Materials and reagents**

FMT, RNT and NZT were kindly provided by Memphis Company for Pharmaceutical and Chemical Industries, Pharco Pharmaceuticals, and Marcyrl Pharmaceutical Industries respectively, which are all based in Egypt.

PNT was imported from Takeda GmbH Pharmaceuticals, Germany.

DOM, MET, and SUL were supplied respectively by Epico, EVA, and Sanofi-aventis Pharmaceutical Companies, Egypt.

Methanol and acetonitrile; HPLC grade were purchased from Fisher Scientific, UK.

Sodium dihydrogen phosphate, ortho phosphoric acid and triethyl amine were supplied by El Nasr Pharmaceutical and Chemical Company, Egypt.

**Chromatographic separation conditions**

Reversed phase chromatographic separation for both mixtures was performed using Knauer® (150 × 4.6 mm) C18 column, supplied with Eurospher®* pre column, SN: EK 207, Batch # KU40612, adopting a flow rate of 1 mL/min. at ambient temperature applying an isotropic elution mode. For mixture I, the mobile phase was composed of methanol: 0.02 M NaH_{2}PO_{4} buffer (60:40, v/v) of pH 6.9, and accompanied with UV detection at 240 nm. Regarding mixture II, the utilized mobile phase was acetonitrile: 0.075 M NaH_{2}PO_{4} buffer (30:70, v/v), of pH 6 where UV detection was carried out at 270 nm. Adjustment of final pH in both cases was accomplished with 2 M o-phosphoric acid and triethylamine. Before analysis, some routine steps were followed including ultra filtration of the mobile phases with Chrom Tech-UK Nylon membrane filters (0.45 μm pore size, and 0.47 mm diameter) followed by sonication for 30 min. Besides, washing the chromatographic system for one
hour was carried out after analysis using a mixture of double distilled water: methanol (1:1, v/v).

Preparation of standard solutions and calibration
Stock solutions of each of the studied drugs were separately prepared as 500.0 μg/mL in methanol. Further dilution with the same solvent was conducted to reach the following concentration ranges: 0.5–150.0, 0.5–200.0, 0.4–300.0, 0.5–250.0, 0.5–200.0, 1.0–200.0, and 2.0–150.0 μg/mL for FMT, RNT, NZT, PNT, DOM, MET, and SUL respectively. Each concentration was injected in triplicates using the previously described chromatographic conditions to obtain average values. Calibration curve for each drug was established by plotting peak areas versus final concentrations (μg/mL).

Analysis of spiked drugs in rat heart blood samples
One mL of control rat whole heart blood samples were transferred into 10 mL centrifuge tubes, and spiked with appropriate volumes of the studied drugs to reach the following concentration ranges: 0.5–10.0, 0.5–15.0, 0.4–5.0, 0.5–10.0, 0.5–15.0, 1.0–20.0, and 2.0–30.0 for FMT, RNT, NZT, PNT, DOM, MET, and SUL respectively. Addition of 2 mL acetonitrile was compensated, after which centrifugation at 3,500 rpm for 10 min was pulled off to precipitate plasma proteins as a sticky mass at the bottom of the tube. The supernatant was then quantitatively transferred to 10 mL volumetric flask, and the volume was completed to the mark with the suitable mobile phase as described previously. The procedure discussed under “Preparation of standard solutions and calibration” was followed to deduce the regression equation of each drug.

Application of the proposed method to the analysis of the studied drugs in vivo
Stock solutions for all of the studied drugs were prepared in 0.9% sodium chloride: propylene glycol (60:40, v/v) as mentioned in a previously published report [28], aiming to reduce the irritating effect of injection on experimental animals. Stock solutions were prepared to be 300.0 μg/mL for both RNT and NZT, 40.0 μg/mL for PNT and FMT, 4.0 mg/mL for SUL, 600.0 μg/mL for MET, and 30.0 μg/mL for DOM. These concentrations were selected as to reach therapeutic doses of 0.75 mg/kg for RNT and NZT, 0.1 mg/kg for PNT and FMT. To study the dose dependent effect of drugs in mixture II, two therapeutic concentrations were targeted for each drug; i.e., 20.0 and 40.0 mg/kg for SUL, 2.0 and 4.0 mg/kg for MET, 0.075 and 0.15 mg/kg for DOM.

The protocol described for the in vivo experiment was performed according to the instructions of the Institutional Animal Care and Use Committee (IACUC) at Faculty of Pharmacy, Delta University for Science and Technology, Egypt. The proposed method carries Ethical Committee approval number FPDU 2/2020.

The authors’ state that they did their best to reduce animals suffering during the experimental work, and that they utilized the least number of rats that guarantee acceptable results.

The experiment was performed using 22 rats, with average weight ranging from 150 to 200 g. The experimental animals were kept in cages each containing two rats under controlled temperature and lighting. The rats were divided into four groups; where the first one consisted of three rats, while the rest of the groups consisted of six rats each, keeping one rat as a control. Each rat in the first group received an intra peritoneal injection of mixture I drugs applying the specified therapeutic doses. Concerning the three other groups, each group was treated with one single drug in mixture II. Each drug was injected separately in two different doses (specified previously), where each injection was repeated in triplicate to estimate the average responses.

All rats were sacrificed after 30 min, and heart blood samples were collected for each rat one at a time, after which, the procedure mentioned under “Analysis of spiked drugs in rat heart blood samples” were followed to calculate the concentration of each drug using its corresponding regression equation.

RESULTS AND DISCUSSION

OFAT (one factor at a time) optimization
OFAT optimization methodology was applied so as to practically optimize each factor individually keeping other factors at constant values. Different factors affecting the efficiency of separation were carefully optimized to yield well separated peaks of the drugs in both mixtures and, achieving maximum sensitivity. Such factors include: type and ratio of organic modifier, molar strength of buffer, pH of the mobile phase, detection wavelength, and flow rate.

Regarding mixture I, methanol was found to be the solvent of choice, as it succeeded to separate the four drugs efficiently. The percentage of methanol was a key factor in optimization. Different ratios of methanol ranging from 30 to 50% were investigated, upon using 30% methanol, H₂ antagonist drugs were separated efficiently, but PNT was retained for more than 30 min. Upon increasing its ratio to 40%, RNT and NZT were poorly separated with significant overlapped peaks. When methanol ratio was raised to reach 50%, all of the four drugs were adequately separated, but asymmetric peak of PNT resulted. Eventually, when the proportion of methanol: NaH₂PO₄ buffer was (60:40, v/v), well separated peaks accompanied with symmetry in a short run were obtained (Fig. 1A). Upon including acetonitrile together with methanol as a part of the organic modifier in the mobile phase (in a ratio of 2:3, v/v for the two solvents respectively), the peaks of the four drugs were well separated, but distorted peak of PNT resulted. As a consequence, a mobile phase composed of methanol: NaH₂PO₄ buffer (60:40, v/v), was used through the study.

The effect of pH of the mobile phase was very critical upon separating the drugs in this mixture. Different pH ranges were studied ranging from 3 to 6.9. At pH values
lower than 6, all H2 antagonists were eluted with the solvent front, only an asymmetric peak of lower sensitivity of PNT resulted. At pH 6, RNT and NZT appeared as a single peak overlapping with FMT, while NZT peak was tailed and still have poor sensitivity. Optimum separation was procured at pH values higher than 6.5, as a consequence, pH of 6.9 was chosen for the assay of this mixture (Fig. 1B).

This behavior could be well explained by knowing the pK\textsubscript{a} values of the studied drugs which are specified to be (7.61 & 7.75 for FMT, 8.4 for RNT, 7.31 for NZT and 9.15 & 3.55 for PNT) \[29\] (Table 1). The alkaline pK\textsubscript{a} values of H2 antagonists keep them ionized over the entire pH range; (2.5 – 6.5), which increases their polarity and results in their elution with the mobile phase. Upon elevating pH values above 6.5, their ionization starts to be partial; hence a decrease in their polarity arises, separating them from the solvent front. PNT, which has two pK\textsubscript{a} values in both the acidic and alkaline side, will also be ionized through the whole pH range, in spite; its peak was well separated but still distorted at pH values lower than 6.5. Once pH was higher than 6.5, its peak appeared earlier with a remarkable symmetric shape. The relative high log \(P\) value of PNT (2.18) relative to corresponding values of – 3.1, 0.98, and 0.77 for FMT, RNT, and NZT respectively \[29\] (Table 1), reflects its lipophilic nature when compared to H2 antagonists, hence it was slightly retained on the stationary phase.

The molar strength of phosphate buffer was also investigated over the range of 0.005 – 0.05 M. Best separation takes place at concentrations (0.005 – 0.03 M), without affecting the sensitivity of any drug. At higher molar strength; 0.04 – 0.05 M, overlap between the peaks of RNT and NZT takes place. As a result, 0.02 M NaH\textsubscript{2}PO\textsubscript{4} was utilized (Fig. 1C).

Figure 1. A: Effect of methanol percentage on retention time of drugs in mixture I. B: Effect of mobile phase final pH on retention time of drugs in mixture I. C: Effect of molar strength of phosphate buffer on retention time of drugs in mixture I. D: Effect of acetonitrile percentage on retention time of drugs in mixture II. E: Effect of mobile phase final pH on retention time of drugs in mixture II. F: Effect of molar strength of phosphate buffer on retention time of drugs in mixture II.
A flow rate of 1 mL/min. was found to be suitable where lower flow rates; 0.5–0.9 mL/min. resulted in broad peaks accompanied with longer retention times, while higher values; 1.2–1.5 mL/min., resulted in poor resolution of H₂ antagonists peaks. Regarding selection of detection wavelength, 240 nm provided best sensitivity for the four drugs; hence it was applied for this mixture.

Concerning mixture II, it was found that acetonitrile succeeded to separate the three drugs when it was in a ratio of 30% of the mobile phase. Upon increasing its percentage (40–60%), only SUL appeared in a reasonable time, while both MET and DOM were eluted with the solvent front (Fig. 1D). An explanation of this behavior could be presented keeping in consideration the log p values of SUL, MET, and DOM are 0.6, 2.6, and 3.9 respectively [29] (Table 1). It is expected then that drugs with a more lipophilic nature (MET and DOM), as revealed from their log p values, have a higher tendency to dissolve in organic solvent portion of mobile phase and as a result higher ratios of acetonitrile eluted them with the solvent front. Meanwhile, SUL was not subjected to this phenomenon, since it bears a less hydrophobic structure. When methanol was included as an organic modifier besides acetonitrile in a ratio of (3:2, acetonitrile: methanol, v/v), the elution order was modified, where MET peak appeared first at 2 min, while SUL and DOM eluted later with an unacceptable overlap.

The pH of the mobile phase was also very critical in maintaining well separated peaks. When the pH was lower than 5.5, peaks of SUL and MET overlapped, so, for best separation efficiency, the pH was kept at values higher than 5.5. Consequently, pH 6 was chosen for best separation of the drugs in this mixture (Fig. 1E). This behavior could be well understood by knowing pkα values of the drugs which are stated to be 8.9, 9.27, and 7.9 for SUL, MET, DOM respectively [29] (Table 1). It is obvious that the alkaline nature of the three drugs keep them ionized through the entire pH range, where both SUL and MET, have a higher ionization tendency than DOM as revealed from their higher pkα values, as a consequence their peaks were liable to overlap at pH values lower than 5.5. Upon raising the mobile phase pH, this overlap was overcome. DOM, on the other hand did not show any overlap with either drugs assuming that it has a lower ionization probability as reflected from its pkα.

The molar strength of NaH₂PO₄ buffer was studied over the range of (0.005–0.1 M), and it was found that concentrations lower than 0.05 M did not achieve separation of SUL and MET, while concentrations ranging from (0.05–0.1 M), managed this challenge, as a consequence, 0.075 M was chosen as an optimum molar concentration of the buffer (Fig. 1F).

Detection wavelength was scanned over the range of 240–280 nm, where 270 nm was selected to obtain maximum sensitivity for all three drugs, while the flow rate was kept at 1 mL/min. for the same reason mentioned in optimization of mixture I.

A representative chromatogram of the drugs in mixture I is illustrated in Fig. 2A using methanol: 0.02 M NaH₂PO₄ buffer (60:40, v/v), of final pH 6.9 adopting UV detection at 240 nm at a flow rate of 1 mL/min. where: (a) FMT, (b) RNT, (c) NZT, (d) PNT. B: Representative chromatogram of the drugs in mixture II, each in a concentration of 100.0 µg/mL, using a mobile phase composed of: methanol: 0.075 M NaH₂PO₄ buffer (30:70, v/v), at pH 6, using 1 mL/min. as a flow rate, and 270 nm as a detection wavelength; where: (a) SUL, (b) MET, (c) DOM.

Design of experiment
Application of OFAT to optimize chromatographic separation, where every factor was studied solitary, consumes a lot of effort and time to achieve optimum separation, keeping in mind that a well planned experiment is introduced [30–32]. These difficulties encourage the authors to conduct design of experiment (DOE) to predict and optimize separation parameters of the two studied mixtures, employing fractional factorial design enable the determination of the noteworthy of the studied factors, while optimization of chromatographic conditions is attained through application of central composite design.
Before carrying out optimization, it is worth to recognize the vital factors influencing the quality of the inferred results. Two level fractional factorial design was utilized to study the significance of six independent factors on separation efficiency. Selection of the factors is based on prelude experiments. The six studied factors are ratio of organic solvent (X1), pH (X2), type of organic solvent (X3), buffer strength (X4), flow rate (X5), and detection wavelength (X6).

Such factors are believed to significantly affect the outcomes. To screen k number of factors, 2^k based two level fractional factorial design was applied according to the following equation which encompasses the mathematical model of the design [33]:

\[ Y = \alpha_0 + \sum_{T=1}^{n} \alpha_T X_T + \sum_{T=1}^{n} \sum_{J=T+1}^{n} \alpha_{TJ} X_T X_J \]

where: X is the investigated factor, Y is the response of the tested factor, n is the number of factors, and \( \alpha_0, \alpha_T, \alpha_{TJ} \) are the coefficients for each major or interaction influence. The proposed method studied six factors over two levels, where upper and lower limits of factors were determined in view of fundamental analyses. All tests were led in randomized request and in triplicate.

To optimize the separation of the studied drugs in both mixtures, each one of the six studied factors was studied over three levels using face centered cube response surface experimental design. Optimization could be performed over the expedient range of the chosen factors listed in Table 2. The number of experiments in two level fractional factorial design = 2^3 experiments (Table 2). This diminished plan permits the main estimation of the chief factor impacts.
### Table 3. Comparison of the separation parameters obtained by experimental procedure and matlab prediction for the studied drugs in both mixtures

| Drug | Mixture I | Mixture II | Mixture I | Mixture II |
|------|-----------|------------|-----------|------------|
|      | FMT | RNT | NZT | PNT | SUL | MET | DOM | FMT | RNT | NZT | PNT | SUL | MET | DOM |
| Rs   | 1.2 | 2.76 | 4.67 | 9.85 | 2.92 | 4.92 | 9.93 | 1.18 | 2.57 | 4.82 | 9.67 | 3.14 | 4.88 | 9.89 |
| NTP  | 211 | 714 | 824 | 1,391 | 630 | 3,838 | 4,045 | 215 | 699 | 832 | 1,388 | 645 | 3,844 | 4,050 |
| HETP | 0.12 | 0.035 | 0.03 | 0.018 | 0.039 | 0.0065 | 0.0062 | 0.114 | 0.038 | 0.029 | 0.014 | 0.038 | 0.0071 | 0.0059 |

### Table 4. Summary of ANOVA results for fractional design and central composite design

| Factors | Mixture I | Mixture II |
|---------|-----------|------------|
|         | $t_{FMT}$ | $t_{PNT}$ | COF | $t_{SUL}$ | $t_{DOM}$ | COF |
| X1      | $6.235$ | $<1 \times 10^{-4}$ | $10.255$ | $<1 \times 10^{-4}$ | $7.815$ | $<1 \times 10^{-4}$ | $7.881$ | $<1 \times 10^{-4}$ | $13.649$ | $<1 \times 10^{-4}$ | $8.924$ | $<1 \times 10^{-4}$ |
| X2      | $5.894$ | $<1 \times 10^{-4}$ | $11.234$ | $<1 \times 10^{-4}$ | $8.129$ | $<1 \times 10^{-4}$ | $8.134$ | $<1 \times 10^{-4}$ | $12.935$ | $<1 \times 10^{-4}$ | $9.034$ | $<1 \times 10^{-4}$ |
| X3      | $3.9 \times 10^{-3}$ | $0.415$ | $7.7 \times 10^{-4}$ | $0.295$ | $3.5 \times 10^{-3}$ | $0.408$ | $9.7 \times 10^{-4}$ | $0.229$ | $6.6 \times 10^{-3}$ | $0.334$ | $2.8 \times 10^{-4}$ | $0.392$ |
| X4      | $1.4 \times 10^{-4}$ | $0.394$ | $5.4 \times 10^{-3}$ | $0.354$ | $2.9 \times 10^{-2}$ | $0.394$ | $8.8 \times 10^{-5}$ | $0.315$ | $5.3 \times 10^{-4}$ | $0.229$ | $7.6 \times 10^{-5}$ | $0.446$ |
| X5      | $8.2 \times 10^{-3}$ | $0.297$ | $9.7 \times 10^{-3}$ | $0.428$ | $6.9 \times 10^{-4}$ | $0.517$ | $7.1 \times 10^{-5}$ | $0.339$ | $8.4 \times 10^{-4}$ | $0.408$ | $1.5 \times 10^{-4}$ | $0.267$ |
| X6      | $6.4 \times 10^{-5}$ | $0.337$ | $5.6 \times 10^{-3}$ | $0.396$ | $7.9 \times 10^{-3}$ | $0.443$ | $6.9 \times 10^{-3}$ | $0.289$ | $3.6 \times 10^{-3}$ | $0.443$ | $3.9 \times 10^{-3}$ | $0.296$ |
| X1X2    | $826$ | $8.2 \times 10^{-4}$ | $962$ | $3.5 \times 10^{-4}$ | $345$ | $2.9 \times 10^{-4}$ | $422$ | $3.3 \times 10^{-4}$ | $881$ | $7.4 \times 10^{-4}$ | $554$ | $8.2 \times 10^{-4}$ |
| R2_adj | 0.9997 | 0.9998 | 0.9996 | 0.9996 | 0.9996 | 0.9995 | 0.9997 | 0.9997 |
| A       | 158 | $<1 \times 10^{-4}$ | $271$ | $<1 \times 10^{-4}$ | $145$ | $<1 \times 10^{-4}$ | $229$ | $<1 \times 10^{-4}$ | $290$ | $<1 \times 10^{-4}$ | $112$ | $<1 \times 10^{-4}$ |
| B       | 118 | $<1 \times 10^{-4}$ | $71$ | $<1 \times 10^{-4}$ | $29$ | $<1 \times 10^{-4}$ | $163$ | $<1 \times 10^{-4}$ | $121$ | $<1 \times 10^{-4}$ | $18$ | $<1 \times 10^{-4}$ |
| AB      | 17 | 0.015 | 11 | 0.068 | 3 | 0.084 | 13 | 0.033 | 10 | 0.089 | 7 | 0.018 |
| R2_adj | 0.982 | 0.961 | 0.966 | 0.978 | 0.938 | 0.978 |
puzzled with the second order interaction; in spite this prompts partial slaying of data. Central replication experiments demonstrated in Table 2; aim to assess practical error and to examine reproducibility. Separation efficiency for the studied drugs in both mixtures was significantly affected by two factors; ratio of organic solvent and pH of the mobile phase. Table 3 summarizes the separation efficiency parameters obtained upon applying both optimization techniques; OFAT and DOE.

To apprehend the factors that remarkably affect separation, a supporting fold over fractional factorial design experiments were carried out (Table 2), which is acquired through reversing the signs of the experiments, listed in the last three columns of the factorial design. The impact of this design lays in its ability to discriminate between primary factors from secondary interaction ones. The results obtained from this design in addition to those attained from fractional design assert the previous conclusion emphasizing that retention of all drugs are mainly affected by mobile phase pH and percentage of organic phase, while other factors has inappreciable effect.

Studying the presence of quadratic effects was possible via calculating the \( F \) value which can make a distinction between retention parameters in central replications and

### Table 5. Summary of the performed experiments and the corresponding responses applying central composite design

| Run | Factors | Mixture I | Responses | Mixture II | Responses |
|-----|---------|-----------|-----------|-----------|-----------|
|     |         | \( t_{\text{FMT}} \) | \( t_{\text{PNT}} \) | COF | \( t_{\text{SUL}} \) | \( t_{\text{DOM}} \) | COF |
| 1   | 30      | 6.9       | 3.25      | 32.86     | 1.82      | 2.13      | 5.4 | 4.51 |
| 2   | 40      | 5         | 1.52      | 15.25     | 2.27      | 2.38      | 1.32 | 2.92 |
| 3   | 60      | 5         | 1.35      | 5.24      | 3.85      | 2.16      | 1.45 | 2.81 |
| 4   | 45      | 6         | 2.05      | 12.74     | 2.95      | 2.03      | 1.52 | 2.71 |
| 5   | 55      | 4.5       | 1.26      | 8.34      | 3.42      | 2.31      | 1.36 | 2.94 |
| 6   | 55      | 6.9       | 1.92      | 6.51      | 3.65      | 1.95      | 1.28 | 1.58 |
| 7   | 60      | 3         | 1.22      | 6.64      | 3.77      | 1.35      | 1.35 | 1.64 |
| 8   | 40      | 6.9       | 2.36      | 12.25     | 2.55      | 2.12      | 1.33 | 2.06 |
| 9   | 45      | 4         | 1.55      | 16.91     | 2.74      | 1.95      | 1.47 | 1.38 |
| 10  | 55      | 5.5       | 1.42      | 7.25      | 3.35      | 1.96      | 1.52 | 2.03 |
| 11  | 40      | 5.5       | 1.22      | 14.29     | 2.28      | 2.12      | 1.56 | 2.14 |
| 12  | 60      | 5.5       | 1.51      | 4.95      | 3.92      | 1.81      | 1.23 | 1.35 |
| 13  | 35      | 6.9       | 3.01      | 28.52     | 1.95      | 2.11      | 4.81 | 3.85 |
| 14  | 50      | 4.5       | 1.42      | 8.47      | 3.22      | 2.34      | 1.34 | 2.16 |
| 15  | 60      | 6.5       | 1.77      | 4.77      | 4.25      | 1.85      | 1.53 | 1.08 |
| 16  | 40      | 6         | 1.82      | 13.55     | 2.38      | 2.15      | 1.29 | 2.11 |
| 17  | 45      | 6.9       | 2.03      | 10.45     | 3.14      | 2.05      | 1.37 | 2.36 |
| 18  | 60      | 6         | 1.93      | 4.83      | 4.18      | 1.82      | 1.33 | 1.06 |
| 19  | 30      | 3.5       | 1.26      | 30.45     | 1.54      | 1.45      | 3.82 | 3.75 |
| 20  | 35      | 3         | 1.14      | 29.74     | 1.65      | 1.38      | 3.61 | 3.66 |

**Figure 3.** Perturbation plots illustrating the effect of the studied factors on the responses, where: (A) \( t_{\text{FMT}} \), (B) \( t_{\text{PNT}} \), (C) \( t_{\text{SUL}} \), (D) \( t_{\text{DOM}} \), (E) COF for mixture I, (F) COF for mixture II
Table 6. Accuracy and precision data of drugs in mixtures I and II applying the proposed methods

| Parameter | Mixture I | Mixture II | Mixture I | Mixture II |
|-----------|-----------|------------|-----------|------------|
|           | FMT | RNT | NZT | PNT | DOM | MET | SUL | FMT | RNT | NZT | PNT | DOM | MET | SUL |
| 0.5       | 0.5 | 0.4 | 0.5 | 0.5 | 1.0 | 2.0 | 100.52 | 99.31 | 100.84 | 99.28 | 100.03 | 99.36 | 100.34 |
| 10.0      | 20.0 | 20.0 | 10.0 | 10.0 | 10.0 | 10.0 | 99.84 | 99.87 | 101.25 | 99.46 | 100.85 | 99.65 | 99.46 |
| 30.0      | 50.0 | 50.0 | 50.0 | 50.0 | 50.0 | 50.0 | 99.31 | 101.16 | 100.37 | 101.16 | 99.46 | 99.84 | 99.84 |
| 50.0      | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.09 | 100.95 | 101.39 | 101.41 | 99.88 | 99.75 | 98.94 |
| 100.0     | 150.0 | 200.0 | 200.0 | 200.0 | 150.0 | 150.0 | 100.47 | 101.06 | 99.64 | 103.37 | 101.45 | 100.28 | 100.45 |
| 150.0     | 200.0 | 300.0 | 250.0 | 200.0 | 200.0 | 200.0 | 100.22 | 100.84 | 99.55 | 99.85 | 101.32 | 101.06 | 101.35 |
| Mean      |          |          |          |          |          |          | 100.08 | 100.37 | 100.13 | 100.09 | 100.49 | 100.16 | 100.06 |
| SD        |          |          |          |          |          |          | 0.45   | 0.71   | 0.82   | 0.78   | 0.83   | 0.53   | 0.84   |
| Reference methods [36–39], % Found |
| Mean      |          |          |          |          |          |          | 99.91  | 99.38  | 99.42  | 100.38 | 100.19 | 100.25 | 99.32  |
| SD        |          |          |          |          |          |          | 0.29   | 0.45   | 0.47   | 0.73   | 0.91   | 0.56   | 0.59   |
| t-test    |          |          |          |          |          |          | *0.95  | 0.72   | 0.09   | 0.37   | 0.83   | 0.38   | 0.24   |
| F test    |          |          |          |          |          |          | *2.4   | 2.5    | 3.1    | 1.1    | 1.2    | 1.1    | 2.1    |

Intra-day precision

|          |          |          |          |          |          |          | 0.5    | 0.5    | 0.4    | 0.5    | 1.0    | 2.0    | 99.25  | 100.58 | 99.68  | 100.78 | 99.68  | 100.47 | 99.78  |
| Mean     |          |          |          |          |          |          | 99.91  | 100.97 | 99.74  | 101.03 | 99.61  | 100.54 | 99.96  |
| SD       |          |          |          |          |          |          | 0.58   | 0.44   | 0.72   | 0.29   | 0.23   | 0.81   | 0.75   |

Inter-day precision

|          |          |          |          |          |          |          | 0.5    | 0.5    | 0.5    | 0.5    | 1.0    | 2.0    | 99.14  | 100.85 | 100.11 | 100.84 | 100.64 | 99.78  | 99.64  |
| Mean     |          |          |          |          |          |          | 100.19 | 100.34 | 100.81 | 100.65 | 101.01 | 99.86  | 99.72  |
| SD       |          |          |          |          |          |          | 0.94   | 1.11   | 0.67   | 0.82   | 0.38   | 0.58   | 0.75   |

The significance of bold is highlighting the obtained results.

*2.015 and 19.3 are tabulated t and F values at P = 0.05 [40].

The fractional design. F is calculated from the following mathematical equation [34]:

\[ F = \frac{(YC - YF)^2}{VC\left(\frac{1}{n_C} - \frac{1}{n_F}\right)} \]

YC and YF are the mean responses of the design experiments; (central replication and fractional factorial respectively), \(n_C\) and \(n_F\) are the corresponding numbers of experiments, and VC is experimental variance.

By calculating F value, it was found to be higher than tabulated values, which led to inclusion of quadratic effect in the models. Consequently, star design experiments were built in to the suggested model yielding a composite design (Table 2).

Variable selection algorithm was applied to obtain optimum models for regression, which revealed \(R^2_{CV} \geq 97.4\%\) and 96.9\% for mixture I and mixture II respectively with corresponding values of \(R^2 \geq 98.12\%\) and 98.10\% manifesting the acceptable predicting behavior of the models. After through study, separation of the drugs in mixture I was carried out using a mobile phase composed of methanol: 0.02 M sodium dihydrogen phosphate (60:40, v/v) of pH 6.9, while pharmaceutical compounds in mixture II were separated using acetonitrile: 0.075 M sodium dihydrogen phosphate (30:70, v/v) of pH 6.

For statistical analysis of the obtained results, analysis of variance (ANOVA), was applied. Selection of the response factors for both mixtures could be summarized as follows: the first factor is the retention times of highly polar drugs which elute first since their separation is critical where elution with solvent front is possible; \(t_{DOM}\) for mixture I and \(t_{SUL}\) for mixture II. Secondly, the retention times of the last eluted drugs as this factor has a direct influence on the length of the chromatographic run; \(t_{PNT}\) for mixture I and \(t_{DOM}\) for mixture II, eventually the last factor is COF i.e., chromatographic optimization function. Numerical value of COF is deliberated from the mathematical expression [33]:

\[ COF = \sum_{i=1}^{K} A_i \ln\left(\frac{R_i}{R_d}\right) + B(t_M - t_L) \]

In this mathematical expression, \(R_i\) and \(R_d\) are actual and desired resolution (=1.5) between certain pair of drugs peaks (ith pair), \(A_i\) and \(B\) are constant values (=1), \(t_L\) and \(t_M\) are the actual retention time of the last eluted drug peak in each mixture, and the desired analysis time (postulated to be
whereas the measured responses are retention time of (X1X2) takes place (Table 4). In of the studied cases interaction between tested factors on two level basis has \( P_{fl} \) the response. Remarkable in eluted drug in each mixture (Eq. 10 min in this study). COF is appealing to researchers as its use minimize the data into one numerical value which render the optimization facile.

By referring to the mathematical expression of COF, it could be realized that its value increases by enhanced resolution between pairs of drug peaks and reduced chromatographic separation optimization. The primary main factors selected were percentage interaction factors, application of central composite design ANOVA are abridged in Table 4. When a studied factor could be applicable for chromatographic separation optimization readily.

By previous knowledge of primary and secondary interaction factors, application of central composite design could be applicable for chromatographic separation optimization. The primary main factors selected were percentage of organic phase (A) and pH of the mobile phase (B), whereas the measured responses are retention time of first eluted drug in each mixture \( t_{FMT} \) for mixture I and \( t_{SUL} \) for mixture II), the retention time of the last eluted drug \( t_{PNT} \) for mixture I and \( t_{DOM} \) for mixture II). Details of the experiments which were performed randomly and their measured responses are listed in Table 5.

By referring to Table 4, it could be realized that for mixture I factor A (ratio of methanol) greatly affects \( t_{PNT} \) and factor B (pH) influences \( t_{FMT} \). Regarding mixture II, factor A has an effect on \( t_{DOM} \) while factor B affects \( t_{SUL} \).

Interaction between the two factors AB also affects the measured responses.

Illustration of the predicted models in the form of perturbation plots is presented in Fig. 3 (A–F). The plot manifests that by deviation of a factor from selected standard point where other factors are kept fixed at this point [35], the response will consecutively change, as steep curvature is an indication of the high sensitivity of a certain response to a studied factor.

**Table 7. Accuracy and precision data for the analysis of spiked drugs in rat heart blood samples applying the proposed methods**

| Parameter | Taken (μg/mL) | % found |
|-----------|---------------|---------|
|           | Mixture I     | Mixture II | Mixture I | Mixture II |
|           | FMT | RNT | NZT | PNT | DOM | MET | SUL | FMT | RNT | NZT | PNT | DOM | MET | SUL |
| 0.5       | 0.5 | 0.4 | 0.5 | 0.5 | 1.0 | 2.0 | 98.25 | 97.74 | 97.85 | 96.66 | 98.25 | 99.85 | 97.61 |
| 1.0       | 1.0 | 1.0 | 1.0 | 1.0 | 5.0 | 5.0 | 99.25 | 97.12 | 96.95 | 97.85 | 97.75 | 99.15 | 98.35 |
| 3.0       | 5.0 | 1.5 | 3.0 | 5.0 | 7.0 | 10.0 | 97.75 | 98.52 | 98.89 | 97.12 | 97.16 | 98.78 | 97.33 |
| 5.0       | 7.0 | 2.5 | 5.0 | 7.0 | 10.0 | 15.0 | 97.75 | 98.52 | 98.89 | 97.12 | 98.95 | 100.68 | 98.22 |
| 7.0       | 10.0 | 3.0 | 7.0 | 10.0 | 15.0 | 25.0 | 100.14 | 100.73 | 99.03 | 97.26 | 100.48 | 100.74 | 100.84 |
| 10.0      | 15.0 | 5.0 | 10.0 | 15.0 | 20.0 | 30.0 | 100.95 | 100.28 | 99.75 | 97.86 | 100.35 | 101.35 | 100.39 |

The significance of bold is highlighting the obtained results.

Statistical validation of the obtained results is thoroughly studied according to USP guidelines [3] focusing in this study on: linearity and range, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness.

Linearity of the proposed method was accomplished over the concentration ranges of 0.5–150.0, 0.5–200.0, 0.4–300.0, 0.5–250.0, 0.5–200.0, 1.0–200.0, and 2.0–150.0 μg/mL for FMT, RNT, NZT, PNT, DOM, MET, and SUL respectively. Regression equation for each drug was concluded by plotting linear relation between the final concentration of the studied pharmaceutical compound in μg/mL and peak area. LOD and LOQ; i.e., the concentrations yielding a peak to noise ratio of 3:1, and 10:1 respectively [3], were determined experimentally. LOD of FMT, RNT, NZT, PNT, DOM, MET, and SUL were found to be 0.32, 0.37, 0.25, 0.31, 0.29, 0.76, and 1.32 μg/mL respectively. While the corresponding LOQ values were
0.44, 0.41, 0.33, 0.44, 0.37, 0.94, and 1.85. These low values emphasize the high sensitivity of the proposed methods.

Accuracy, which measures the closeness between the true values and the measured values, was evaluated by analyzing different concentrations of each drug in both mixtures – within the linearity ranges – and statistically comparing the results obtained with those procured by the reference methods [36–39]. Agreement between proposed and reference methods was evident as reflected from small t and F values [40], Table 6.

Precision, which judge the imminence between repeated measured values, was estimated by analyzing three different concentrations of each drug within the same day or on three different days (Table 6). Precision of the proposed method was obvious as disclosed from the small values of SD. Moreover, precision was also apparent when the application to spiked rat blood was performed, as divulged from Table 7.

Robustness of the proposed method was manifested when minor changes in chromatographic conditions had no effect on separation efficiency or sensitivity of the separated peaks. As for example the molar strength of phosphate buffer in the range of (0.005–0.03 M) for mixture I, and (0.05–0.1 M) for mixture II, and the pH of the mobile phase having values higher than 6.5 and 5.5 for mixtures I and II respectively.

VALIDATION OF THE BIO-ANALYTICAL ASSAY METHODS IN SPIKED WHOLE BLOOD SAMPLES

It is well known that before applying the analytical methods to analyze pharmaceuticals in biological matrices, the method should be subjected to appropriate setup and

Figure 4. A: Representative chromatogram of drugs in mixture I in rat heart blood after their injection in their therapeutic doses (0.75 mg/kg for RNT, and NZT, and 0.1 mg/kg for PNT) where: (a) RNT (0.61 µg/mL), (b) NZT (0.55 µg/mL), (c) PNT (1.74 µg/mL). B and C: Representative chromatograms of DOM in rat heart blood after its injection in two therapeutic doses [0.075 mg/kg (3B), 0.15 mg/kg (3C)] where: (a) DOM; 0.63 µg/mL (3B), 1.49 µg/mL (3C). D and E: Representative chromatograms of MET in rat heart blood after its injection in two therapeutic doses [2.0 mg/kg (3D), 4.0 mg/kg (3E)] where: (b) MET; 2.19 µg/mL (3D), 33.15 µg/mL (3E). F and G: Representative chromatograms of SUL in rat heart blood after its injection in two therapeutic doses [20.0 mg/kg (3F), 40.0 mg/kg (3G)] where: (c) SUL; 5.28 µg/mL (3F), 8.58 µg/mL (3G). H: Representative chromatogram of control rat heart blood.
The precision of the bioassay as revealed by Table 7 prove the precision of the bio analysis as revealed by three times of its value. The obtained results abridged in Table 8 was selected so as to be close to the LOQ but does not exceed this point of concern for analysts in future research papers.

Meanwhile, the dose dependent effect of the drugs in mixture II could be simply proved by injecting the laboratory animals (three rats for each dose of the selected drug to get average values) with two different doses of each drug. Higher heart blood concentration of each drug was revealed upon increasing its injected dose. Heart blood concentrations of the two doses of DOM (0.075, and 0.15 mg/kg) were 0.65, and 0.98, while those of SUL (20.0, 4.0 mg/kg) were 2.19, and 33.42 mg/mL, respectively. FMT, on the other hand, could not be quantified, owing to its short elution time (1.8 min.), where it was found to slightly overlapped with the solvent front – that showed a large peak of soluble plasma proteins – when the method was applied in vivo. Hence, quantitative analysis was not possible, and in spite that other reported extraction techniques were attempted, no improvement in its quantitation was detected. The short analysis time and utility of an easily prepared mobile phase in this research, in addition to accomplishment of many parameters included in validation, were advantageous, so that quantitative determination of FMT in vivo was sacrificed, leaving this challenge to be a point of concern for analysts in future research papers.

### Table 8. Application of the proposed method to the analysis of the studied drugs in both mixtures in vivo

| Injected dose | RNT | NZT | PNT |
|--------------|-----|-----|-----|
| Rat#1        | 0.74| 0.57| 1.58|
| Rat#2        | 0.66| 0.49| 1.62|
| Rat#3        | 0.61| 0.55| 1.74|
| Mean         | 0.67| 0.54| 1.65|
| SD           | 0.07| 0.04| 0.08|
| Rat#1        | 5.36| 2.19| 0.65|
| Rat#2        | 5.39| 1.92| 0.58|
| Rat#3        | 5.28| 2.42| 0.63|
| Mean         | 5.36| 2.19| 0.65|
| SD           | 0.07| 0.25| 0.09|
| Rat#1        | 8.58| 34.25| 0.98|
| Rat#2        | 9.24| 33.15| 1.58|
| Rat#3        | 7.88| 32.85| 1.49|
| Mean         | 8.57| 33.42| 1.35|
| SD           | 0.68| 0.74| 0.32|

The significance of bold is highlighting the obtained results.

The high sensitivity and accuracy of the proposed methods qualify them to be applied in vivo. Pharmaceutical compounds in mixture I were injected (three animals for each drug) in their specified therapeutic doses – 0.75 mg/kg for RNT and NZT, 0.1 mg/kg for PNT and FMT – and their concentrations in heart blood samples were calculated according to the regression equations obtained from spiked rat blood experiments (Fig. 4A, Table 8). The average concentrations of RNT, NZT, and PNT were 0.67, 0.54, and 1.65 µg/mL respectively. FMT, on the other hand, could not be quantified, owing to its short elution time (1.8 min.), where it was found to slightly overlapped with the solvent front – that showed a large peak of soluble plasma proteins – when the method was applied in vivo. Hence, quantitative analysis was not possible, and in spite that other reported extraction techniques were attempted, no improvement in its quantitation was detected. The short analysis time and utility of an easily prepared mobile phase in this research, in addition to accomplishment of many parameters included in validation, were advantageous, so that quantitative determination of FMT in vivo was sacrificed, leaving this challenge to be a point of concern for analysts in future research papers.

In vivo analysis of the drugs in both mixtures

The high sensitivity and accuracy of the proposed methods qualify them to be applied in vivo. Pharmaceutical compounds in mixture I were injected (three animals for each drug) in their specified therapeutic doses – 0.75 mg/kg for RNT and NZT, 0.1 mg/kg for PNT and FMT – and their concentrations in heart blood samples were calculated according to the regression equations obtained from spiked rat blood experiments (Fig. 4A, Table 8). The average concentrations of RNT, NZT, and PNT were 0.67, 0.54, and 1.65 µg/mL respectively. FMT, on the other hand, could not be quantified, owing to its short elution time (1.8 min.), where it was found to slightly overlapped with the solvent front – that showed a large peak of soluble plasma proteins – when the method was applied in vivo. Hence, quantitative analysis was not possible, and in spite that other reported extraction techniques were attempted, no improvement in its quantitation was detected. The short analysis time and utility of an easily prepared mobile phase in this research, in addition to accomplishment of many parameters included in validation, were advantageous, so that quantitative determination of FMT in vivo was sacrificed, leaving this challenge to be a point of concern for analysts in future research papers.

Meanwhile, the dose dependent effect of the drugs in mixture II could be simply proved by injecting the laboratory animals (three rats for each dose of the selected drug to get average values) with two different doses of each drug. Higher heart blood concentration of each drug was revealed upon increasing its injected dose. Heart blood concentrations of the two doses of DOM (0.075, and 0.15 mg/kg) were 0.65, and 0.98, while those of SUL (20.0, 4.0 mg/kg) were 2.19, and 33.42 mg/mL, respectively. FMT, on the other hand, could not be quantified, owing to its short elution time (1.8 min.), where it was found to slightly overlapped with the solvent front – that showed a large peak of soluble plasma proteins – when the method was applied in vivo. Hence, quantitative analysis was not possible, and in spite that other reported extraction techniques were attempted, no improvement in its quantitation was detected. The short analysis time and utility of an easily prepared mobile phase in this research, in addition to accomplishment of many parameters included in validation, were advantageous, so that quantitative determination of FMT in vivo was sacrificed, leaving this challenge to be a point of concern for analysts in future research papers.

Meanwhile, the dose dependent effect of the drugs in mixture II could be simply proved by injecting the laboratory animals (three rats for each dose of the selected drug to get average values) with two different doses of each drug. Higher heart blood concentration of each drug was revealed upon increasing its injected dose. Heart blood concentrations of the two doses of DOM (0.075, and 0.15 mg/kg) were 0.65, and 1.35 µg/mL. Corresponding values of MET (2.0, and 4.0 mg/kg) were 2.19, and 33.42 µg/mL, while those of SUL (20.0, and 40.0 mg/kg) were 5.36 and 8.57 µg/mL. These conclusions could be manifested from Fig. 4B, C, D and Table 8). It is obvious from the obtained results that by doubling the injected dose of each drug, the concentration of DOM in rat heart blood increased by a two fold time, while that of SUL by...
one and a half value. Concerning MET, a significant increase of 15 times in concentration was observed. By analyzing these data, it is assumed that high doses or prolonged use of the three pharmaceuticals for maternal mothers may lead to complicated cardiovascular effects (especially in case of MET) and hence their administration should be controlled under guidance of a specialized physician.

**CONCLUSION**

The proposed liquid chromatographic methods succeeded to separate and quantify the pharmaceutical agents included in two mixtures used during pregnancy and lactation. Well resolved peaks were obtained upon using simply prepared mobile phases in short chromatographic runs; 6 min. Both optimization protocols (OFAT and DOE) were in accordance regarding selection of optimum separation conditions. Conduction of full validation parameters was performed, which enables application of the proposed chromatographic methods in vivo to measure the concentrations of the studied drugs in rat heart blood, and to study the dose dependent cardiovascular effects of drugs in mixture II. The methods carry many advantages being simple, sensitive, rapid and relatively economic, which make them suitable in quality control laboratories as a monitoring guide for blood sample analysis in maternal females taking any of these medications and liable to negative cardiovascular complications.

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