# Development and validation of a stability-indicating analytical method for simultaneous determination of sodium phenylbutyrate and taurursodiol in bulk and formulation using reverse phase ultra-performance liquid chromatography

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**Background**
Amyotrophic Lateral Sclerosis, often known as Lou Gehrig's disease, is typically treated with a combination therapy that includes both sodium phenylbutyrate (SPB) and taurursodiol (TRS).
**Objectives**
To assess both SPB and TRS in bulk and their dosage form concurrently, a stability-indicating analytical method was developed and validated using Ultra-Performance Liquid Chromatography.
**Patients and methods**
The chromatographic separation was carried out on a Waters $C_{18}$ Column, with dimensions of 150×4.6 mm i.d. and a particle size of 2 μm. A mobile phase consisting of a phosphate buffer pH 2.5 with methanol in the ratio of 45 : 65 v/v was, then delivered at a flow rate of 1 ml/min. Detection of the analytes occurred at 285 nm using a photo diode array detector. An auto sampler injected a 10 μl sample into the column, and the column was maintained at a temperature of 30°C.
**Results and conclusion**
SPB and TRS were eluted at 1.483 and 2.492 nm, respectively. Linearity was established in the range of 567–1701 μg/ml for SPB and 189–567 μg/ml for TRS. The robustness of the method was assessed by intentionally modifying parameters such as flow rate, detection wavelength, and column temperature. Furthermore, studies on forced degradation under various stress conditions, including acid, base, peroxide, heat, and ultra violet exposure, indicated the method's capability to identify stable materials. In summary, the developed analytical approach for simultaneously determining SPB and TRS in bulk and their formulation was found to be specific, accurate, precise, and reliable.

**Keywords:**
amyotrophic lateral sclerosis, Lou Gehrig's disease, sodium phenylbutyrate, taurursodiol, ultra-performance liquid chromatography

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## Introduction

Amyotrophic Lateral Sclerosis (ALS) is a condition marked by the gradual degeneration of motor neurons in the motor cortex and spinal cord, leading to a progressive weakening of muscles [1,2]. The average life expectancy after the onset of symptoms is typically around 2 to 3 years, with respiratory failure being the primary cause of death. At present, ALS treatment primarily revolves around symptom management [3]. Both Riluzole [4] and Edaravone [5] have shown the potential to influence the progression of ALS and have received food and drug administration (FDA) approval [6]. A fixed-dose oral combination of sodium phenylbutyrate (SPB) and Taurursodiol (TRS), also known as tauroursodeoxycholic acid, has been developed to mitigate neuronal loss in ALS patients by simultaneously addressing endoplasmic reticulum stress and mitochondrial dysfunction [7–9]. Figure 1a and b illustrates the analytes.

Upon conducting an extensive review of the existing literature, it became evident that there were no previously reported analytical methods for the quantification of SPB and TRS. As far as the author is aware, no stability-indicating assay method for the determination of SPB and TRS, whether in their pure form or within their dosage form, has been documented in the literature. Hence, the main goal of this study is to develop and validate a fast and



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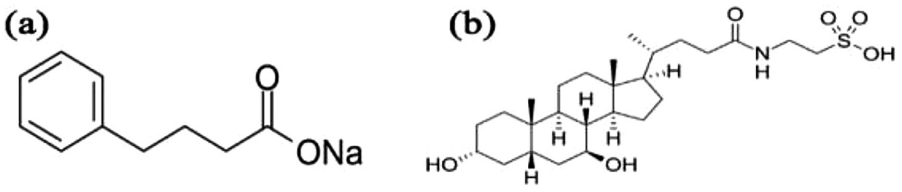
**Figure 1**




Chemical structures of (a) Sodium phenylbutyrate;(b) Taurursodiol.



straightforward Reverse Phase Ultra-Performance Liquid Chromatography with Photodiode Array detection (RP-UPLC-PDA) method for the concurrent quantification of SPB and TRS. The validation of this newly developed method adhered to the specifications outlined in the Q2 guidelines of the International Conference on Harmonization (ICH) [10,11].

## Patients and methods

### Chemicals and reagents

Gift samples of SPB and TRS were generously provided by Torrent Pharmaceuticals, located in Ahmedabad, India. We obtained High-Performance Liquid Chromatography (HPLC) grade Methanol, water, and acetonitrile from Sigma-Aldrich. Additionally, we acquired analytical grade sodium hydroxide (NaOH), hydrogen peroxide ($H_2O_2$), hydrochloric acid (HCl), and a 0.22 mm membrane filter from Sigma-Aldrich. Relyvrio, an oral suspension powder pack from Amylyx Pharmaceuticals, containing SPB and TRS as labeled with 3 g of SPB and 1 g of TRS, was purchased from the local pharmaceutical market. High-purity water was produced using a Millipore Milli Q water purification system. All chemicals used in this study were either analytical or LC grade.

### UPLC instrumental conditions

Data acquisition was performed using an Acquity UPLC system from Waters, headquartered in Milford, MA, USA. This system was equipped with a model 2996 PDA detector and utilized Empower software for both control and analysis. The UPLC separation of the two drugs was accomplished using a Waters $C_{18}$ Column with dimensions of 150 mm×4.6 mm inner diameter and a particle size of 2 µm. An isocratic mobile phase, consisting of Methanol and Phosphate Buffer at pH 2.5 (in a 45 : 65, v/v ratio), was employed and maintained at a flow rate of 1.0 ml/min. The column was operated at room temperature. Monitoring of the two drugs was carried out using the PDA detector at an isosbestic wavelength of 285 nm. Prior to usage, the solvents underwent filtration through a 0.22 mm membrane filter and were degassed in an ultrasonic bath. The analytical method was optimized using a preanalytical standard, with the mobile phase used as the diluent.

### Preparation of standard solutions

To prepare the standard solutions, a mixed stock solution was carefully prepared, consisting of SPB at a concentration of 2000 µg/ml and TRS at 1000 µg/ml. This involved the precise weighing of 50 mg of SPB and 25 mg of TRS, which were then placed into a 25 ml volumetric flask. Subsequently, 10 ml of methanol was added to the flask, and sonication was employed to ensure the complete dissolution of the contents. The volume in the flask was carefully adjusted to the mark using the same methanol solvent. As a result, the flask contained a stock solution with concentrations of 2000 µg/ml for SPB and 1000 µg/ml for TRS. For further analysis, aliquots of this mixed standard solution were transferred into 10 ml volumetric flasks, each of which was filled with 5 ml of diluent (mobile phase). These samples were subjected to sonication for a duration of 5 min, and the remaining volume in each flask was topped up with the diluent to achieve final concentrations of 1134 µg/ml for SPB and 378 µg/ml for TRS.

### Analysis of formulation

To analyze the Relyvrio formulation, we began by accurately weighing ten individual single-dose packets and then grinding them into a fine powder using a mortar. From this powdered formulation, an amount equivalent to 25 mg of TRS was carefully transferred into a volumetric flask. Subsequently, 5 ml of the diluent (methanol) was added to aid in solubility, and the mixture was sonicated for 10 mins until complete solubility was achieved. The volume was then adjusted to 25 ml to create the primary stock solution. From this primary stock solution, we withdrew an aliquot of 3.78 ml and transferred it into a 10 ml volumetric flask. The volume was adjusted using the mobile phase to attain concentrations of 1134 µg/ml for SPB and 378 µg/ml

for TRS. If necessary, the resulting solution was filtered through a 0.45 µm Millipore nylon filter paper.

### Method validation

The validation of the chromatographic method followed the guidelines outlined in the ICH Guidelines, specifically ICH Guidelines, Q2 (R1), 2005 [10,11].

### System suitability

The performance of the system was confirmed by evaluating the system suitability parameters. Six successive injections of the same standard preparation were used to gauge precision, with an emphasis on crucial elements such peak area, peak resolution, and theoretical plate number.

### Accuracy (Recovery)

Accuracy was assessed through recovery experiments conducted at three distinct levels: 50%, 100%, and 150% of the target concentration. Chromatograms were analyzed to evaluate the accuracy of the method.

### Precision

The precision of the analytical technique, both intraday and interday, was established by carrying out six replicate injections of optimized concentrations of SPB and TRS. The averages and % RSD (Relative Standard Deviation) of peak area and assay were calculated from the chromatograms.

### Specificity

Specificity of the developed method was examined by introducing a working placebo solution (blank) that lacked SPB and TRS, as well as a standard solution with concentrations of 1134 µg/ml for SPB and 378 µg/ml for TRS, into the UPLC system. Additionally, formulations were analyzed, and chromatograms were generated to assess specificity.

### Linearity

By preparing and examining pure analytical standards at five different concentrations, linearity was verified. In the defined concentration ranges, the new method displayed remarkable linearity in the ranges of 567.00, 850.50, 1134.00, 1417.50, and 1701.00 µg/ml for SPB and 189.00, 283.50, 378.00, 472.50, and 567.00 µg/ml for TRS.

### LOD and LOQ

The determination of LOD and LOQ for both SPB and TRS was based on the standard deviation (SD) of the response and the slope. LOD was calculated as 3.3 times the SD divided by the slope, whereas LOQ was determined as 10 times the SD divided by the slope.

### Robustness

The method's robustness, which measures its ability to withstand minor but intentional alterations in chromatographic conditions, was assessed. This evaluation included testing the impact of slight variations in the flow rate of the mobile phase (±0.2 units), changes in column temperature (±5°C), and variations in wavelength (±2%).

### Stress studies

We carried out stress testing to understand the inherent stability features of the active compounds by International Council for Harmonization (ICH) recommendations for stability testing for new medicinal substances and products. As part of this project, stress degradation experiments on SPB and TRS were carried out with the suggested method [11].

### Acidic hydrolysis

A process was used to look at how acidic environments affected SPB and TRS. 3.78 ml of the primary stock solution were initially poured into a 25 ml flask. To establish an acidic environment, 1 ml of 0.1 N HCL was added to one of the standard flasks. The contents of the flask were then brought up to the mark using the mobile phase and refluxed at 75°C for 5 h. To neutralize the acidic solution, 1 ml of 0.1 N NaOH was added after cooling. A 0.22 mm syringe filter was used to filter the finished product before being injected into the UPLC system for analysis.

### Alkaline hydrolysis

A 3.78 ml of the primary standard solution was mixed with 3 ml of 0.1 N NaOH in a 25 ml volumetric flask to examine the effects of alkaline conditions. The mobile phase was then used to regulate the volume. Two heating cycles of 10 h at 70°C and 10 h at 65°C were applied to these standard flasks. After the resultant solution cooled to room temperature, 3 ml of 0.1 N HCl was added to neutralize the base. The solution was then put into vials for analysis in the UPLC machine after being filtered using a 0.22 mm syringe filter.

### Thermally induced degradation

To investigate thermally induced degradation, 3.78 ml of the primary stock solution was transferred to a 25 ml standard flask and subjected to reflux at 80°C for a period of 12 h. The sample was then diluted with diluents (mobile phase) and adjusted to a total volume of 25 ml, resulting in concentrations of 1134 µg/ml for SPB and 378 µg/ml for TRS. After cooling to room temperature, the solution was filtered using a 0.22 mm syringe filter and introduced into vials for analysis in the UPLC system.

### Oxidative degradation

A 3.78 ml of the primary stock solution and 1 ml of 3% (w/v) hydrogen peroxide were mixed in a 25 ml standard flask to conduct oxidative degradation investigations. Diluents were added to the volume to adjust the concentrations to 1134 µg/ml for SPB and 378 µg/ml for TRS. After that, the typical flask was kept at room temperature for 8 h. A 0.22 mm syringe filter was used to filter the resultant solution before being added to vials for UPLC system analysis.

### Photo degradation

A 3.78 ml of the primary stock solution was pipetted into a 25 ml standard flask to measure the effects of photodegradation. The concentrations of SPB and TRS were 1134 µg/ml and 378 µg/ml, respectively. These samples were subjected to 1.2 million lux and 200 Wh/$m^2$ of ultra violet radiation for 30 h in a photostability chamber. The solution was then filtered using a 0.22 mm syringe filter and added to vials for UPLC system analysis.

## Results

### System suitability study

A system Suitability test was performed to confirm the accuracy and validity of the analytical method. We carefully analyzed several important parameters, including theoretical plate number (N), resolution, retention time (Rt), and tailing factor. Table 1 presents a summary of the findings of this analysis.

### Specificity

In order to evaluate the specificity of the method, various solutions were introduced into the UPLC system, including a blank solution, SPB, and TRS standards with concentrations of 1134 and 378 µg/ml, respectively, as well as the formulations under investigation. During this analysis, no discernible peaks were observed in the blank solution, and the Rts for the standards and samples were consistent. Representative chromatograms depicting these results are presented in Fig. 2a-c.

### Accuracy (Recovery)

The method was assessed at three different levels for accuracy: 50%, 100%, and 150%. Table 2 presents a summary of the outcomes. At these levels, the mean percentage recoveries for SPB were found to be 100.29%, 99.62%, and 99.78%, respectively. This indicates that the technique can produce reliable results at a variety of concentrations.

### Precision

The methods precision was assessed by intraday and interday variations with the concentrations of 1134 µg/ml for SPB and 378 µg/ml for TRS. This assessment involved performing six replicate injections at each concentration level. Detailed results for precision are presented in Table 3. The inter and intraday precision of SPB was found to be 0.75% and 0.20%, respectively. Whereas for TRS inter and intraday precision was 0.14% and 0.46%, respectively.

### Linearity

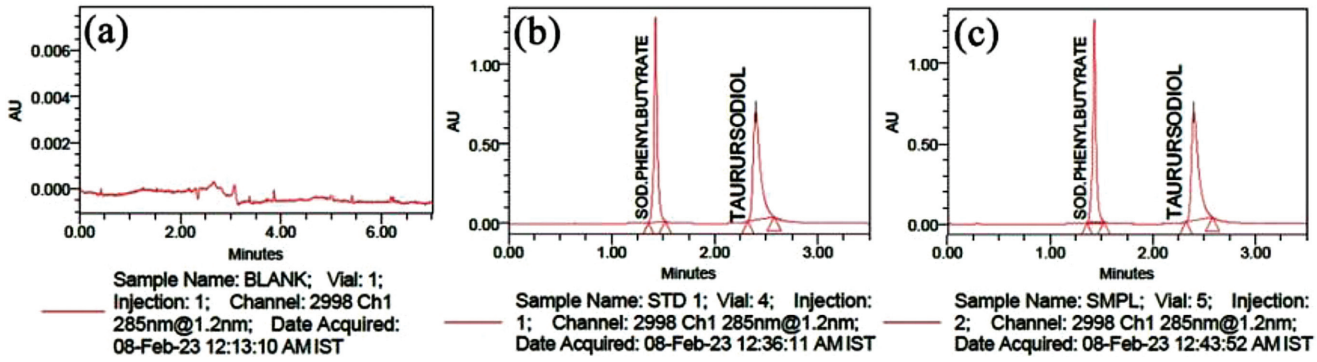
To establish the linearity of the method, linearity curves were constructed by plotting peak areas on

**Table 1 System suitability data**

| S. No | Parameter* | Sodium phenylbutyrate | Taurursodiol |
|---|---|---|---|
| 1 | Theoretical Plate Count | 5739 | 7893 |
| 2 | Average Peak Area | 6481227 | 5570645 |
| 3 | Retention time (Rt), min | 1.483 | 2.492 |
| 4 | Tailing | 1.01 | 1.21 |
| 5 | Resolution | – | 12.33 |
| 6 | Signal to Noise ratio | 1669.82 | 464.25 |

*Average of six replicates.

**Figure 2**




Representative chromatograms of (a) blank;(b) Standard; (c) Sample.

**Table 2 Accuracy data**

| Sodium Phenylbutyrate | | | | | | |
|---|---|---|---|---|---|---|
| S. No | Accuracy level | Wt. of sample (mg) | Peak area* | Amount added (mg) | Amount found (mg) | % recovery |
| 1 | 50% | 51.28 | 3227302 | 563.01 | 564.67 | 100.29 |
| 2 | 100% | 102.55 | 6411434 | 1126.03 | 1121.79 | 99.62 |
| 3 | 150% | 153.83 | 9632433 | 1689.04 | 1685.36 | 99.78 |
| Taurursodiol | | | | | | |
| 4 | 50% | 51.28 | 2745336 | 188.03 | 186.29 | 99.07 |
| 5 | 100% | 102.55 | 5528876 | 376.06 | 375.17 | 99.76 |
| 6 | 150% | 153.83 | 8297883 | 564.08 | 563.06 | 99.82 |

*Mean of three determinations at each level.

**Table 3 Precision data**

| | Mean peak area* | | % RSD* | | Mean %assay* | | % RSD* | |
|---|---|---|---|---|---|---|---|---|
| Precision | Sodium phenylbutyrate | Taurursodiol | Sodium phenylbutyrate | Taurursodiol | Sodium phenylbutyrate | Taurursodiol | Sodium phenylbutyrate | Taurursodiol |
| Intraday | 6476226.83 | 5570645.17 | 0.75 | 0.20 | 99.92 | 100.00 | 0.75 | 0.20 |
| Interday | 6435663.40 | 5541998.95 | 0.46 | 0.14 | 99.30 | 99.49 | 0.46 | 0.14 |

*Mean of six replicates.

the X-axis against drug concentrations on the Y-axis. Regression equations were computed based on these curves. The methods linearity was established between 567 and 1701 µg/ml for SPB and 189 and 567 µg/ml for TRS. Visual representations of the linearity curves for SPB and TRS can be observed in Fig. 3a and b, respectively.

### Limit of detection (LOD) and limit of quantitation (LOQ)

Calculating the LOD (Limit of Detection) and LOQ (Limit of Quantification) values allowed us to determine the sensitivity of the method. The LOD and LOQ for SPB were determined to be 1.56 and 5.19 µg/ml, respectively. The LOD and LOQ, on the other hand, were found to be 1.48 µg/ml and 4.95 µg/ml for TRS, respectively.

### Robustness

The robustness study indicated minimal deviations in the chromatograms when compared to the optimized conditions. The findings of this study are summarized in Table 4.

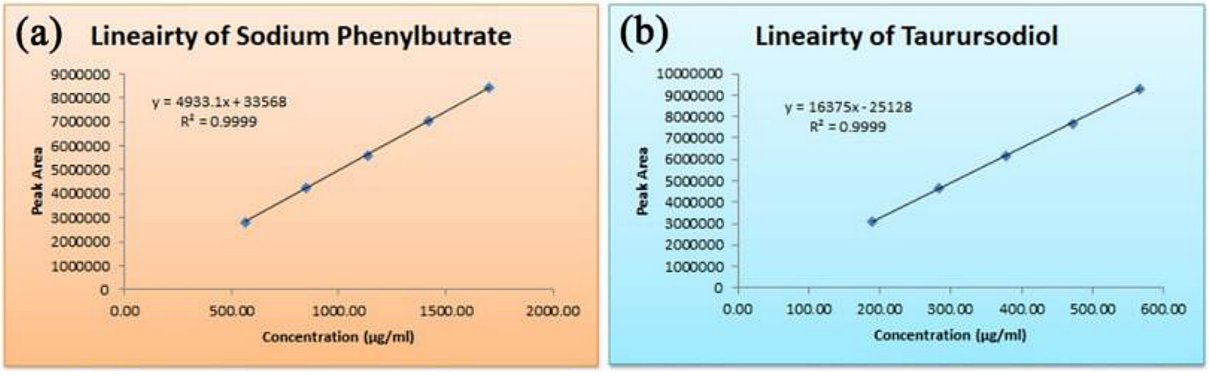
### Assay

The developed method was effectively employed for the quantification of analytes in the powder for suspension formulation. For SPB and TRS, the average % assay was found to be 99.22% and 100.00%, respectively. Detailed assay results are provided in Table 3.

### Stress studies

Method specificity was evaluated by forced degradation studies in presence of degradation products, both bulk and pharmaceutical dosage forms. These studies covered five different stress conditions. The degradation might have occurred due to the catalysis of ionizable groups within the analyte. A couple of degradation products were found in acidic and alkaline conditions, but no additional degrading peaks were

**Figure 3**




Linearity chromatograms of (a) Sodium phenylbutyrate and (b) Taurursodiol.

**Table 4 Results of robustness**

| | | Sodium phenylbutyrate | | | Taurursodiol | | |
|---|---|---|---|---|---|---|---|
| Parameter | Condition | Retention time (minimum) | Peak Area | % Assay | Retention time (minimum) | Peak Area | % Assay |
| Flow | 0.8 ml/min | 2.523 | 6976665 | 107.64 | 3.902 | 5564947 | 99.90 |
| | 1.0 ml/min | 1.483 | 6481227 | 100.00 | 2.492 | 5570645 | 100.00 |
| | 1.2 ml/min | 1.387 | 6406666 | 98.85 | 2.037 | 5610579 | 100.72 |
| Temp | 25°C | 2.845 | 6498542 | 100.27 | 2.795 | 5547497 | 99.58 |
| | 30°C | 1.483 | 6481227 | 100.00 | 2.492 | 5570645 | 100.00 |
| | 25°C | 2.842 | 6449293 | 99.51 | 1.756 | 5597781 | 100.49 |
| Wavelength | 283 nm | 1.414 | 6597131 | 101.79 | 2.220 | 5552436 | 99.67 |
| | 285 nm | 1.483 | 6481227 | 100.00 | 2.492 | 5570645 | 100.00 |
| | 287 nm | 1.481 | 6556765 | 101.17 | 2.263 | 5543379 | 99.51 |

**Table 5 Results of stress study**

| | Sodium phenylbutyrate | | | Taurursodiol | | |
|---|---|---|---|---|---|---|
| Condition | Peak area | % assay | % degradation | Peak area | % assay | % degradation |
| Acid | 632872 | 9.76 | 90.24 | 4977483 | 89.35 | 10.65 |
| Base | 636378 | 9.82 | 90.18 | 5024376 | 90.19 | 9.81 |
| Peroxide | 623382 | 9.62 | 90.38 | 5019497 | 90.11 | 9.89 |
| Thermal | 635809 | 9.81 | 90.19 | 5019492 | 90.11 | 9.89 |
| UV | 639459 | 9.87 | 90.13 | 5117124 | 91.86 | 8.14 |

observed within the Rt of SPB and TRS. It's worth noting that both drugs exhibited more significant degradation in acidic conditions compared to alkaline conditions.

In oxidative degradation studies, three degradation products were identified, but none of them appeared within the Rt of SPB and TRS. The higher degradation observed in peroxide conditions could be attributed to the electron transfer mechanism, leading to the formation of additional peaks.

In photolytic stress, degradation may have occurred through photooxidation via free radical mechanisms, while in heat stress conditions, the degradation can be explained based on the Arrhenius equation. The results of these stress studies are summarized in Table 5. This suggests that SPB and TRS are susceptible to the mentioned stress conditions but remain stable in the proposed technique, even under stress conditions for a specified period (as depicted in Fig. 4a-e).

## Discussion

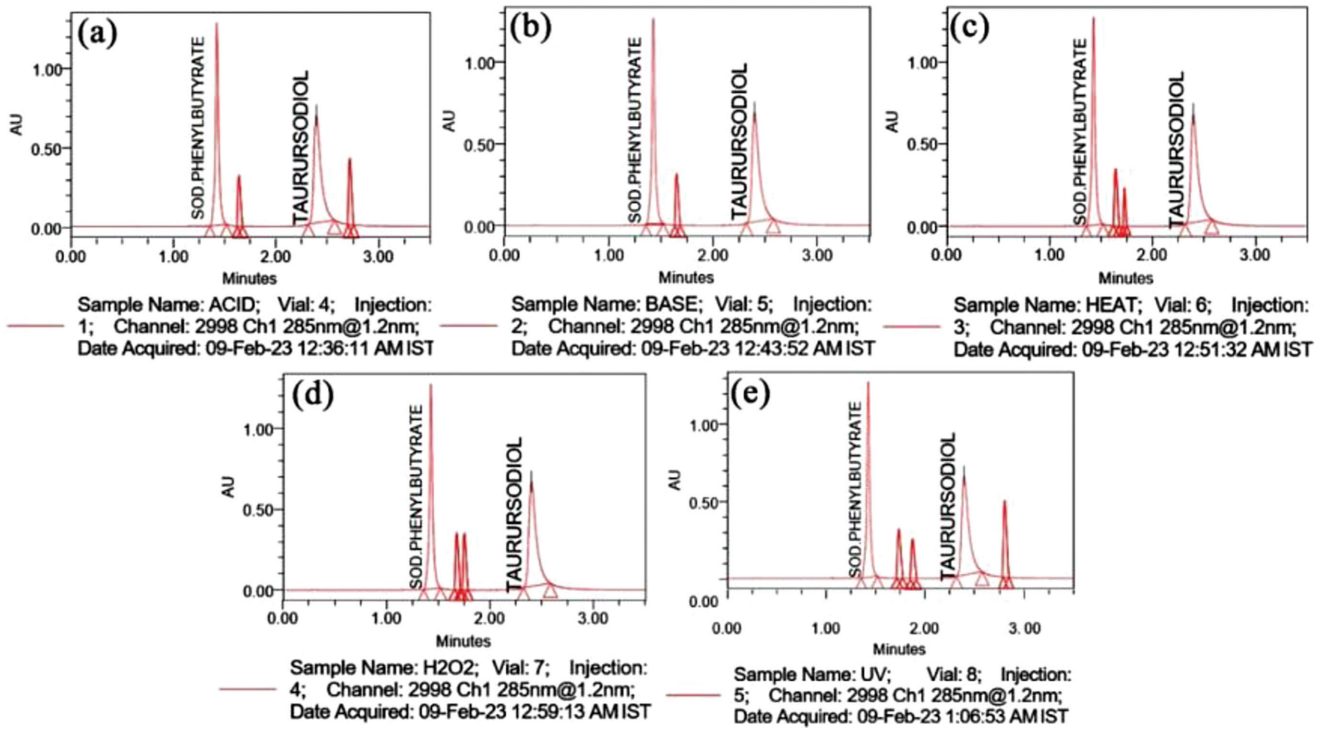
The implementation of the acquity UPLC system in this study has unveiled substantial advantages, notably in terms of cost reduction and time efficiency when compared to conventional HPLC methods. UPLC, empowers analysts to operate at an advanced level by offering broader linear speeds, and reduced system back pressures [12,13]. These attributes collectively make UPLC an invaluable tool for modern analytical chemistry.

In light of the escalating global demand for the drugs under investigation, the development of a novel, uncomplicated, cost-effective, and precise UPLC method for concurrent quantification in pharmaceutical formulations assumes paramount significance. Such a method not only facilitates routine drug analysis but also contributes to cost savings and heightened productivity in pharmaceutical quality control laboratories.

Furthermore, the developed UPLC method was extended to explore the degradation behavior of these drugs under diverse stress conditions. This comprehensive investigation serves to confirm the method's specificity and offers valuable insights into the stability profiles of the drugs, thereby ensuring the safety and efficacy of pharmaceutical products. This aligns perfectly with the stringent standards and regulations governing drug manufacturing and quality control, ultimately elevating the overall quality assurance standards within the pharmaceutical industry.

The main aim of this research work was to develop an analytical method for the quantification of SPB and TRS utilizing the UPLC-PDA chromatography system. The method underwent meticulous optimization, resulting in significantly reduced Rts

**Figure 4**




Chromatograms of forced degradation conditions; (a) Acidic; (b) Basic; (c) Heat;. (d) Peroxide; (e) Ultra violet light.

of 1.483 min for SPB and 2.492 min for TRS. This optimization process yielded exceptional sensitivity, well-defined peak shapes, and adequate resolution. The inclusion of the PDA detector, operating at a specific wavelength of 285 nm, substantially augmented detection sensitivity for all analytes. The ultra-sensitive instrument enabled a substantial reduction in total analysis time to as little as 3.00 min. These optimized conditions were subsequently utilized in the validation studies.

The developed method demonstrated accuracy, evident from the % recovery of all analytes falling within acceptable limits. Precision was firmly established through % RSD values in both intra and interday precision studies, all of which remained below 2%, affirming the method's precision. The Ultra Performance liquid chromatography system exhibited suitability for analyzing all drugs, as indicated by the system suitability study (Table 1), which showcased % RSD values below 2% for all parameters.

The LOD and LOQ study results underscored the method's remarkable sensitivity, while the robustness study demonstrated that deliberate alterations in various parameters, including flow rate, pH, and temperature, did not significantly affect the results. Additionally, the % assay for the marketed formulation yielded values of 99.92% for SPB and 100.00% for TRS, further validating the effectiveness of the developed test procedures.

Empirical evidence underscores the novelty of this method, which delivers both speed and cost-efficiency benefits. By reducing the total analysis time to a mere 3.00 min and substituting costly acetonitrile with methanol, it emerges as a cost-effective solution. Furthermore, the method achieves remarkably low detection limits and quantification limits, underscoring its exceptional sensitivity. The validation parameters produced results well within the limits stipulated by the ICH Q2B guidelines [14], further affirming its reliability and suitability for pharmaceutical analysis.

## Conclusion

The developed UPLC method represents a reliable, reproducible, accurate, and specific approach for quantifying Sodium Phenylbutyrate and Taurursodiol in bulk and tablet formulations. This newly established method has undergone rigorous validation in accordance with regulatory requirements, demonstrating acceptable levels of accuracy, precision, and sensitivity. Consequently, this method can be readily employed for routine analysis of these specified drugs within Quality Control laboratories.

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Nil.

**Conflicts of interest**

There are no conflicts of interest.

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