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Research article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR THE SIMULTANEOUS QUANTIFICATION OF ACETAMINOPHEN AND TRAMADOL IN COMBINED PHARMACEUTICAL DOSAGE FORMS WITH UV DETECTION

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ABSTRACT: A new high performance liquid chromatography (HPLC) with ultraviolet detection method is developed for the simultaneous quantification of acetaminophen and tramadol in bulk and in its combined pharmaceutical dosage form. The chromatographic separation was performed on Waters symmetry C8 column (250 mm × 4.6 mm I.D., 5 µm particle size) using isocratic elution. The optimized mobile phase consists of phosphate buffer (pH 6.8) and methanol (80:20, v/v). The eluted analytes are monitored at 215 nm wavelength using a UV detector. The developed method separates acetaminophen and tramadol within a run time of 6 min. The developed method was validated as per International Conference of Harmonization guidelines with respect to linearity, sensitivity (limit of detection and limit of quantification), selectivity, accuracy, precision and robustness. The developed and validated method was successfully applied to the determination of acetaminophen and tramadol in combined pharmaceutical dosage forms without any interference from the excipients with good recovery, precision and accuracy.
Key words: acetaminophen, tramadol, simultaneous estimation, C8 column, combined dosage form.

INTRODUCTION

Acetaminophen, chemically known as N-(4-hydroxyphenyl) acetamide**,** is a non-opiate, non-salicylate analgesic agent used to treat conditions such as headache, muscle aches, arthritis, backache, toothaches, colds and fevers (Graham GG et al, 2013, Toussaint K et al, 2010). Tramadol hydrochloride, chemically known as (±) cis-2-[(dimethyl amino) methyl]-1-(3methoxyphenyl) cyclohexanol hydrochloride, is a centrally acting synthetic opioid analgesic agent used to treat moderate to severe pain (Leppert, W, 2009). The combination of acetaminophen and tramadol HCl is used in the management of acute pain (Dhillon S, 2010). There are some analytical techniques reported in the literature for the simultaneous analysis of AMN and TDL in combined dosage formulation, human plasma, human urine and human serum samples. The reported techniques are spectrophotometry (Toral Ponceet al, 2008, Vikas Jain, Rajesh Sharma, 2010, Rajesh Shukla et al, 2001, Chaudhari Bharat G, Rami Ruchita R. 2014), voltametry (Fatemeh et al, 2010, Bankim J et al, 2011), LC-MS (Tian Zhu et al, 2007) and GC-MS (Belal T et al, 2009). The spectrophotometry methods are less selective as it involves absorption in the ultraviolet region where the absorbance of excipients is significant. The reported voltametry, LC-MS, GC-MS and HPTLC methods are time consuming, expensive, cumbersome and tedious. There are already few methods of the use of HPLC with UV detection for the simultaneous analysis of AMN and TDL in combined dosage formulation (Belal T et al, 2009, Arunadevi S et al, 2010, Rajesh M et al, 2011, K.Shivaramakrishna et al, 2014).

However the reported HPLC with UV detection methods suffer from disadvantages like narrow range of linearity, lack of sensitivity, less selective, more retention time, less precise and accurate. In most of the HPLC methods, acetonitrile is used in the preparation of mobile. Acetonitrile is a toxic chemical can cause environmental pollution and health hazards.

Therefore, the aim of the present study is to develop a rapid, sensitive, selective, accurate, precise and least time consuming HPLC method with UV detection for the simultaneous determination of AMN and TDL in the bulk drug samples and in its tablet formulations.

MATERIAL AND METHODS

Apparatus

During the method development and validation, Waters Alliance HPLC system equipped with 1525 separation modules having 2487 ultraviolet detector was used. Data acquisition, analysis and processing were done using Empower II software. The analytical column used for the separation was 250 mm × 4.6 mm I.D., 5 µm particle size, waters symmetry C8 column.

Chemicals

All chemicals were of HPLC grade quality. Milli-Q-water, potassium dihydrogen phosphate, methanol, orthophosphoric acid used in the process was obtained from Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India.

1 M Phosphate buffer (pH-6.8)

The 1M phosphate buffer was prepared by dissolving 136.09 gm of potassium dihydrogen phosphate in 1000 ml deionized water. The pH of the buffer was adjusted to 6.8 with orthophosphoric acid.

Diluent

Diluent was prepared by mixing phosphate buffer and methanol in the ratio of 50:50 *v/v*

Mobile Phase

The mobile phase consisted of phosphate buffer, pH 6.8 and methanol (80:20, *v/v*).

Stock standard solution:

Acetaminophen: 325 mg of AMN was accurately weighed, transferred into 100 ml volumetric flask, dissolved with 10 ml diluent, and diluted up to the mark with the same solvent (3250 µg/ml).

Tramadol: 37.5 mg of TDL was accurately weighed, transferred into a 100 ml volumetric flask, dissolved with 10 ml diluent, and diluted up to mark with same solvent (375 µg/ ml).

Mixed standard solutions for calibration curve:

From the stock solutions, aliquot volumes containing AMN and TDL were quantitatively transferred into a series of 10 ml volumetric flasks, so that the final concentration were in the range of 32.5–195 µg mL^{-1} (i.e., 32.5, 65, 97.5, 130, 162.5 and 195 µg mL^{-1}) for AMN and 3.75–22.5 µg mL^{-1} (i.e., 3.75, 7.5, 11.25, 15, 18.75 and 22.50 µg mL^{-1}) for TDL. The solutions were completed to the volume with the diluent.

Preparation of sample solution:

The tablet dosage form, Ultracet (Janssen Pharms, Mumbai), containing 325 and 37.5 mg of acetaminophen and tramadol hydrochloride, respectively was purchased from the local pharmacy and used in the present investigation. Ten tablets were weighed and their average weight was determined. The tablets were finely powdered into homogenous mixture. A quantity of powder equivalent to 325 mg AMN and 37.5 mg TDL was transferred into a conical flask containing 30 ml of diluent and sonicated for 10 min with continuous shaking. The resulting solution was filtered through a 0.45 µm membrane filter into 100 ml volumetric flask and completed to volume with same solvent. This solution was appropriately diluted with the diluent to acquire a concentration 195 µg mL^{-1} of AMN and 22.50 µg mL^{1} of TDL.

Preparation of placebo blank:

The placebo blank was prepared by mixing starch (10 mg), acacia (15 mg), hydroxyl cellulose (10 mg), sodium citrate (10 mg), talc (20 mg), magnesium stearate (15 mg) and sodium alginate (10 mg) and its solution was prepared as described under "preparation of sample solution".

Chromatographic conditions

In the present study phosphate buffer (pH-6.8) and methanol in the ratio of 80:20 *v/v* is used as mobile phase. Before use the mobile phase was filtered through a 0.45 µg membrane filter and degassed for about 15 min. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1.0 ml/min. the injection volume was 10 µl and the column temperature was maintained at 40^0C.The eluents were monitored at 215 nm.

General Procedure

Analysis of laboratory prepared mixture of AMN and TDL:

Ten µl aliquot of each solution prepared in the section "Mixed standard solutions for calibration curve" was injected automatically on to the column in triplicate. The chromatograms were recorded. The calibration curve was constructed by plotting the peak area against the final concentration of the drug (µg/ml). The corresponding regression equations were derived.

Analysis of tablet sample:

Ten µl aliquot of solution prepared in the section "Preparation of sample solution" was injected automatically onto the column in triplicate. The chromatograms were recorded and the peak areas were determined. The nominal contents of the tablet were calculated using either the corresponding calibration curve or corresponding regression equation.

RESULTS AND DISCUSSION

Development of the method

To select the appropriate column for good separation, well shaped symmetrical peaks and high resolution, three different columns (Develosil ODS C18 column (150mm × 4.6 mm I.D., × 5µm particle size),Thermo Hypersil BDS C18 column (250mm × 4.6 mm I.D., × 5µm particle size), Waters symmetry C8 column (250mm × 4.6 mm I.D., × 5µm particle size) were tested primarily. The studies revealed that the Waters symmetry C8 column was the best one, because it produced good separation of the selected drugs, well shaped symmetrical peaks with high resolution. The UV spectra of the AMN and TDL were assessed. It was observed from the UV spectra that AMN and TDL have considerable absorbance at 215 nm. Therefore, 215 nm was selected as the detection wavelength. In order to achieve satisfactory peak symmetry and separation with good resolution, various combinations of methanol, water and phosphate buffers of different pH were tried systematically on Waters symmetry C8 column. Preliminary experiments indicated that use of different combinations of phosphate buffer or methanol with water was not able to separate the peaks of AMN & TDL and to obtain appropriate retention times & peak symmetry. Finally, a mobile phase consisting of phosphate buffer of pH 6.8 (adjusted with orthophosphoric acid) and methanol in a ratio of 80:20 *v/v* was selected to achieve better resolution and acceptable peak symmetry. Flow rates between 0.5 and 1.5 ml/min were tried. Flow rate of 1.0 ml/min was observed to be adequate to get both the drugs (AMN and TDL) eluted within less than 5 min. The column temperature was set at 40°C. Under the optimized chromatographic conditions, the retention times for AMN and TDL were 2.391 min and 4.602 min, respectively. No interference was found among the two peaks. A typical chromatogram is shown in Figure 1.

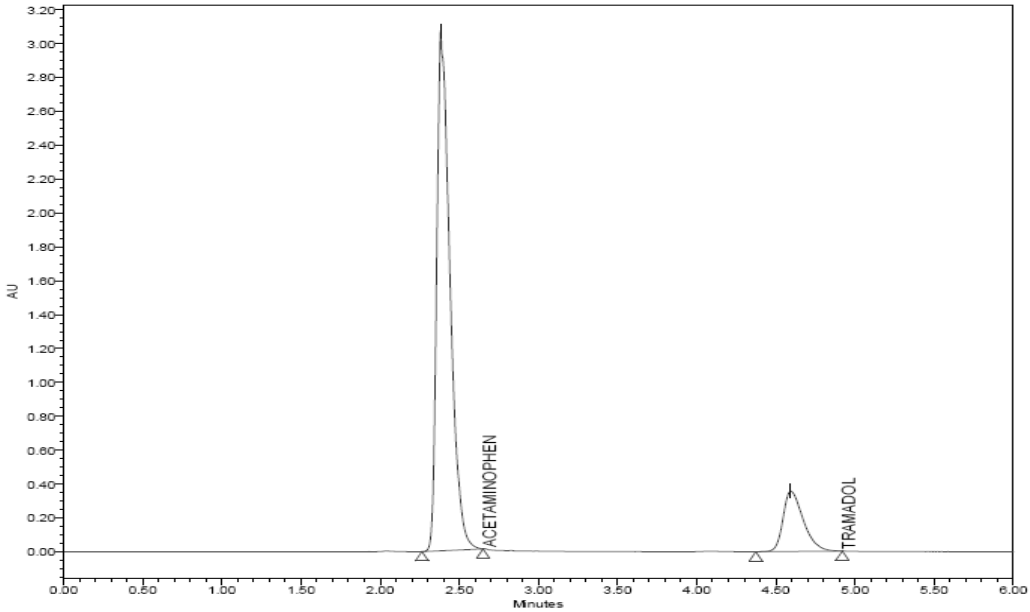

Figure 1: Chromatogram of acetaminophen and tramadol

System suitability

In order to assess the system suitability, five replicate injections of mixed standard solutions (AMN-130 µg/ml, TDL-15 µg/ml) were injected into the HPLC system. The suitability parameters like relative standard deviation of retention time, peak area, theoretical plates, and plates per meter and peak asymmetry were calculated. Results are shown in Table 1. The values are within the acceptable range and are enough for the analysis to be done.

Table 1: System suitability parameters

Parameters	AMN[a]	%RSD	TDL[a]	%RSD
Retention time (min)	2.391	0.728	4.602	0.482
Peak area	12548354	0.564	2152226	0.839
Theoretical plates	3985.6	0.435	6414	0.928
Plates per meter	15942.4	0.532	25656	1.010
Peak asymmetry	0.989	0.238	1.100	0.421

[a] Average of five values

Selectivity

The selectivity of the method was verified by comparing the chromatograms of standard mixed solution with the analytes concentration of 130 µg mL^{-1} (AMN) and 15 µg mL^{-1} (TDL) with those of blank, placebo and tablet samples. The chromatograms are represented in Figures 2-5. There is no peak interference of blank and placebo at the retention time of AMN and TDL. The results indicated that there is no drug-drug interaction or drug-excipient interaction. Therefore, the method is selective for the determination of AMN and TDL in their combined pharmaceutical dosage form.

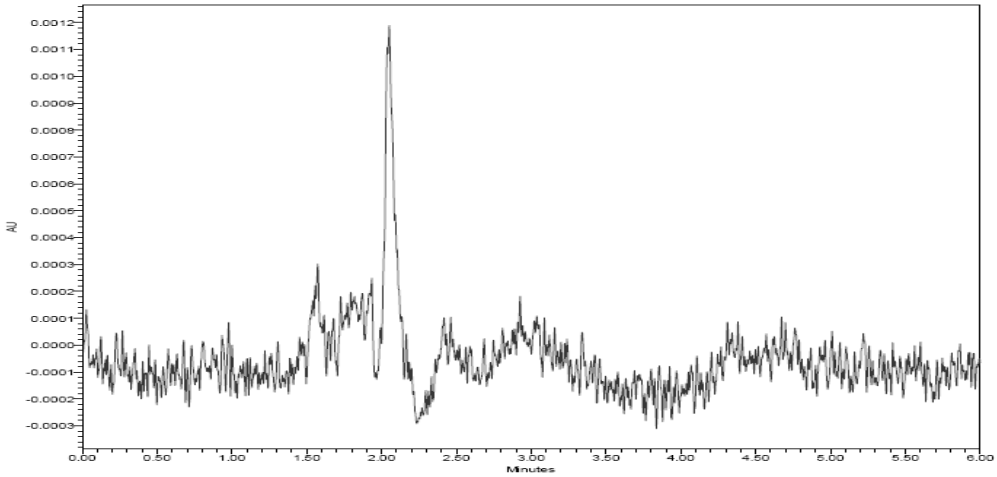

Figure 2: Chromatogram of placebo blank

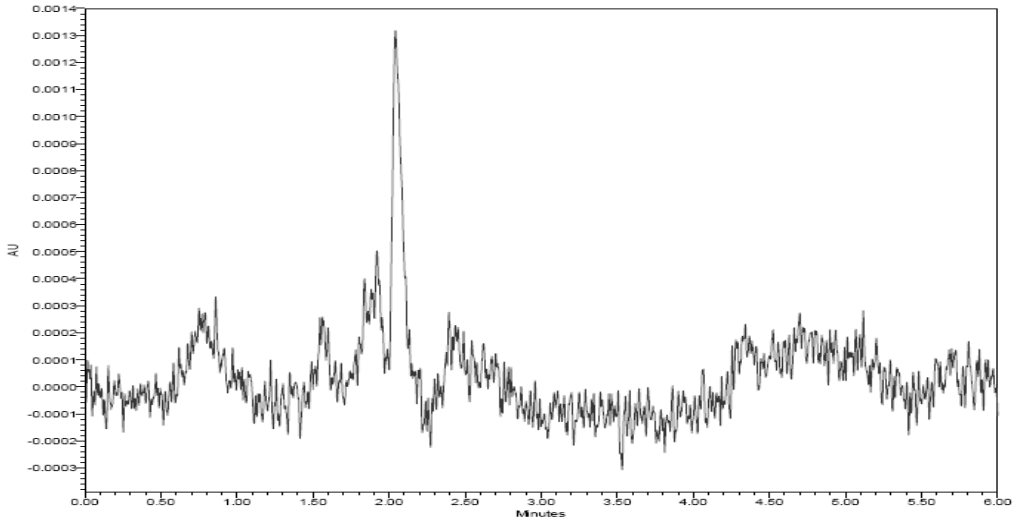

Figure 3: Chromatogram of mobile phase blank

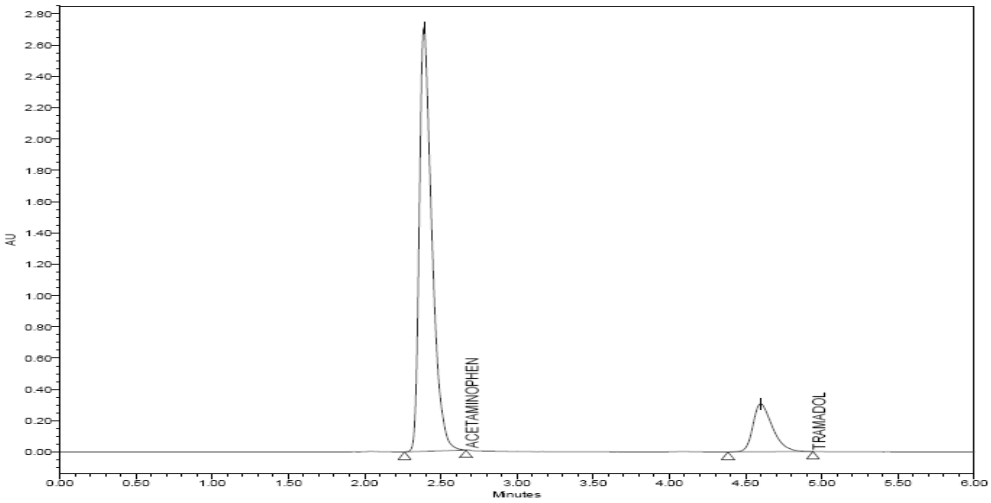

Figure 4: Chromatogram of Tablet Sample Solution (Acetaminophen - 130 µg mL^{-1}; Tramadol -15 µg mL^{-1})

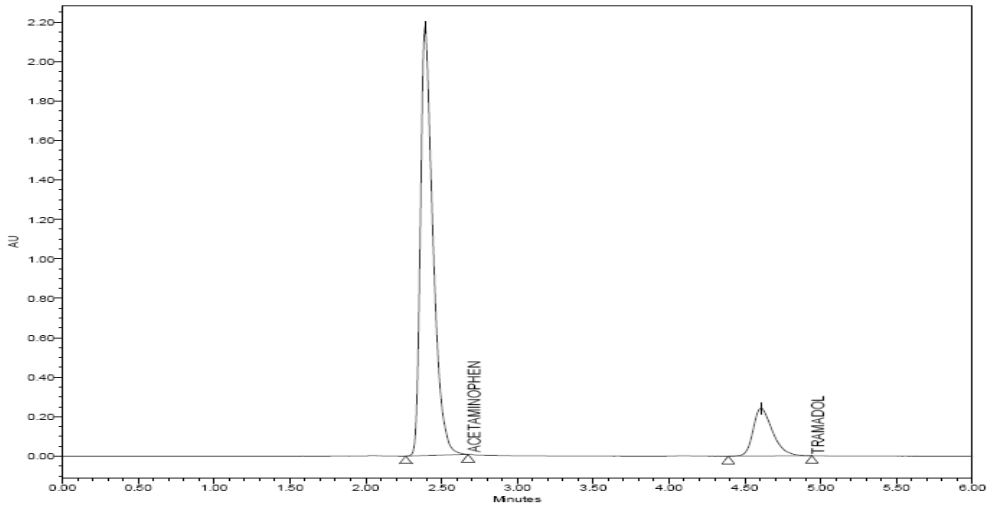

Figure 5: Chromatogram of Standard Acetaminophen (130 µg mL^{-1}) And Tramadol (15 µg mL^{-1})

Linearity

A linear relationship was established by plotting the peak area against the drug concentration. The relationship was found to be linear over the range 32.5–195 µg/ml and 3.75–22.5 µg/ml for AMN and TDL respectively. The results are summarized in Table 2. The results show that good correlation existed between the peak area and concentration of the studied drugs.

Table 2: Linearity of the method

Parameter	AMN	TDL
Linearity (µg/ml)	32.5–195	3.75–22.5
Regression equation (P^a= a + mc^b)	P^a= - 458 + 95973 c^b	P^a= -9101.1+ 143092 c^b
Slope (m)	95973	143092
Intercept (a)	- 458	-9101.1
%RSD of Slope	0.426	0.571
%RSD of intercept	0.382	0.437
Regression coefficient (R^2)	0.9999	0.9999

[a] Peak area [b] Concentration of drug in µg/ml

Sensitivity

The sensitivity of the method was expressed in terms of limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were determined according to 3:1 and 10:1 signal/noise ratios, respectively. For this purpose, five replicate injections of dilute solutions with known concentration (AMN-32.5 µg mL^{-1} and TDL-3.75 µg mL^{-1}) were injected into the HPLC system. The limit of detection of AMN and TDL was 9.75 and 1.131 µg mL^{-1}, respectively. The limit of quantification of AMN and TDL was 32.5 and 3.77 µg mL^{-1}, respectively.

Precision

Repeatability of the method was assessed by determination of intra-day and inter-day precision. Intra- and inter-day precision was assessed by injecting five mixed standard solutions of three different concentrations (within linearity range) on the same day and for three consecutive days, respectively. Relative standard deviation of the peak area was then calculated to represent precision. The results of intra-day and inter-day precision are shown in Table 3. The relative standard deviation was found to be <1, which proves that the method is adequately precise.

Table 3: Intra-day and inter-day precision

AMN			TDL		
Concentration (µg mL^{-1})	**Mean peak area[a]**	**%RSD**	**Concentration (µg mL^{-1})**	**Mean peak area[a]**	**%RSD**
Intra-day precision					
32.5	3114986	0.324	3.75	523728	0.438
97.5	9479768	0.449	11.25	1737283	0.336
195	18739719	0.968	22.5	3180692	0.579
Inter-day precision					
32.5	3115099	0.394	3.75	523819	0.279
97.5	9482479	0.314	11.25	1744380	0.271
195	18740118	0.338	22.5	3183884	0.219

[a] Average of five values

Accuracy

Accuracy of the method was assessed by recovery studies through standard addition method. In the standard addition method known quantities of AMN and TDL at three different concentration levels (50, 100 and 150% of the labeled claim) were supplemented to the tablet sample solution previously analyzed. The solutions were once again analyzed by the proposed method. The percentage recoveries and relative standard deviations for AMN and TDL were calculated and are given in Table 4. Recovery studies showed the method to be adequately accurate and suitable for the simultaneous determination of AMN and TDL.

Table 4: Recovery studies through standard addition method

Labeled claim (mg/tablet)		% Level		% Recovered[a]		% RSD	
AMN	TDL	AMN	TDL	AMN	TDL	AMN	TDL
325	37.5	50	50	99.08	100.02	0.624	0.927
325	37.5	100	100	99.94	99.95	0.600	0.931
325	37.5	150	150	99.89	99.91	0.578	0.954

[a] Average of five values

Robustness

Robustness of the method was assessed by deliberately varying certain parameters like mobile phase ratio, Buffer pH, Flow rate and Temperature. Each parameter was studied at three levels. In these experiments, one parameter was changed while the others were kept unchanged. The peak area and relative standard deviation was calculated each time. The assay was carried out at two different concentration levels of AMN (32.5 and 195 µg/ml) and TDL (3.75–22.5 µg/ml). The results are summarized in Table 5. The results indicated that small variation in the experimental variables did not significantly affect the analytical performance of the method.

Table 5: Robustness of the method

Parameter	AMN			TDL		
	Conc. (µg/mL)	Peak area[e]	% RSD	Conc. (µg/mL)	Peak area[e]	% RSD
Mobile phase ratio[a]	32.5	3102674	0.924	3.75	522330	0.950
	195	18048250	0.776	22.5	3179970	0.888
Buffer pH[b]	32.5	3101162	0.849	3.75	522280	0.721
	195	18049918	0.778	22.5	3181452	0.842
Flow rate[c]	32.5	3101231	1.063	3.75	522577	1.025
	195	18050281	1.045	22.5	3181862	1.004
Temperature[d]	32.5	3101281	0.817	3.75	522243	0.578
	195	18049719	0.934	22.5	3153533	0.872

[a.] Phosphate buffer and methanol ratios (*v/v*) - 82:18, 80:20 and 78:22
[b.] Buffer pH – 6.7, 6.8 and 6.9
[c.] Flow rate (ml/min) – 0.9, 1.0 and 1.1
[d.] Temperature (^oC) – 38, 40 and 42
[e] Mean of three values

Application of the proposed method to pharmaceutical dosage forms:

The proposed method was successfully applied to analyze AMN and TDL in commercial tablet formulations. A clear separation of the drugs was achieved in tablet with no interference from excipients. The assay results are shown in Table 6. The results of the quantitative analysis of tablet formulations indicate that the proposed method can be used for quantization and routine quality control analysis of AMN and TDL in pharmaceutical dosage forms.

Table 6: Assay of pharmaceutical dosage form

Drug	Labeled claim (mg/tablet)	% Recovered[a]	% RSD
AMN	325	99.95	0.357
TDL	37.5	100.04	0.491

[a] Average of five values

CONCLUSION

An isocratic HPLC with ultra violet detection method was successfully developed for the simultaneous analysis of acetaminophen and tramadol. The method validation results have proven that the method is adequately sensitive, precise, accurate, linear and robust. The method is selective for the acetaminophen and tramadol and free from the interference of the placebo and components of mobile phase. The short run time (6 min) enables rapid determination of the acetaminophen and tramadol. Further more, the method was suitable for the routine quality control of acetaminophen and tramadol in combined dosage forms.

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