

Scientific paper

# Optimization and Validation of a Sensitive Method for HPLC–PDA Simultaneous Determination of Torasemide and Spironolactone in Human Plasma using Central Composite Design

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Received: 03-12-2014

## Abstract

A sensitive, accurate, precise and rapid HPLC-PDA method was developed and validated for the simultaneous determination of torasemide and spironolactone in human plasma using Design of experiments. Central composite design was used to optimize the method using content of acetonitrile, concentration of buffer and pH of mobile phase as independent variables, while the retention factor of spironolactone, resolution between torasemide and phenobarbitone; and retention time of phenobarbitone were chosen as dependent variables. The chromatographic separation was achieved on Phenomenex C<sub>18</sub> column and the mobile phase comprising 20 mM potassium dihydrogen *ortho* phosphate buffer (pH-3.2) and acetonitrile in 82.5:17.5 v/v pumped at a flow rate of 1.0 mL min<sup>-1</sup>. The method was validated according to USFDA guidelines in terms of selectivity, linearity, accuracy, precision, recovery and stability. The limit of quantitation values were 80 and 50 ng mL<sup>-1</sup> for torasemide and spironolactone respectively. Furthermore, the sensitivity and simplicity of the method suggests the validity of method for routine clinical studies.

**Keywords:** Column liquid Chromatography; Torasemide; Spironolactone; Human plasma; Bioanalytical method validation; Central Composite Design

## 1. Introduction

Hypertension (HT) or high blood pressure is a major contributor to the growing global pandemic of cardiovascular diseases, its control is essential in reducing death from stroke.<sup>1</sup> Although, a mild raise in blood pressure can be controlled by dietetic management and modified lifestyle. It is indeed, often necessary to control the HT by anti-hypertensive drug therapy (AHDT). Diuretic based AHDT is considered to be first line of treatment in control of HT. Diuretics are the class of drugs which helps the body to get rid of excess of water and salt by promoting the excretion of urine, which in turn lowers the HT and helps the heart to pump efficiently. The classification of diuretics and their role in management of HT is described elsewhere.<sup>2</sup>

Torasemide (TOR), (Fig.1.a), designated as [N-(isopropylamino)carbonyl]-4-[(3-methyl phenyl)amino]pyri-

dine-3-sulfonamide] belongs to the class of pyridine-sulfonyl urea type loop diuretics which is used in the treatment of HT. Although, TOR shows less diuretic effect than the other drugs of same class (furosemide) it still causes hypokalemia.<sup>3</sup> Spironolactone (SPI), (Fig. 1.b), designated as [7 $\alpha$ -acetylthio-3-oxo-17 $\alpha$ -pregn-4-ene-21,17-carbolactone] belongs to the class of potassium sparing diuretics which are specifically used in combination with thiazide diuretics in AHDT. SPI controls HT by inhibiting the effect of mineralocorticoids, which decreases the reabsorption of sodium and water in kidneys, while decreasing the excretion of potassium. Recently, a combination based on SPI and TOR has been introduced for the treatment of hypertension. Formulation of TOR with SPI synergizes the anti-hypertensive activity and also reduces the loss of potassium by diuresis. The monitoring of plasmatic concentrations of these drugs is indeed, quite essential for drug-drug interaction studies, therapeutic drug monitoring and other

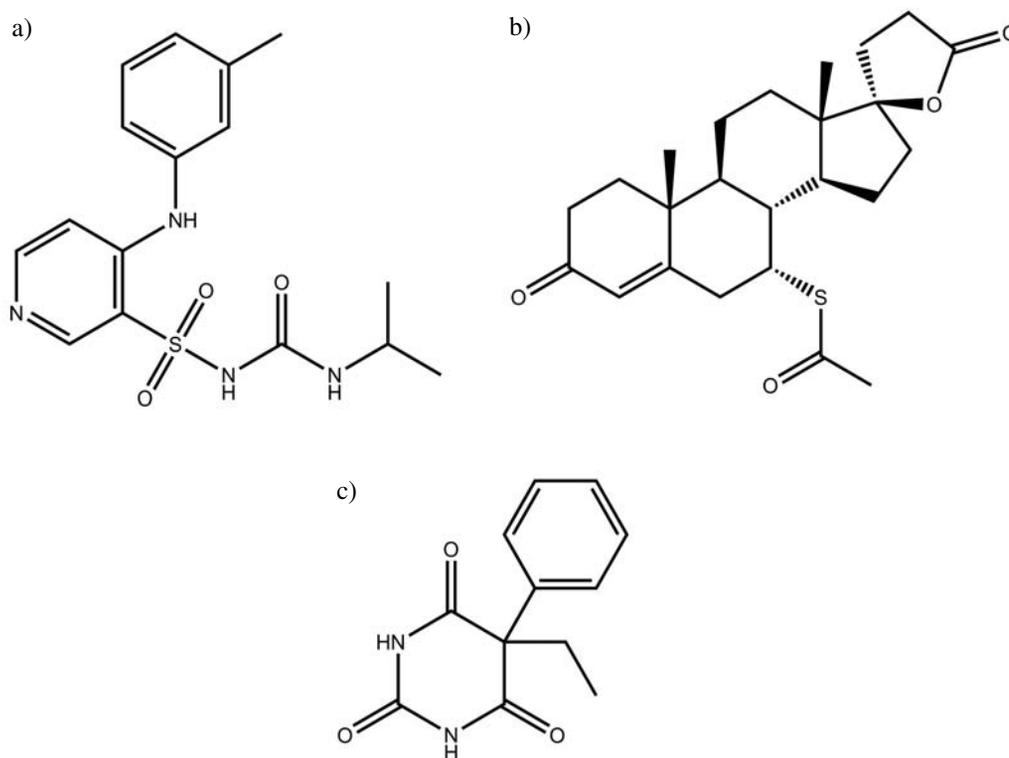


Fig. 1. Chemical structures of (a) Torasemide; (b) Spironolactone; (c) Phenobarbitone (IS)

routine clinical investigations. HPLC has proved to be a substantial tool for clinical investigations based on the accuracy in results and economic considerations. Phenobarbitone (PhB), (Fig. 1.c), designated as [5-ethyl-5-phenylpyrimidine-2,4,6(1H,3H,5H)-trione] is used as Internal standard (IS) in this study as it presented acceptable resolution and retention time with TOR and SPI.

In literature, several methods have been reported for the determination of TOR in pharmaceutical formulation and biological matrices. These methods include quantification by spectrophotometry,<sup>4,5</sup> HPLC,<sup>6–12</sup> and capillary electrophoresis.<sup>13</sup> Although, there are no published analytical methods available for determination of SPI alone; but a thorough literature review revealed quite a few methods available for the determination of SPI in combination with its metabolites,<sup>14–19</sup> degradation products<sup>20</sup> and other drugs.<sup>21–27</sup> However, an intensive literature search revealed to the best of our knowledge that only eight methods are available for simultaneous determination of TOR and SPI. These include their determination by UV spectrophotometry<sup>28,29</sup> by HPLC<sup>30–34</sup> and by Micellar liquid chromatography<sup>35</sup> in bulk drugs and pharmaceutical formulations. However, much of the reported methods are focused on quantification of these drugs in raw material and pharmaceutical formulation and involves a simple trial and error approach in method development. Furthermore, the reported methods are not sensitive enough for the determination of these drugs in biological matrices such as human plasma. Together, this de-

mands the necessity of a sensitive, selective and robust method for the quantification of TOR and SPI in human plasma. In this paper, an approach which includes Design of Experiments (DoE) methodology was employed in the development of a new HPLC method for the analysis of TOR and SPI in human plasma. The application of DoE methodology in HPLC method development offers a detailed view of the analyzed system and enables a mathematical description of the system behavior, after performing a minimal number of experiments.<sup>36</sup> Following this strategy, a bioanalytical method for the simultaneous determination of TOR and SPI in human plasma was developed which can be employed for routine clinical investigations. Finally, the suitability of the developed method was tested by validating the proposed method by following USFDA-CDER guidelines.<sup>37</sup>

## 2. Experimental

### 2.1. Instrumentation

Chromatographic analysis was performed on Shimadzu HPLC comprising LC 20AT pump with SPD M20A PDA detector, a rheodyne injector valve with a 20  $\mu$ l loop (Shimadzu, Kyoto, Japan). Chromatographic data collection and processing was performed using LC Solutions software (SP-1.11). The mobile phase was degassed using Branson sonicator (Branson Ultrasonic's Corporation, USA). The chromatographic separation was

carried out by Phenomenex Luna C<sub>18</sub> (Phenomenex, USA) column (150 mm × 4.6 mm, i.d., 5μ).

## 2. 2. Chemicals and Reagents

Working standards of pure Torasemide (98.9%) and Spironolactone (99.4%) from Cipla Ltd (Mumbai, India) and Phenobarbitone (99.1%) from Bondane Pharma Ltd (Mumbai, India) were used in this study. Acetonitrile of HPLC grade, potassium di-hydrogen *ortho* phosphate, phosphoric acid and triethylamine of analytical reagent grade were procured from SD Fine Chemicals (Mumbai, India). HPLC grade water was prepared by using Milli-Q Academic system, Millipore (Bangalore, India).

## 2. 3. Plasma Sampling

Blank human blood was collected from healthy human volunteers. Plasma was obtained by centrifugation of blood treated with sodium citrate (anticoagulant) and was stored at -20 °C until further use.

## 2. 4. Preparation of Solutions

Standard stock solutions of TOR and SPI at 1000 μg mL<sup>-1</sup> and PhB at 1600 μg mL<sup>-1</sup> were prepared by dissolving appropriate amounts of each compound separately in a mixture of acetonitrile and water (1:1). Calibration standards were prepared in the range of 0.3–6.0 μg mL<sup>-1</sup> for TOR and 0.1–2.0 μg mL<sup>-1</sup> by spiking the appropriate amount of analytes in blank human plasma and stored at -20 ± 2 °C until further analysis. Quality Control (QC) samples at three concentration levels Lower QC, Middle QC and Higher QC for TOR (0.6, 2.4 and 6.0 μg mL<sup>-1</sup>) and SPI (0.2, 0.8 and 2.0 μg mL<sup>-1</sup>) were prepared by diluting the stock solutions in the mobile phase.

## 2. 5. Plasma Sample Treatment

Prior to the extraction, drug free plasma samples were removed from the deep freezer and were allowed to thaw. To 275 μl of plasma sample, fixed aliquots of 200 μl working standard solutions and 25 μl of PhB (Internal standard, (IS)) was added. To the mixture, 500 μl of acetonitrile was added and was vortex mixed for 2.0 min; and then precipitated proteins were separated by centrifugation at 6000 rpm for 15 min. The supernatant was collected and filtered through 0.22 μm membrane filter and 20 μl aliquot of the filtrate was injected into the HPLC system.

## 2. 6. Design of Experiments

Experiments were performed according to the matrix of experiments created by Central Composite

Design (CCD). The selection of factors and their levels was based on preliminary experiments and prior knowledge of literature. The variables and the selected ranges were acetonitrile concentration (A) from 15% to 20% v/v, buffer concentration (B) from 10 to 20 mM and pH (C) from 3.0 to 4.0. The experiments were performed in duplicate and the matrix of experiments is presented in Table 1.

## 2. 7. Software

Experimental design, data analysis and desirability function calculations were performed by using JMP trial version 10.0.0. (Stat-Ease Inc., Minneapolis).

## 2. 8. Chromatographic Conditions

Chromatographic separation was carried out on a C<sub>18</sub> column by employing an isocratic mobile phase comprising 20 mM potassium dihydrogen *ortho* phosphate buffer (pH-3.2) and acetonitrile in 82.5:17.5 v/v. The analytes were detected at 240 nm at a flow rate of 1.0 mL min<sup>-1</sup>. The injection volume of the sample was 20 μl. The HPLC analysis was performed at an ambient temperature of 25 ± 2 °C.

## 2. 9. Validation

The proposed method was validated in compliance with USFDA-CDER guidelines.<sup>37</sup> The method was validated for the following parameters – selectivity, linearity, plasma recovery, accuracy and precision, limit of detection and quantification and freeze thaw stability.

### 2. 9. 1. Selectivity

Selectivity of the method was assessed by analyzing six lots of blank plasma samples without analytes and screened for any interfering signals from endogenous compounds (matrix effect) with that of analytes. Interference from other commonly co-prescribed drugs like warfarin, aspirin, fenofibric acid, valsartan, amlodipine, hydrochlorothiazide and furosemide at a concentration of 1 μg mL<sup>-1</sup> was also tested.

### 2. 9. 2. Linearity

Linearity of the proposed method was assessed in the range of 0.3–6.0 μg mL<sup>-1</sup> for TOR and 0.1–2.0 μg mL<sup>-1</sup> for SPI using the calibration standards prepared on five separate days (n = 5) at eight non-zero concentration levels. Calibration curves were plotted by using peak area ratio's (analyte peak area/IS peak area) vs. nominal concentration and was analyzed using linear least square regression method.

### 2. 9. 3. Plasma Recovery

Recovery of TOR and SPI from spiked human plasma was evaluated at three concentration levels (0.6, 2.4 and 6.0  $\mu\text{g mL}^{-1}$  for TOR and 0.2, 0.8 and 2.0  $\mu\text{g mL}^{-1}$  for SPI) in six replicates. The recovery of the analytes was calculated by comparing the analyte/IS peak area ratio of processed plasma samples with the corresponding ratio obtained from pure aqueous solutions at the same concentrations. Recovery of the IS was also evaluated by calculating the peak area ratio of the IS from the spiked processed plasma samples and aqueous solutions at equivalent concentrations.<sup>38</sup>

### 2. 9. 4. Accuracy and Precision

The accuracy, intra-day and inter-day precision was assessed by analyzing the prepared QC standards at three concentration levels (0.6, 2.4 and 6.0  $\mu\text{g mL}^{-1}$  for TOR and 0.2, 0.8 and 2.0  $\mu\text{g mL}^{-1}$  for SPI) in five replicates, representing the entire range of calibration curve. The precision (coefficient of variation, CV%) at each concentration level from the nominal concentration was expected to be not more than 15% and accuracy (% bias value) within  $\pm 15\%$ .

### 2. 9. 5. Limits of Detection and Quantification

Both limit of detection (LOD) and limit of quantification (LOQ) of the developed method for determination of TOR and SPI in plasma were computed by the 3.3s and 10s criterion, respectively.<sup>37</sup>

### 2. 9. 6. Stability

Stability of analytes TOR and SPI in human plasma was evaluated at two concentration levels (0.6 and 6.0  $\mu\text{g mL}^{-1}$  for TOR and 0.2 and 2.0  $\mu\text{g mL}^{-1}$  for SPI), at room temperature for 3h, at 4 °C for 24h and at  $-20^\circ\text{C}$  for three weeks in order to simulate sample handling and storage time in the freezer before the analysis. The stability of the drugs in human plasma was also investigated for three freeze thaw cycles. A stability/reference analyte concentration ratio of 85–115 % was accepted as the stability criterion ( $n = 5$ ).<sup>37</sup>

### 2. 9. 7. Robustness

To demonstrate the reliability of the developed method for routine use, robustness of the developed method was tested by duplicate injections ( $n = 3$ ) of a standard solution containing all analytes (including IS) at 1  $\mu\text{g mL}^{-1}$  concentration with deliberate modifications to the following method parameters (range): potassium dihydrogen *ortho*-phosphate buffer concentration (16.5–18.5 mM), pH (3.1–3.3), flow rate (0.95–1.05  $\text{mL min}^{-1}$ ) and wavelength (235–245 nm).

## 3. Results and Discussion

### 3. 1. Optimization of Chromatographic Conditions

The preliminary chromatographic conditions (stationary phase, pH-range, choice of buffer and wavelength) were chosen based on experience and prior knowledge from literature. The optimization goal was to increase the resolution and decrease the analysis time. For the optimization, Central Composite Design (CCD) was preferred as it is ideal for chromatographic trialing and allows relatively controlled range of experiments to outline the factors that have an effect on the chromatographic behavior of investigated substances.<sup>36</sup> CCD is an imbedded full factorial design ( $2^k$  for  $k$  number of factors investigated) with the addition of a group of star ( $2^k$ ) and central point's. In the present study, an orthogonal CCD was used. In this type of design the star points are equal to  $\pm (2k)^{1/4}$  and the information is equally generated from all directions, i.e., the variance of the estimated responses is the same at all points on a sphere centered at the origin.<sup>40</sup> In order to assess the quality of the method under different conditions, the following responses of interest were defined (i) capacity factor of the first eluted peak ( $k_1$ ), (ii) resolution between the 2<sup>nd</sup> and 3<sup>rd</sup> peak ( $Rs_{2,3}$ ), and (iii) retention time of the last peak, ( $tR_3$ ). Table 1 summarizes the conducted experiments viz., ( $n = 14 + 6$ ) six replicates at center point and the responses.

**Table 1.** Plan of experiments for Central Composite Design and results obtained for the responses

Design Points	Factor levels			Responses		
	A	B	C	$k_1$	$Rs_{2,3}$	$tR_3$
1	15	10	3	2.47	9.71	12.21
2	20	10	3	1.76	6.08	6.65
3	15	20	3	2.88	7.39	13.69
4	15	10	4	1.47	9.57	6.45
5	20	20	3	2.07	5.89	6.88
6	20	10	4	1.88	5.39	5.39
7	15	20	4	1.77	9.97	8.78
8	20	20	4	1.17	5.59	5.97
9	17.5	15	3.5	1.67	6.69	7.90
10	17.5	15	3.5	1.66	6.99	7.76
11	17.5	15	3.5	1.70	6.78	7.70
12	17.5	15	3.5	1.73	6.79	7.79
13	17.5	15	3.5	1.75	6.96	7.74
14	17.5	15	3.5	1.76	6.11	7.77
15	17.5	15	2.69	3.01	6.78	8.68
16	17.5	15	4.31	1.34	6.64	4.42
17	17.5	6.59	3.5	1.57	8.34	7.39
18	17.5	23.41	3.5	1.76	5.47	7.98
19	13.3	15	3.5	2.68	12.69	16.74
20	21.7	15	3.5	1.39	6.26	6.43

\*A – content of acetonitrile (%); B – buffer concentration; C – pH  
 \*\* $k_1$  – retention factor of first peak;  $Rs_{2,3}$  – resolution between 2<sup>nd</sup> and 3<sup>rd</sup> peak;  $tR_3$  – retention time of 3<sup>rd</sup> peak

Table 2. Response models and statistical parameters obtained from ANOVA for CCD

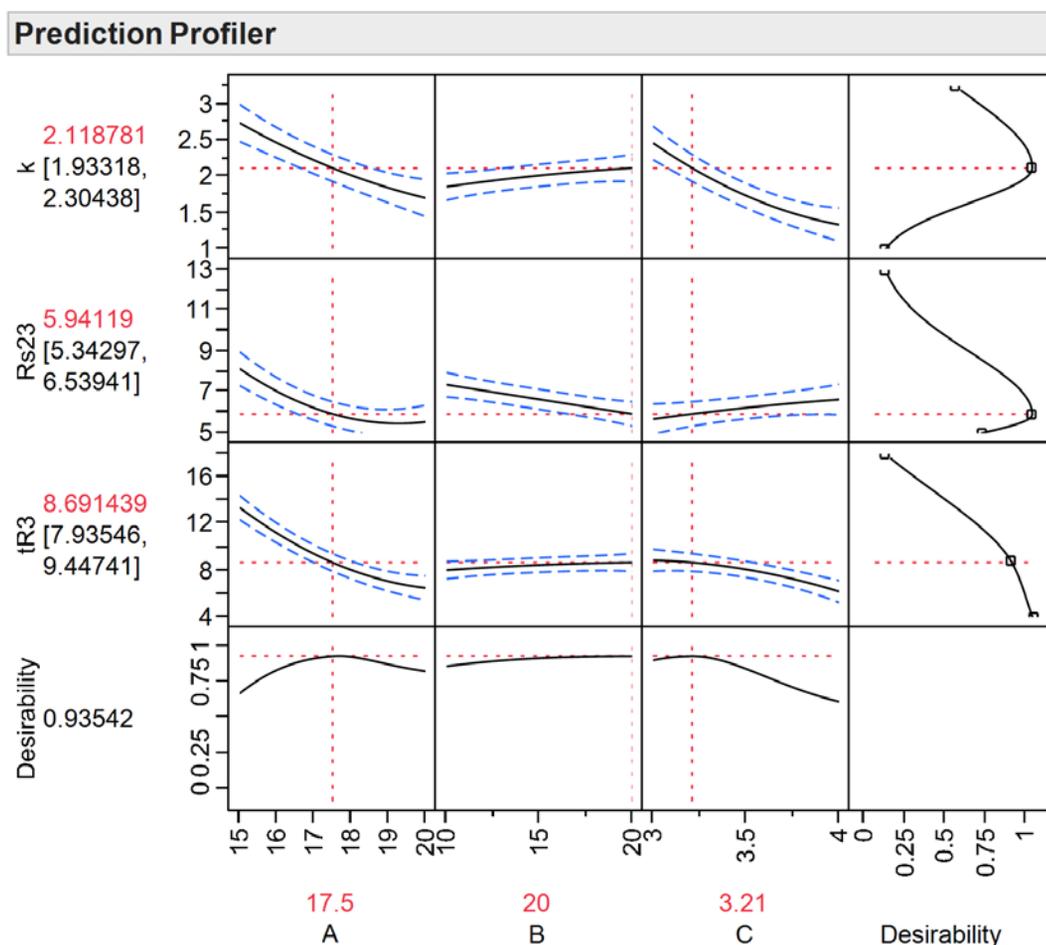
Responses	Regression model	Adjusted R <sup>2</sup>	Model p-value	% C.V	Adequate precision
$k_1$	+1.70 – 0.28 A + 0.047 B – 0.42 C – 0.14 AB + 0.16 AC – 0.14 BC + 0.11 A <sup>2</sup> + 0.16 C <sup>2</sup>	0.8984	<0.0001	8.54	18.223
$Rs_{23}$	+ 6.68 – 1.79 AB – 0.49B + 0.92 A <sup>2</sup>	0.8855	<0.0001	8.63	24.755
$tR_3$	+ 6.63 – 2.89 A – 1.32 C + 0.54 AC + 1.11A <sup>2</sup>	0.9244	<0.0001	9.67	26.711

\* $k_1$  – retention factor of first peak;  $Rs_{23}$  – resolution between 2<sup>nd</sup> and 3<sup>rd</sup> peak;  $tR_3$  – retention time of 3<sup>rd</sup> peak

All experiments were conducted in randomized order and in duplicate to minimize the effects of uncontrolled variables that may introduce a bias on the measurements. Furthermore, the results were statistically evaluated by using “Standard least squares” model. The obtained values for coefficients and other statistical data are given in Table 2.

To obtain a simple and realistic model, the insignificant terms ( $p > 0.05$ ) were eliminated from the model through a backward elimination process. The adjusted R<sup>2</sup> values were well within the acceptable limits of  $R^2 \geq$

0.80.<sup>41</sup> The adequate precision values were found to be in the range of 18.22–26.71, which indicates an adequate signal and therefore the model is significant for the separation process.<sup>41</sup> The coefficient of variation (CV %) measures the reproducibility of the model; a value less than 10% is desirable. Furthermore, in all three generated models the coefficients corresponding to acetonitrile content (A) had minus sign, which means that higher values of acetonitrile influence the decrease of all three analyzed responses. Likewise, the positive interaction between A

Fig 2. Prediction Profiler obtained for the responses  $k_1$ ,  $Rs_{23}$  and  $tR_3$

and C is statistically significant ( $p < 0.0001$ ) for the  $tR_3$  model. The model also reveals that changing the fraction of acetonitrile content from low (-1) to high (+1) results in a rapid improvement in  $tR_3$  both at synergistically with low (-1) and high level (+1) of pH (C).

In order to find the optimum chromatographic conditions, the selected responses were simultaneously optimized by employing a desirability function. Global optimization based on desirability function was achieved by using JMP prediction profiler given in Fig. 2. Desirability function transfers the response variable to a 0 to 1 scale. A response of 0 represents a completely undesirable response and 1 represents the most desirable response. The targeted criterion for the optimization was to maximize the retention factor ( $k_1$ ), minimize the resolution between the second and third peak pair ( $Rs_{23}$ ) and reduce the analysis time ( $tR_3$ ). Fig. 2 depicts that as Factor (A), % acetonitrile increases;  $k_1$  also increases but at the same time it also lead to excess resolution ( $Rs_{23}$ ). Additionally, factor (B) concentration of buffer showed a good impact on response  $k_1$ .

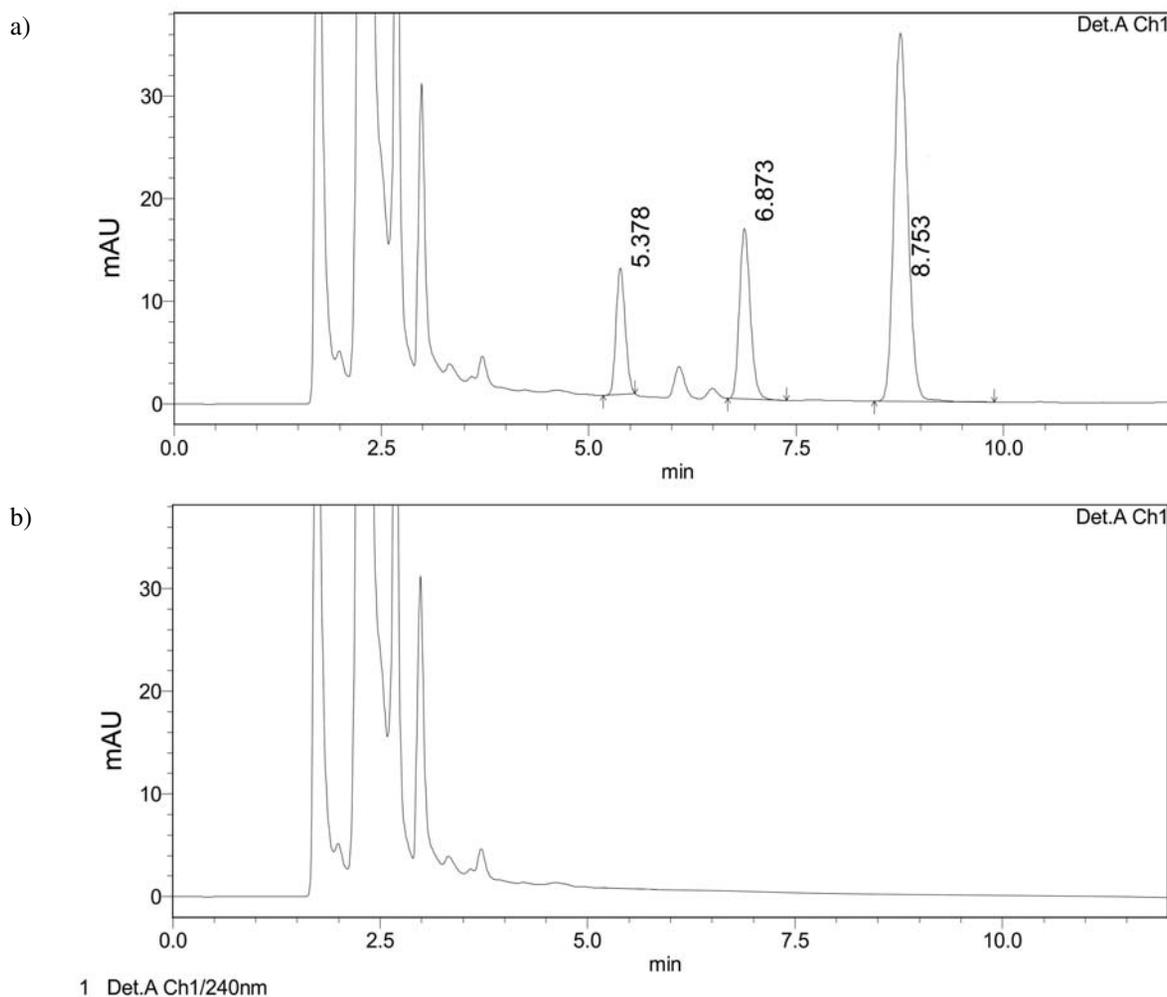
Factor (C), pH had a negative impact on  $k_1$  value. However, the effect of pH on other responses was not significant as it showed less curvature for both  $Rs_{23}$  and  $tR_3$ . The operating conditions were chosen to achieve the maximum overall desirability ( $D = 0.935$ ); all the responses were optimized simultaneously. The set of coordinates producing high desirability value at acetonitrile concentration of 17.5 % v/v, 20mM buffer concentration and pH-3.2. The predicted response values corresponding to the latter value of D were:  $k_1 = 2.11$ ,  $Rs_{23} = 5.94$  and  $tR_3 = 8.69$  min.

Fig 3.a shows a typical chromatogram obtained by the analysis of  $0.3 \mu\text{g mL}^{-1}$  mixture of TOR, SPI and IS. The individual retention times of SPI, TOR and IS were 5.3, 6.8 and 8.7 respectively.

## 3. 2. Method Validation

### 3. 2. 1. Selectivity

The chromatogram from Fig. 3.b indicates that the peaks of analytes and IS are well resolved from plasma



**Fig. 3.** Representative chromatograms corresponding to (a) Torasemide and Spironolactone with Phenobarbitone as internal standard (IS) in spiked human plasma; (b) processed blank human plasma

endogenous compounds. Similarly, none of the tested co-prescribed drugs were interfering with that of the retention time of the analytes. So, the developed method is selective for plasma endogenous compounds and tested commonly co-prescribed drugs.

### 3. 2. 2. Linearity

The calibration curves showed a good linearity ( $r^2 \geq 0.9993$ ) over the range of 0.3 – 6.0  $\mu\text{g mL}^{-1}$  for TOR and 0.1 – 2.0  $\mu\text{g mL}^{-1}$  for SPI. The regression equations for plotted calibration curves were  $y = 0.018x + 0.026$  for TOR and  $y = 0.011x + 0.019$  for SPI.

### 3. 2. 3. Limits of Detection and Quantification

The computed LOD values were 25  $\text{ng mL}^{-1}$  for TOR and 15  $\text{ng mL}^{-1}$  for SPI, and their LOQ values were 80 and 50  $\text{ng mL}^{-1}$ , respectively. These results suggest that the developed method is adequate sensitive to detect TOR and SPI in plasma for clinical purposes.

### 3. 2. 4. Accuracy and Precision

The accuracy, intra and inter-day precision of the method was measured and coefficient of variation (CV) values was  $<5\%$  at all times (Table 3). These results suggest that the developed method is suitable for the

routine analysis of TOR and SPI, since CV did not exceed 5% and the bias value was within the limit of  $\pm 15\%$  (Table 3).<sup>37</sup>

### 3. 2. 5. Plasma Recovery

From Table 3, it can be seen that the mean recoveries of TOR and SPI were ranged from 96.08–98.19% and 94.84–96.47%, respectively. The recovery of the IS was also evaluated and a mean value of 97.4% was obtained.

### 3. 2. 6. Stability

The stability of TOR and SPI was assessed at aiming different circumstances expected to be encountered during the analytical process and sample storage, by analyzing five replicates ( $n = 5$ ) of low, medium and high QC samples. The obtained stability study results (Table 4) showed that the selected drugs were stable in plasma when stored in a frozen state.

### 3. 2. 7. Robustness

The deliberate variations in the chromatographic conditions didn't showed significant effect on retention time ( $<3.7\%$ ) and peak area ( $<2.9\%$ ). So, the method can be considered robust and can be employed in routine analysis of these drugs in plasma.

Table 3. Precision, Accuracy and Recovery studies for Torasemide and Spironolactone ( $n = 5$ )

Nominal Concentration ( $\mu\text{g/ml}$ )	Intra-day		Inter-day		Plasma recovery	
	Precision (% CV)	Accuracy (%)	Precision (% CV)	Accuracy (%)	(%)	(% CV)
TOR						
0.6	2.34	98.47	1.85	97.89	96.08	3.51
2.4	1.76	99.15	2.19	98.96	98.19	2.04
6.0	1.48	98.46	0.95	99.15	97.07	2.33
SPI						
0.2	4.37	97.67	2.72	99.41	94.84	2.74
0.8	2.03	98.04	1.14	98.86	95.15	3.01
2.0	2.81	98.92	1.52	98.17	96.47	1.52

Table 4. Stability studies of Torasemide and Spironolactone in human plasma

Analyte	Stability	Mean		CV (%)	
	Concentration ( $\mu\text{g/ml}$ )	0.6	6.0	0.6	6.0
TOR	Freeze thaw (3 cycles at $-20 \pm 2$ °C)	0.57	5.95	2.19	1.89
	Short term (25 °C)	0.59	5.84	1.21	2.94
	Long term ( $-20 \pm 2$ °C)	0.57	5.99	1.68	1.01
	Concentration ( $\mu\text{g/ml}$ )	0.2	2.0	0.2	2.0
SPI	Freeze thaw (3 cycles at $-20 \pm 2$ °C)	0.19	1.87	2.43	3.39
	Short term (at 25 °C)	0.19	1.95	1.17	1.62
	Long term ( $-20 \pm 2$ °C)	0.20	1.99	0.97	1.25

### 3. 3. Advantages of the Developed Method

The developed method presents several important bioanalytical advantages. First of all, it was demonstrated to be an accurate, precise and highly selective method for the determination of TOR and SPI in human plasma. This method employs simple protein precipitation technique for extraction of selected drugs from plasma which showed good recovery. The proposed method enables to detect the drugs TOR and SPI in nano gram level, which satisfies the demand of quantifying the same in biological matrices such as human plasma. Furthermore, the proposed method is economical and has low environmental impact as it consumes minimal amount of toxic organic solvents.

## 4. Conclusion

In this study, a new LC method for simultaneous determination of TOR and SPI in human plasma by employing Design of Experiments methodology is presented. The developed plasma extraction procedure was simple and effective (>96% of recovery). The run time required for the determination of analytes in plasma samples was only 9 min. Furthermore, the results from the validation suggest that the method is adequate in terms of selectivity, linearity, accuracy, precision, robustness and sensitivity at the ng mL<sup>-1</sup> level for the determination of TOR and SPI in plasma samples. Moreover, the sensitivity and simplicity of the method make it suitable for routine clinical studies.

## 5. Acknowledgments

The authors are grateful to University Grants Commission (UGC), New Delhi, India for providing UGC-BSR fellowship and to UGC-SAP DRS Phase-II sponsored Department of Pharmacy, Annamalai University, Tamil nadu, India for providing the facilities to carry out this research.

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## Povzetek

S pomočjo eksperimentalnega načrta smo razvili in validirali občutljivo, točno, natančno in hitro HPLC-PDA metodo za hkratno določanje torasemida in spironolaktona v človeški plazmi. Za optimizacijo smo uporabili centralno kompozitni načrt in spreminjali vsebnost acetonitrila, koncentracijo pufru in pH mobilne faze kot neodvisnih spremenljivk. Za odvisne spremenljivke smo izbrali retencijski faktor spironolaktona, ločljivost med torasemidom in fenobarbitonom ter retencijski čas fenobarbitona. Kromatografsko ločbo smo dosegli na koloni Phenomenex C<sub>18</sub> in z mobilno fazo iz 20 mM kalijevega dihidrogen orto-fosfatnega pufru (pH 3,2) in acetonitrila v razmerju 82,5:17,5 v/v in s pretokom 1,0 mL min<sup>-1</sup>. Metodo smo validirali v skladu z USFDA smernicami; preverili smo selektivnost, linearnost, točnost, natančnost, izkoristek in stabilnost. Meja določitve je bila 80 ng mL<sup>-1</sup> za torasemid in 50 ng mL<sup>-1</sup> za spironolakton. Dobra občutljivost in preprostost metode nakazuje, da bo uporabna za rutinske klinične študije.