

# Stability-Indicating Chromatographic Methods for the Determination of a Skeletal Muscle Relaxant and an Analgesic in their Combined Dosage Form

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## Abstract

Two stability indicating chromatographic methods have been proposed for the determination of Dantrolene sodium (DNT) and Paracetamol (PAR). TLC was applied for the separation of the proposed drugs and their degradation products. The method employed silica gel as stationary phase and chloroform-methanol-glacial acetic acid (9:1:0.35 by volume) as the mobile phase, and the chromatograms were scanned at 230 nm. Determination of DNT and PAR was successfully achieved over the concentration ranges of 0.3–8 µg/band and 5–36 µg/band with mean percentage recoveries of  $100.04 \pm 0.970$  and  $100.23 \pm 0.922$  for DNT and PAR, respectively. In addition, an isocratic RP-HPLC method was developed for the separation of the studied components on a C18 column using phosphate buffer pH 3.0-methanol (35:65 by volume) at ambient temperature. The chromatograms were detected at 230 nm for optimum sensitivity of both drugs with running time less than 10 min. DNT and PAR were determined by HPLC in concentration ranges of 0.5–50 and 1–150 µg mL<sup>-1</sup> with mean percentage recoveries of  $100.33 \pm 0.843$  and  $100.14 \pm 1.084$ , respectively. Both TLC and HPLC were applied successfully for the assay of pharmaceutical dosage form. The proposed chromatographic methods were validated as per ICH guidelines and statistically compared with a reported gradient RP-HPLC method.

**Keywords:** Dantrolene sodium; Paracetamol; Stability indicating method; TLC; HPLC

## Introduction

Musculoskeletal disorders are better treated with combination of skeletal muscle relaxant (SMR) and non-steroidal anti-inflammatory drugs (NSAID) rather than single agent [1]. Dantrolene Sodium (DNT), the hemiheptahydrate of the sodium salt of 1-[5-(4-nitrophenyl) furfurylideneamino] imidazolidine-2, 4-dione [2], (Figure 1a), is a muscle relaxant with a direct action on skeletal muscle. It is also given for the treatment of malignant hyperthermia [3]. Paracetamol (PAR), 4'-Hydroxyacetanilide; N-(4-Hydroxyphenyl) acetamide [2], (Figure 1b), has analgesic, antipyretic properties and weak anti-inflammatory activity. The mechanism of analgesic action remains to be fully elucidated, but may be due to inhibition of prostaglandin synthesis both centrally and peripherally [3].

Literature review revealed that several electrochemical methods [4–6] and HPLC methods [7–14] have been reported for the determination of DNT either in pharmaceutical or biological samples. Degradation behavior of DNT was reported in different buffers and temperature [15]. The study showed that base catalysis is more predominant where degradation of the neutral species is catalyzed by hydroxyl ion. Hydrolysis of hydantoin ring took place to form an opened ring structure (DNT-DEG); 2-(1-carbamoyl-2-((5-(4-nitrophenyl) furan-2-yl) methylene) hydrazinyl) acetic acid [15], (Figure 1c). Several methods for the determination of PAR in combination with other drugs; such as spectrophotometric [16–24], electrochemical [25,26], HPLC [27–30] and TLC [31–33] methods have been reported. Para-Aminophenol (PAP), (Figure 1d) was reported as a primary hydrolytic degradation product of PAR or it may be originated from the synthesis [17].

Few analytical methods have been described for the simultaneous determination of DNT and PAR including spectrophotometric [34–36] and HPLC [36,37] methods. Concerning stability indicating methods for determination of DNT and PAR binary mixture, there was only a reported gradient RP-HPLC method [37]. The reported method

separated DNT and PAR from their degradation products using HS C18 analytical column with gradient elution using a mixture of 50 mM sodium dihydrogen phosphate, 5 mM heptane sulfonic acid sodium salt, pH 4.2 and acetonitrile as mobile phase. The flow rate was 1.5 mL/min and detection at 214 nm with 30 min run time. Since no reported TLC, the aim of our study was to develop inexpensive stability indicating TLC method. In addition, the work proposed an isocratic HPLC method as stability indicating assay with better sensitivity and shorter run time for the determination of DNT and PAR.

## Experimental

### Materials and reagents

**Pure authentic sample:** Dantrolene sodium authentic sample was kindly supplied by Chemipharm Pharmaceutical Industries, 6th October, Giza, Egypt. Its purity was found to be  $99.67 \pm 0.913$  according to a reported RP-HPLC method [37].

- Paracetamol authentic sample was obtained from El-Nile pharmaceutical Company, Cairo, Egypt. Its purity was checked to be  $100.04 \pm 0.641$  according to a reported RP-HPLC method [37].

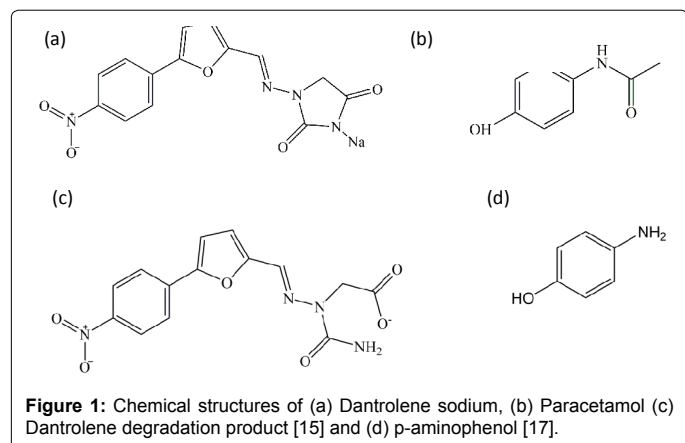
- Para-Aminophenol Pure sample, labeled with 99% purity, was purchased from Sigma Aldrich, Germany.

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**Figure 1:** Chemical structures of (a) Dantrolene sodium, (b) Paracetamol (c) Dantrolene degradation product [15] and (d) p-aminophenol [17].

**Degradation product:** Degradation product of dantrolene was laboratory prepared by dissolving 50 mg of pure DNT in 50 mL of 2 M aqueous NaOH then reflux in a 500-mL flask for 2 hr and neutralized with 2 M aqueous HCl. The formed precipitate was filtered, washed with distilled water and dried. The dried residue was used as the DNT-DEG.

**Pharmaceutical dosage form:** Dantrelax Compound<sup>®</sup> capsules manufactured by Chemipharm Pharmaceutical Industries, 6th October, Giza, Egypt, batch No. 130859A and 131186A, and labeled to contain 25 mg Dantrolene sodium salt and 300 mg Paracetamol per capsule.

**Chemicals and reagents:** All chemicals used were of pure spectroscopic analytical grade or HPLC grade including methanol and chloroform (Sigma Aldrich, Germany), Glacial acetic acid (Adwic, Egypt), hydrochloric acid and sodium hydroxide (Merck, Germany), sodium dihydrogen phosphate (El-Nasr Pharmaceutical Chemicals Co., Egypt), and double distilled deionized water.

### Instrument and software

Thin Layer Chromatography (TLC) system consisted of a Camag Linomat auto sampler (Muttentzl, Switzerland), a Camag micro syringe (100 mL) and a Camag 35/N/30319 TLC scanner with winCATS software; an ultraviolet (UV) lamp with a short wavelength at 254 nm (Desaga, Wiesloch, Germany); and TLC plates pre-coated with silica gel G.F254 10 × 20 cm, 0.25 mm thickness (Merck, Darmstadt, Germany).

The HPLC system consisted of Agilent pump (model 1100 series; Agilent, Germany) equipped with a variable wavelength detector and a 20  $\mu$ L injection loop. Agilent C18 column (250 mm × 4.6 mm I.D., particle size 5  $\mu$ m) was used as stationary phase. The samples were injected with a 50  $\mu$ L Hamilton analytical syringe. The detector was operating at 230 nm and isocratic elution with 1 ml/min flow rate.

### Solutions

**Standard solutions:** Stock standard solution (1.0 mg mL<sup>-1</sup>) of DNT was prepared by accurately weighing 25 mg of its bulk powder into 25-mL volumetric flask. About 10 mL methanol was added, sonicated for few minutes, and diluted to the volume with methanol.

Stock standard solution (10.0 mg mL<sup>-1</sup>) of PAR was prepared by accurately weighing 250 mg of its bulk powder into 25-mL volumetric flask. About 10 mL methanol was added, sonicated for few minutes, and diluted to the volume with methanol.

Working standard solutions (100.0  $\mu$ g mL<sup>-1</sup>) of DNT and PAR were prepared by suitable dilutions of their corresponding stock standard solutions.

**Pharmaceutical formulation solution:** Twenty Dantrelax Compound<sup>®</sup> capsules were weighed, carefully evacuated and reweighed again. The contents of capsules were mixed then accurate amount of the powder equivalent to one capsule was transferred into 100-mL beaker; 25 mL methanol was added, sonicated for 30 minutes then filtered into 50-mL volumetric flask. The residue was washed three times each using 5 mL methanol and the solutions were completed to the mark with the same solvent to have a dosage form solution containing 0.5 mg mL<sup>-1</sup> of DNT and 6.0 mg mL<sup>-1</sup> of PAR.

### Procedures

**TLC-spectrodensitometric method:** Aliquots equivalent to 0.3–8.0 mg of DNT and 5.0–36.0 mg of PAR were transferred from their respective stock standard solutions into separate series of 10-mL volumetric flasks; the volumes were completed to the mark with methanol. A 10- $\mu$ L aliquot of each solution was applied to a TLC plate using 50  $\mu$ L Hamilton syringe. Bands of 6 mm width were spaced 1 cm from the bottom edge of the plate. The plate was developed in a chromatographic tank, previously saturated for at least 1 hr with the developing mobile phase; to a distance of about 8 cm by the ascending technique using chloroform: methanol: glacial acetic acid (9:1:0.35 by volume) as the mobile phase. The plate was removed, air-dried, and the bands were visualized under a UV lamp at 254 nm. The chromatogram was scanned at 230 nm and calibration curves representing the relationship between the recorded area under the peak and the corresponding concentrations of DNT and PAR in micrograms per band were plotted and the regression equations were computed.

**HPLC:** Suitable aliquots were accurately transferred from DNT and PAR standard solutions into 10-mL volumetric flasks and the volumes were completed to the mark with mobile phase to construct calibrations for DNT and PAR in the range of 0.5–50.0  $\mu$ g mL<sup>-1</sup> and 1.0–150.0  $\mu$ g mL<sup>-1</sup>, respectively. Twenty microliters of these solutions were injected in triplet into the HPLC system. Agilent C18 column was used as stationary phase with isocratic elution using a mobile phase consisting of phosphate buffer pH 3.0 (50 mM of sodium dihydrogen phosphate adjusted to pH 3 using ortho-phosphoric acid) - methanol (35:65 by volume) at flow rate 1 ml/min. The chromatograms were recorded at 230 nm and calibration curves were plotted between peak area and the corresponding concentrations of each drug.

**Assay of synthetic mixtures:** Synthetic mixtures containing different ratios of DNT and PAR were prepared from their respective standard solutions. The previously described chromatographic conditions were followed for the analysis of these mixtures by TLC or HPLC. The concentrations of DNT and PAR were calculated from the corresponding regression equations.

**Assay of pharmaceutical formulation:** For TLC assay, 2.0  $\mu$ L of the pharmaceutical solution was directly applied, whereas 0.2 mL aliquot was accurately transferred into 10-mL volumetric flask and diluted to the volume with the mobile phase for HPLC assay. The described chromatographic conditions under the calibration procedure of each method were followed for the determination of DNT and PAR.

### Results and Discussion

Many pharmaceutical compounds undergo degradation during storage or even during the different processes of their manufacture. Several chemical or physical factors can lead to the degradation of drugs [38]. Without exception, the studied drugs are liable to hydrolysis, where DNT is liable to alkaline degradation [15] and PAP is reported as a primary hydrolytic degradation product of PAR or it may be

originated from the synthesis [17]. So in this work stability indicating methods were developed. The applied chromatographic systems provided simple and accurate methods for quantitative determination of the studied drugs in presence of their degradation products.

## TLC

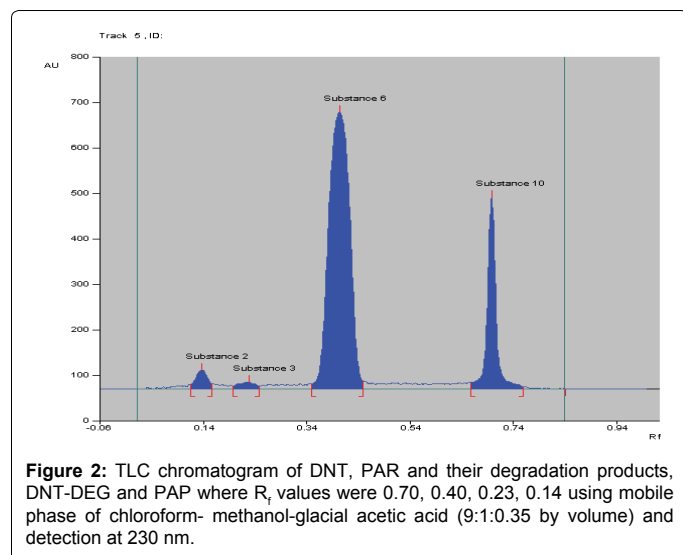
Densitometry offers a simple way of quantifying directly on a TLC plate by measuring the optical density of the separated bands. The amounts of compounds are determined by comparing them to a standard curve from reference materials chromatographed simultaneously under the same condition [39]. In this study, TLC was applied for the separation of DNT and PAR from their degradation products depending on the difference in  $R_f$  values.

Several developing systems were applied for the separation of the proposed components. First attempt using toluene-ethyl acetate mixture in different ratios provided poor separation particularly for DNT and PAP bands. Sufficient separation was achieved upon using chloroform-methanol as a developing system; however PAR and PAP showed overlapped bands. Additions of ethyl acetate to the latter system worsen the separation and PAP showed a tailed band. Finally, optimum separation was achieved when chloroform-methanol-glacial acetic acid (9:1:0.35 by volume) was used as a developing system. Four well resolved bands of DNT, PAR, DNT-DEG and PAP were obtained having  $R_f$  values of 0.70, 0.40, 0.23 and 0.14, respectively, (Figure 2).

A polynomial relationship was found to exist between the integrated area under the peak of the separated bands at the selected wavelength (230 nm) and the corresponding concentration of DNT (0.3–8.0  $\mu\text{g}/\text{band}$ ) and PAR (5.0–36.0  $\mu\text{g}/\text{band}$ ) (Figure 3).

## RP-HPLC method

Looking for simple isocratic RP-HPLC method with optimum separation parameters necessitated studying of several critical factors that may affect the separation of DNT and PAR in the presence of their degradation products. Several trials were done using buffer pH 3.0 with several organic modifiers (methanol and acetonitrile) as mobile phase on Agilent C18 column. The elution order in all cases was PAP, PAR, DNT-DEG then DNT. Among these trials, the last eluted peak required significantly large retention volume with acetonitrile in the mobile phase, since it is the least polar organic modifier in our trials. Further trials revealed that buffer and methanol mixture was appropriate for



**Figure 2:** TLC chromatogram of DNT, PAR and their degradation products, DNT-DEG and PAP where  $R_f$  values were 0.70, 0.40, 0.23, 0.14 using mobile phase of chloroform- methanol-glacial acetic acid (9:1:0.35 by volume) and detection at 230 nm.

such separation. However buffer ratio revealed to play a fundamental role in the separation. At high buffer ratio, resolution between PAR and its degradation product increased but high retention for DNT was observed. For instance, using phosphate buffer pH 3.0-methanol (50:50 by volume) as mobile phase increased both resolution of PAR and capacity factor of DNT. In order to have optimum resolution of PAR along with lower DNT capacity factor, stepwise decrease in buffer ratio was done. Trials revealed that using phosphate buffer pH 3.0-methanol (35:65 by volume) as mobile phase achieve sufficient PAR resolution (1.57) and least applicable DNT capacity factor (3.85) with minimum run time. Since the studied drugs contain ionizable function groups with  $pK_a$  7.5 and 9.5 for DNT and PAR, respectively, buffer pH should be considered in optimization. The optimum buffer ratio for separation was then tried using different buffer pH values (3.0, 4.0 and 5.0). Changes in separation performance were observed, where symmetry of PAP peak and capacity factor of DNT were enhanced using buffer pH 3.0. Also the effect of heptane sulfonic acid sodium salt was studied, negligible improvement in peak symmetry was observed. Therefore it was excluded in the proposed system.

Finally, optimization provided that using phosphate buffer pH 3.0-methanol (35:65 by volume) as mobile phase with 1 mL/min flow rate, UV detection at 230 nm, on Agilent C18 column was optimum for the separation of studied components, (Figure 4). The suggested chromatographic system allows complete baseline separation in less than 10 min run time. It is worth noting that the previously mentioned optimum chromatographic conditions was applied on different commercially available C18 columns (Kinetex<sup>®</sup> C18 and XBridge<sup>®</sup> C18) having the same dimensions of the used Agilent C18. Same separation profiles were obtained without significant changes in chromatographic parameters.

Robustness is a validation parameter that should be tested for HPLC method. Minor changes in buffer ratio, buffer pH and flow rate was studied. Levels of buffer ratio in mobile phase ( $35 \pm 3$  mL), buffer pH ( $3.0 \pm 0.2$ ) and the flow rate ( $1.0 \pm 0.05$  units) were varied while keeping the other chromatographic conditions at optimal specified levels. Robustness was expressed by RSD% values of PAR and DNT peak areas as quantitative responses. All values were less than 5%. These results agree with the method robustness for the quantitative analysis of studied drugs. System suitability parameters [40] was checked and method performance parameters including capacity factor ( $k$ ), selectivity ( $\alpha$ ), resolution ( $R_s$ ), tailing factor ( $T$ ) and column efficiency ( $N$ ) are listed in Table 1. All calculated parameters are satisfactory to indicate good specificity of the method for the stability assessment of DNT and PAR.

The results obtained during analysis of laboratory prepared mixtures, (Table 2), show that the proposed chromatographic methods were valid for the determination DNT and PAR in different laboratory prepared mixtures. Validation of the proposed methods was performed according to ICH guidelines [41]. Selectivity of the proposed methods was assisted by synthetic mixture analyses. Good mean recoveries were obtained, (Table 3). Linear regression parameters, concentration ranges and satisfactory results of the accuracy of the proposed methods along with LOD and LOQ were shown in Table 3. The proposed methods repeatability and intermediate precision were evaluated and satisfactory RSD% values were obtained in the same table. The proposed chromatographic methods were applied successfully for the determination of DNT and PAR in Dantrelax Compound<sup>®</sup> capsules, (Table 4). Moreover, standard addition technique has been applied and the obtained results proved the validity of the method and absence of interference of excipients, (Table 4). A statistical comparison of the results obtained by the proposed and a reported [37] methods for

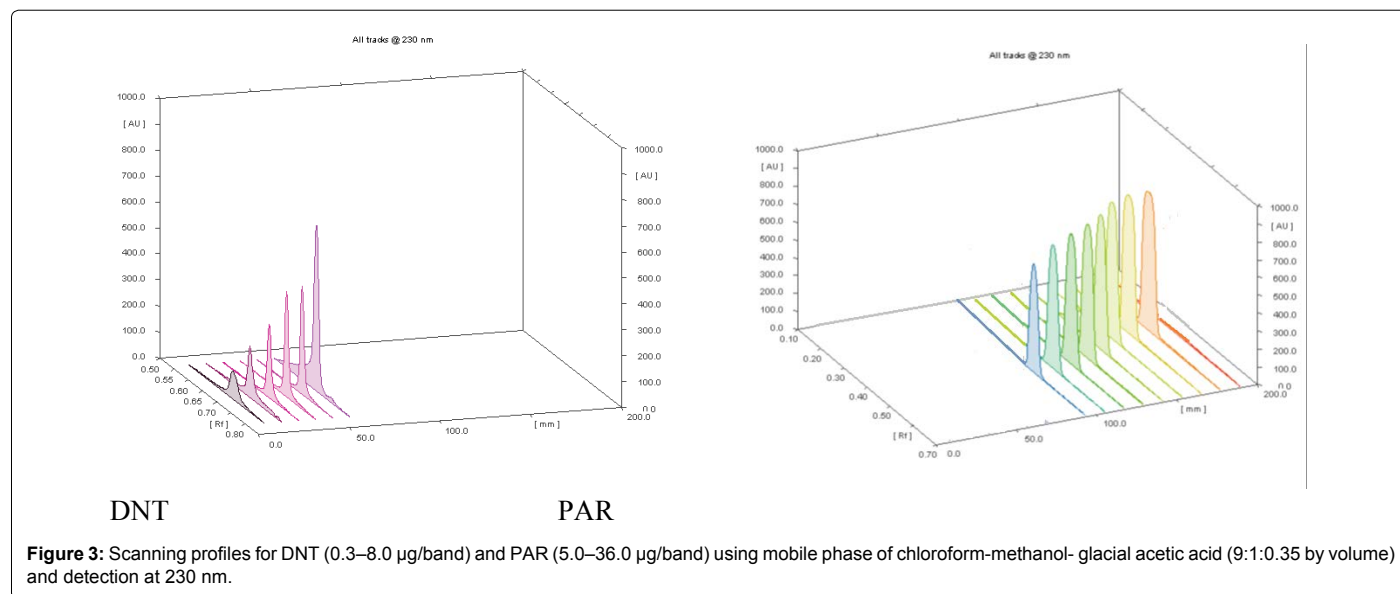


Figure 3: Scanning profiles for DNT (0.3–8.0 µg/band) and PAR (5.0–36.0 µg/band) using mobile phase of chloroform-methanol- glacial acetic acid (9:1:0.35 by volume) and detection at 230 nm.

| Parameter                                    | TLC    |        |        |        | HPLC   |        |        |        | Reference value <sup>a</sup> [40]                       |
|--|--------|--------|--------|--------|--------|--------|--------|--------|---|
|  | PAP    | DEG    | PAR    | DNT    | PAP    | PAR    | DEG    | DNT    |   |
| Retention factor (mm) / Retention time (min) | 0.14   | 0.23   | 0.40   | 0.70   | 2.89   | 3.18   | 5.26   | 7.28   | -----   |
| Capacity factor (k)                          | 6.14   | 3.35   | 1.50   | 0.43   | 0.93   | 1.12   | 2.51   | 3.85   | 1–10 acceptable   |
| Selectivity (α)                              | -----  | 1.84   | 2.23   | 3.50   | -----  | 1.2    | 2.2    | 1.5    | > 1   |
| Resolution (R <sub>s</sub> )                 | -----  | 2.14   | 2.51   | 3.69   | -----  | 1.57   | 7.46   | 5.95   | > 1.5   |
| Tailing factor (T)                           | 1.00   | 1.00   | 1.00   | 1.00   | 1.00   | 1.00   | 1.00   | 1.20   | T=1 for a typical symmetric peak                        |
| Efficiency (N)                               | 169    | 324    | 272    | 2079   | 2791   | 8049   | 2558   | 12453  | Increases with efficiency of the separation             |
| Height equivalent to theoretical plates (mm) | 0.5917 | 0.3086 | 0.3673 | 0.0480 | 0.0896 | 0.0031 | 0.0098 | 0.0020 | The smaller the value. The higher the column efficiency |

<sup>a</sup>Reference values for HPLC system suitability parameters.

Table 1: The system suitability test results of the developed RP-HPLC method.

| TLC                                  |      |                |               | HPLC                                 |      |                |               |
|--------------------------------------|------|----------------|---------------|--------------------------------------|------|----------------|---------------|
| Concentration (µg mL <sup>-1</sup> ) |      | Recovery%      |               | Concentration (µg mL <sup>-1</sup> ) |      | Recovery%      |               |
| DNT                                  | PAR  | DNT            | PAR           | DNT                                  | PAR  | DNT            | PAR           |
| 1.0                                  | 12.0 | 100.23         | 99.75         | 40.0                                 | 20.0 | 99.63          | 100.13        |
| 2.0                                  | 24.0 | 101.53         | 98.74         | 1.0                                  | 10.0 | 101.91         | 99.34         |
| 6.0                                  | 12.0 | 98.72          | 101.05        | 10.0                                 | 10.0 | 102.01         | 99.34         |
| 1.0                                  | 4.0  | 101.76         | 98.28         | 10.0                                 | 20.0 | 102.01         | 99.15         |
| 1.0                                  | 24.0 | 100.23         | 99.49         | 20.0                                 | 10   | 100.47         | 100.49        |
| 6.0                                  | 3.0  | 100.47         | 100.57        | 10.0                                 | 40.0 | 102.01         | 99.10         |
| 5.0                                  | 5.0  | 99.85          | 99.49         |                                      |      |                |               |
| <b>Mean ± RSD%</b>                   |      | 100.39 ± 1.021 | 99.62 ± 0.966 | <b>Mean ± RSD%</b>                   |      | 101.68 ± 0.677 | 99.59 ± 0.580 |

Table 2: Determination of DNT and PAR in laboratory-prepared mixtures by the proposed methods.

DNT and PAR is shown in Table 5. The calculated t and F values were less than the corresponding tabulated ones, which revealed that there was no significant difference with respect to accuracy and precision between the proposed methods and the reported one.

## Conclusion

The developed methods were simple, accurate and precise for the determination DNT and PAR in presence of their degradation products. Good resolution between studied components and their degradation justified the importance of the proposed liquid

| Parameters                                       | TLC            |               | HPLC           |                |
|--|----------------|---------------|----------------|----------------|
|  | DNT            | PAR           | DNT            | PAR            |
| <b>Selectivity</b><br>(Mean ± RSD%)              | 100.39 ± 1.021 | 99.62 ± 0.966 | 101.68 ± 0.677 | 99.59 ± 0.580  |
| <b>Linearity</b><br>Range (µg mL <sup>-1</sup> ) | 0.3- 8.0       | 5.0-36.0      | 0.5-50.0       | 1.0-150.0      |
| Intercept  | 2419.3         | 14212         | - 1.911        | 2.1276         |
| SE of intercept                                  | 0.0094         | 0.0079        | 1.4508         | 3.2087         |
| Slope (X coefficient)                            | 4082.1         | 1478.6        | 7.9703         | 11             |
| SE of slope (X coefficient)                      | 0.0054         | 0.0009        | 0.0517         | 0.0402         |
| Slope (X <sup>2</sup> coefficient)               | -186.33        | -21.5         | 0              | 0              |
| SE of slope(X <sup>2</sup> coefficient)          | 0.0006         | 0.00002       | -----          | -----          |
| Correlation coefficient (r)                      | 0.9997         | 0.9997        | 0.9997         | 0.9999         |
| <b>Accuracy</b><br>(Mean ± RSD%)                 | 100.66 ± 0.608 | 99.43 ± 0.621 | 100.33 ± 0.843 | 100.14 ± 1.084 |
| <b>Precision (RSD %)</b><br>Repeatability        | 0.593          | 0.299         | 0.308          | 0.753          |
| Intermediate precision                           | 1.267          | 0.874         | 0.814          | 0.988          |
| <b>LOD</b> (µg mL <sup>-1</sup> )                | 0.01           | 0.27          | 0.19           | 0.32           |
| <b>LOQ</b> (µg mL <sup>-1</sup> )                | 0.02           | 0.83          | 0.25           | 0.94           |

Table 3: Validation parameters for the proposed chromatographic methods.

chromatographic methods as stability indication one. The proposed TLC offered an inexpensive simple liquid chromatographic technique for the separation. Additionally, the proposed HPLC with isocratic elution showed higher sensitivity and shorter retention time compared to the reported method.

| TLC                   |  |           |                       |  |           | HPLC                  |  |           |                       |  |           |
|-----------------------|--|-----------|-----------------------|--|-----------|-----------------------|--|-----------|-----------------------|--|-----------|
| DNT                   |  |           | PAR                   |  |           | DNT                   |  |           | PAR                   |  |           |
| Assay Mean $\pm$ RSD% | Standard addition  |           | Assay Mean $\pm$ RSD% | Standard addition  |           | Assay Mean $\pm$ RSD% | Standard addition  |           | Assay Mean $\pm$ RSD% | Standard addition  |           |
|                       | Added concentration <sup>a</sup> ( $\mu\text{g}/\text{band}$ ) | Recovery% |                       | Added concentration <sup>a</sup> ( $\mu\text{g}/\text{band}$ ) | Recovery% |                       | Added concentration <sup>b</sup> ( $\mu\text{g mL}^{-1}$ ) | Recovery% |                       | Added concentration <sup>b</sup> ( $\mu\text{g mL}^{-1}$ ) | Recovery% |
| 101.12 $\pm$ 0.701    | 0.5  | 101.11    | 100.24 $\pm$ 1.678    | 6.0  | 101.14    | 100.78 $\pm$ 0.090    | 5.0  | 100.81    | 99.38 $\pm$ 0.581     | 60.0 <sup>c</sup>  | 102.10    |
|                       | 1.0  | 98.61     |                       | 12.0   | 102.33    |                       | 10.0   | 101.41    |                       | 120.0 <sup>c</sup>   | 100.45    |
|                       | 2.0  | 98.33     |                       | 24.0   | 98.90     |                       | 15.0   | 100.00    |                       | 180.0 <sup>c</sup>   | 99.84     |
|                       | Mean   | 99.35     |                       | Mean   | 100.79    |                       | Mean   | 100.74    |                       | Mean   | 100.80    |
|                       | $\pm$ RSD%   | 1.540     |                       | $\pm$ RSD%   | 1.728     |                       | $\pm$ RSD%   | 0.704     |                       | $\pm$ RSD%   | 1.160     |

<sup>a</sup>Added concentration to pharmaceutical formulation containing 1.0 and 12.0  $\mu\text{g}/\text{band}$  of DNT and PAR, respectively.

<sup>b</sup>Added concentration to pharmaceutical formulation containing 10.0 and 120.0  $\mu\text{g mL}^{-1}$  of DNT and PAR, respectively.

<sup>c</sup>Two fold dilutions were applied to the analysed solutions.

**Table 4:** Assay results and application of standard addition technique for the analysis of DNT and PAR in their pharmaceutical dosage form by the proposed chromatographic methods.

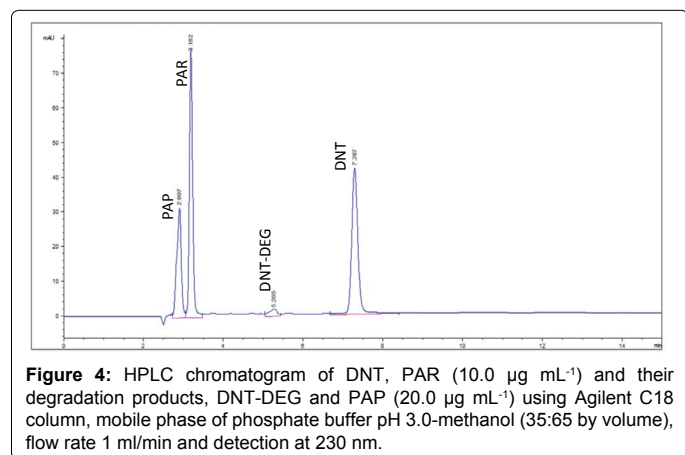
| Value            | TLC                  |                      | HPLC                 |                      | Reported method [37] <sup>a</sup> |        |
|------------------|----------------------|----------------------|----------------------|----------------------|-----------------------------------|--------|
|                  | DNT                  | PAR                  | DNT                  | PAR                  | DNT                               | PAR    |
| Mean             | 100.66               | 99.43                | 100.33               | 100.14               | 99.67                             | 100.04 |
| SD               | 0.612                | 0.617                | 0.846                | 1.086                | 0.910                             | 0.641  |
| RSD%             | 0.608                | 0.621                | 0.843                | 1.084                | 0.913                             | 0.641  |
| n                | 5                    | 5                    | 6                    | 7                    | 5                                 | 5      |
| V(Variance)      | 0.374                | 0.381                | 0.716                | 1.180                | 0.828                             | 0.411  |
| Student's t Test | 2.019                | 1.533                | 1.236                | 2.00                 | -----                             | -----  |
|                  | (2.306) <sup>b</sup> | (2.306) <sup>b</sup> | (2.262) <sup>b</sup> | (2.228) <sup>b</sup> | -----                             | -----  |
|                  | 2.21                 | 1.07                 | 1.15                 | 2.87                 | -----                             | -----  |
| F-value          | (5.05) <sup>c</sup>  | (5.05) <sup>c</sup>  | (6.26) <sup>c</sup>  | (6.16) <sup>c</sup>  | -----                             | -----  |

<sup>a</sup>Hadad et al.; RP-HPLC using HS C18 column (250 mm  $\times$  4.6 mm i.d., 5  $\mu\text{m}$  particle size), with gradient mobile phase; consisting of (A) 50 mmol L<sup>-1</sup> sodium dihydrogen phosphate, 5 mmol L<sup>-1</sup> heptane sulfonic acid sodium salt, pH 4.2 and (B) acetonitrile. The flow rate was 1.5 mL/min, UV detection at 214 nm and run time for 30 min.

<sup>b</sup>The corresponding theoretical values of t at (P=0.05)

<sup>c</sup>The corresponding theoretical values of F at (P=0.05)

**Table 5:** Statistical comparison between the proposed and reported HPLC method for the determination of DNT and PAR in their bulk powder.



**Figure 4:** HPLC chromatogram of DNT, PAR (10.0  $\mu\text{g mL}^{-1}$ ) and their degradation products, DNT-DEG and PAP (20.0  $\mu\text{g mL}^{-1}$ ) using Agilent C18 column, mobile phase of phosphate buffer pH 3.0-methanol (35:65 by volume), flow rate 1 ml/min and detection at 230 nm.

In conclusion, the proposed methods were successfully applied and validated for simultaneous determination of DNT and PAR in pure powder form and in pharmaceutical formulation and therefore can be used as stability-indicating procedures in quality control laboratories where economy and time are essential.

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