Research Article

International Journal of Bioassays
ISSN: 2278-778X
www.ijbio.com

DEVELOPMENT OF A KINETIC REACTION RATE LIMITED STUDY ON DEGRADATION OF IMPURITY PROFILE FOR THE ESTIMATION OF ACETAMINOPHEN IN ACIDIC AQUEOUS SOLUTION FOR PHARMACEUTICAL DOSAGE FORM BY HIGH PERFORMANCES LIQUID CHROMATOGRAPHY.

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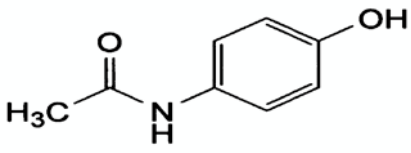
Received for publication: June 05, 2013; **Revised:** June 21, 2013; **Accepted:** July 16, 2013

Abstract: A reaction-limited model for impurity profile is developed for the acetaminophen which consists of degradation impurity as 4- Amino phenol including ten other potential impurities. As many ways of the synthetic routes for acetaminophen it has been identified the impurities are of eleven. Out of which many degrading impurities, 4-Amino phenol is one of the major degradant. And remaining Organic Impurities that may appear in acetaminophen preparations are process-related impurities. Impurity evolution process is considered from Reverse-phase liquid chromatography (RPLC) which is the method of choice for the analysis of basic compounds. The chromatographic behavior of acidic and basic substances is closely related to their structure and is easily influenced by many factors, Such as pH, molarity and Composition of the Mobile Phase. Determination of kinetic reaction for acetaminophen and its main degradation of impurity as 4- aminophenol was developed and method have been validated for the same through the High Performances Liquid Chromatography for all potential eleven impurities. Chromatographic separation has been obtained on an Intersil ODS 3V using gradient elution of having mobile phase A&B. In Mobile phase-A of 0.01M Di-potassium hydrogen phosphate adjusted pH 7.0 diluted with ortho-phosphoric acid and Mobile phase- B is the mixture of 800ml methanol and 200ml of 0.01M Di-potassium hydrogen phosphate adjusted pH 7.0 diluted with ortho-phosphoric acid. Analysis time did not exceed 75minutes and good resolution peak shapes and asymmetric have retained. The method has been successfully obtained for the study of kinetic degradation of acetaminophen with respect to its degradation impurity of the 4-aminophenoland other process related impurities. A kinetic investigation of acidic hydrolysis of acetaminophen was carried out in hydrochloride solutions of 0.3N, 0.5N and 1.0N by monitoring the parent compound itself with respect to its main degradant. The order of acetaminophen followed pseudo-first order kinetics. The activation energy could be extended from the Arrhenius plot and it was found to be 21.2Kcal/mol.

Keywords: High Performance Liquid Chromatography; Reaction-Limited Model; Impurity Profile Evaluation.

INTRODUCTION

Acetaminophen (N-acetyl-p-amino-phenol) is a white crystalline powder, soluble in alcohol, sparingly soluble in water, very slightly soluble in ether and in methylene chloride. It is an antipyretic and analgesic accepted as an effective treatment for the relief of pain and fever.



Structure of Acetaminophen

Impurities of acetaminophen depends upon the choice of synthesis route of drug substances preparations. Impurities are classified as Organic impurities & Degrading Impurities. And run down of impurities is influenced by the adoption of quality of starting raw materials, reagents and solvents.

Moreover, Impurity profile depends up on design of process equipment and final purification. An influential impurity of acetaminophen is 4-aminophenol which reveals its impact at the stage of pharmaceutical preparations as an aftereffect of both synthesis and degradation during storage. And the acceptance criteria limit is 50µg/mL.

A magnitude number of analytical methods are adapted for assaying the principal matrix of acetaminophen with respect to its eleven impurities. Literature intelligence tells that less digit in notifying the analytical method of reverse phase chromatography with the elution of all potential impurities. Analytical methodology aims to elicit all main eleven impurities and there is represented with their IUPAC names as follows:

Imp-A: [N-(2-hydroxyphenyl) acetamide], Imp-B: [N-(4- hydroxyphenyl) propanamide],Imp-C: [N-(3-

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chloro-4-hydroxyphenyl) acetamide], Imp-D: [N-phenylacetamide], Imp-E:[1-(4-hydroxyphenyl) ethanone], Imp-F: [4- nitrophenol], Imp-G: [1-(4-hydroxyphenyl) ethanoneoxime].Imp-H: [4-(acetyl amino) phenyl acetate], Imp-I:[1-(2-hydroxyphenyl) ethanone], Imp-J: [N-(4- chlorophenyl) acetamide (chloroacetanilide)], Imp-K: [4- aminophenol].

In general factor effecting chemical stability of Drug substances/ Drug products include intrinsic factors such as the molecular structure of the drug itself and environment factors such as temperature, pH, buffer species, ionic strength, light, oxygen, moisture, additives and excipients. In the case of solid state degradation the solid state properties of the drug such as melting point, crystalinity and hygroscopicity are very important by applying well established kinetic concepts it is possible not only to summarize numerically. The role that each variable might play in altering the kinetic of degradation but also to provide valuable insight into the mechanism of degradation.

More generally, the degradation rate of a drug D depends on the Drug concentration [D], and the concentrations of chemical species participating in the reaction [A], [B]……….

The rate at which [D] decreases,-d [D]/dt, is described by summing terms for all the reaction that D might undergo.

$$-\frac{d[D]}{dt} = \{K_{O,}AB......[A]^l[B]^m.... K_{o,}EF.....[E]^o[F]^p ....+\} [D]^n \text{...} \quad (1)$$

Where K_O, AB……….and K_O,EF………etc…., are constants of reaction in which species AB…and species EF…..respectively, react with D. The terms n, l, m, o and p are reaction order for each species and sum of these is considered to be one.

With respect to the degradation of Drug substances/Drug products in solution, any observed rate or rate constant can be calculated according to rate laws & Arrhenius equation. In other words, the stability off Drug substance/Drug product does not change unless key parameters appearing in this principle matrix change owing to changes in reaction conditions. (media, etc.)

On the other hand most of the studies haven't focused study of Kinetics rate limited model with the separation of acetaminophen and its impurities by differentiating the Process impurities, Starting Impurities, Raw material Impurities, Intermediate Impurities as well as Degradation Impurities using Reverse-Phase chromatographic technique. The goal of the present study to Develop analytical method for the kinetic study on the degradation of Acetaminophen in Acidic Aqueous Solutions with a feasible, sensitive and specific in presence of its degradation product.

This paper also reports the validation of a new reliable HPLC method for the simultaneously of determination of all impurities namely Imp-A, Imp-B, Imp-C, Imp-D, Imp- F, Imp-G, Imp-H, Imp-I, Imp-J as well as degradant impurity Imp-K mixtures containing acetaminophen.

Moreover, Kinetic studies and accelerated stability experiments to predict expiry dates of pharmaceutical products necessitate such methods.

Theory:

Drug substance undergo chemical degradation by various pathways and mechanisms, depends on their chemical structure. The rate of chemical degradation is determined by various factors contributing to the rate reactions.

A kinetic model is selected to describe the degradation curve and a rate constant is calculated by fitting the observed curve to a suitable rate equation according to the assumed model.

Kinetic model to describe drug degradation in solution:

The generalized rate expression for drug degradation is represented by the rate equation of (2).When a drug substance D degrades via a certain mechanism in which reactants A, B … Participate, the degradation rate generally depends on the concentrations of the various reactants A, B……… and D according to the equation, assuming that all the reactants are involved directly or indirectly in the rate controlling step.

$$-\frac{d[D]}{dt} = K_O \ [D]^n[A]^l[B]^m \quad \text{............................} \ (2)$$

When the concentration of A, B ………. are maintained constant, that is, when the change in their concentrations during the reaction is negligible occurring to their being present at much higher concentrations then drug d, or when there species are components that are maintained constant through the use of buffers. Such as hydronium ion, the degradation rate is often described by

$$\frac{d[D]}{dt} = K[D]^n \text{...} \ (3)$$

When 'n' equals 0, 1, or 2 the reaction is said to

be the pseudo-zero, pseudo-first or pseudo-second order reaction respectively if the concentration of an additional reactant other than drug D is not constant during the reaction order becomes 'n+1'.

Kinetic models generally used for drug stability production usually follow pseudo-zero, pseudo-first or pseudo-second order kinetics.

Here in this study drug product sample considers pseudo-first order reaction:

$$D \xrightarrow{K} P \quad \ldots\ldots\ldots\ldots\ldots(4)$$

The differential rate reaction for a pseudo-first order reaction is

$$-\frac{d[o]}{dt} = k[o] \quad \ldots\ldots\ldots\ldots\ldots(5)$$

The integrated form of this equation is

$$[D]=[D]_o \exp(-kt) \quad \ldots\ldots\ldots\ldots..(6)$$

When [D] is the interact concentration of the drug, from the above equations, the degradation rate is seen to be proportional to drug concentration.

Temperature: General principles:

Temperature is one of the primary factors effecting drug stability. The rate constant/ temperature relationship has traditionally been described by the Arrhenius equation

$$k = A \exp\left(\frac{-E_a}{RT}\right) \quad \ldots\ldots\ldots\ldots\ldots(7)$$

Where E_a is the activation energy and A is the frequency factor. This equation is a variant of the equation describing the effect of temperature on equilibrium processes that was developed by van't Hoff in 1887. Arrhenius applied his equation to various reaction processes. The frequency factor A in the Arrehenius equation corresponds to the product of the universal collision and entropy terms in equation (1). Because a plot of the logarithm of K against the reciprocal of absolute temperature generally yields a linear relationship (Arrehenius plots), the frequency factor and activation energy are regarded as independent of temperature, and the activation energy, E_a, is used as a measure of the temperature dependence of the rate constant. Observed linear Arrhenius plots can be explained by the much larger temperature dependency of the exponential term in Equ (7).

Quantitation of the Temperature Dependency of Degradation Rate Constants:

Estimation of an appropriate rate or rate constant for drug degradation is an important step in predicting the stability of pharmaceuticals. Knowing how such a rate or rate constant changes with temperature in a quantitative way may allow one to predict the stability at other temperatures. Even if a rate or rate constant cannot be estimated by fitting the data to a theoretical or empirical equation, constants such as time required for 10% degradation (t_{90})can be utilized instead of rate constants.. Taking the logarithm of both sides of Equation (7) yields

$$\ln k = \frac{-E_a}{RT} + \ln A \quad \ldots\ldots\ldots\ldots(8)$$

So-called Arrhenius plots in which the logarithm of rate (rate constant) is plotted against the reciprocal of absolute temperature (degrees Kelvin) have been used to validate the conformity of the degradation rates of various drugs to this equation. A linear Arrhenius plot indicates that, once degradation rates are obtained at several temperature levels, the degradation rate at some other specific temperature can be estimated. Thus, the Arrhenius equation has been successfully applied to the prediction of the stability of various pharmaceuticals. The Arrhenius equation is valid in the temperature range where both A and E_a in Equation can be regarded as constant. Changes in the degradation mechanism with temperature may result in nonlinear Arrhenius plots.

MATERIALS AND METHODS

Chemicals: Acetaminophen (≥99.0%), 4-Amino phenol (97.8% as is basis), N-(4-chlorophenyl) acetamide (chloroacetanilide)] (99.6% as is basis), 4-Nitro phenol (99.7% as is basis), N-(2-hydroxyphenyl) acetamide (99.2% as is basis), N-(4-hydroxyphenyl) propanamide (99.4% as is basis), [N-(3-chloro-4- hydroxyphenyl) acetamide] (98.9%),N-phenylacetamide (98.7% as is basis), 1-(4-hydroxyphenyl) ethanone (98.9% as is basis),1-(4-hydroxyphenyl) ethanoneoxime (98.3% as is basis),4-(acetyl amino) phenyl acetate (99.1% as is basis),1-(2-hydroxyphenyl) ethanone (99.3%, as is basis),Disodium hydrogenPhosphate, Sodium Dihydrogen Phosphate , Tetrabutyl Ammonium Hydroxide, Dipotassium Hydrogen Phosphate were purchased from SD-Fine Chemicals. Phosphoric acid (85%), Hydrochloric Acid (36.5%-38.0%), Extra Pure Sodium hydroxide Pellets (99.5%), Methanol (99.7%) and Acetonitrile (99.8%) both HPLC grade were purchased from Merck. Acetaminophen Tablets produced by Sanfovi Aventis were brought from the market. Each tablet 500 mg of acetaminophen and the following excipients are:-

Apparatus: Chromatographic analysis was performed on a HPLC Waters e Alliance 2695 Separations module composed of a quaternary pump, an automatic injector, column thermostat, temperature controlled sample trays, an on-liner degasser, Waters 2998 photodiode Array (PDA) detector and waters 2489 UV/Visible detector. The control was done by the Empower Software.

Milli pure water was produced using a PURE LAB Option Q DV-25, ELGA. The pH of the buffer solutions was measured using a pH/ mV-meter Polmon LP-135M. Weighing of Impurities & working standard were balanced with the instrument Mettler Toledo PB 303-S/ FACT.

Solutions for HPLC Analysis:

A Stock solution of Acetaminophen containing 200µg/mL was prepared in a 10 mL volumetric flask. The flask was filled up to the mark with the diluent.

Each Individual Stock solutions containing 1000µg/mL of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G, Imp- H, Imp-I, Imp-J and Imp-K in a 10 mL volumetric flask. Each flask was filled up to the mark with the diluent.

Working solutions containing all the analytes were prepared in the range of 10µg/mL for the impurities Imp-A, Imp-B, Imp-C, Imp-D, Imp-E, Imp-F, Imp-G, Imp-H, Imp- I & Imp-J. And for Impurity-K & Acetaminophen solutions in the same above containing flask were 50 µg/mL, 20µg/mL respectively. All analytical solutions were prepared daily and kept at 4◦c before and between injections to prevent the sample degradation.

Trails have been initiated with the preparation of mobile Phase containing the mixed salt buffers with various ratios [375:375:250] & [375:375:300] by dissolving 17.9 gm/L solution of Disodium Hydrogen Phosphate, 7.8 gm/L solution of sodium Dihydrogen Phosphate & 4.6 gm/L of a 400gm/L of solution Tetra butyl Ammonium Hydroxide.

A mobile phase-A with Monosalt buffer was prepared containing 0.01M Dipotassium Hydrogen Phosphate at pH 7.0 with diluted orthophosphoric acid.

A mobile phase-B is prepared by 800mL methanol and 200 mL of Purified water mixed and degassed. A mobile phase-B is prepared by 800mL methanol and 200 mL of Buffer containing 0.01 M Dipotassium Hydrogen Phosphate at pH 7.0 with diluted orthophosphoricacid.

Analysis of Acetaminophen Tablets preparations:

Commercially available acetaminophen tablets from Micro labs Limited., India having Batch no:- DOGD0117 reported to contain only acetaminophen and excipients were brought from pharmacy and analysed using the validated chromatographic method as described in text under validation parameter.

Ten tablets of acetaminophen were accurately weighed and then pulverized in a mortar. And transferred an accurately weighed quantity of about 0.200 gm and then dissolved in diluent in a 10.0 mL volumetric flask. Make volume up to the mark with diluent. This solution was filtered through a 0.45µm syringe filter and the same was injected into chromatographic system.

Chromatographic Conditions:

Chromatographic studies were performed using aIntersil ODS 3V with dimensions 250* 4.6mm and 5µm particle size. The injection volume is 20µL. The final method used a Mobile phase-A containing 0.01M Dipotassium Hydrogen Phosphate with pH 7.0 and Mobile phase-B containing 800 mL Methanol and 200 mL of Buffer mixed with degassing and the following gradient program: from 0 to 10 min the percent of Mobile phase-A is 70%, decreases the Mobile phase- A to 65% for time programmer at 15-30 minutes, Mobile phase-A is optimized with 50.0 % from 45 – 65minutes and again mobile phase–A is restrained at 70% for time programmer from 70- 75 minutes. Flow rate was set at 1.0mL/min for the entire time programmer at 0- 75 minutes. Detection was made at 245 nm. Sample holder and the column oven were kept at 25◦c & 30◦c respectively.

Kinetic Studies:

For Studying the Kinetic Order of the reaction: In a 100 ml volumetric flask, dissolve 1000 mg of acetaminophen in 1N Hydrochloric acid and complete to the mark with the same solvent. Transfer this solution into another clean dry conical flask and reflux in a thermostatically controlled water bath at 80◦c for 120 minutes. Take 5.0 mL samples at 20, 40, 60, and 80,100 & 120 minutes intervals, place into 50mL volumetric flasks half-filled with diluent, neutralize with 5mL of 1N Sodium Hydrochloride, and complete the volume with diluent. Inject the solutions in the liquid chromatograph using the chromatographic conditions described above and the same were presented in Fig 10. The concentration of acetaminophen was calculated from the regression equation. Plot the log % of acetaminophen remains against time.

For Studying the Effect of Hydrochloride acid concentration on the Reaction Rate: Into a series of 100mL volumetric flasks dissolve 1000mg of acetaminophen in 0.3, 0.5 and 1.0 N HcL and complete to the mark with the same solvent. Transfer these solutions in to clean dry conical flasks and then reflux in a thermostatically controlled water bath at 90◦c for 120 minutes. Take 5.0mL samples at 20, 40, 60, 80, 100 & 120 minutes intervals and then complete as described in Sec. for studying the Kinetic Order of the reaction.

Plot the log of % acetaminophen remaining against time for different normalities of HcL and calculate the rate constant and $t_{1/2}$.

For studying the Effect of the Temperature on the Reaction Rate : Dissolve three portions each of 1000 mg of acetaminophen in 100 mL volumetric flasks and complete the volume with 0.3,0.5 and 1.0 N HcL respectively. Transfer these solutions into other clean dry conical flasks and then reflux

HPLC Method Development approach:

Optimization of a separation of all eleven impurities from acetaminophen is principally directed by keeping in the view that analytical methodology should encompass the following parameters:-

1. To separate Better higher resolution
2. To Obtain lower detection Limit

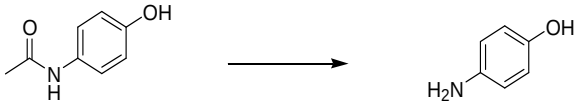
A mixture of acetaminophen impurities represents the similar structures of parent molecule acetaminophen containing functional groups of primary amines and alcohols with same solvent strengths. A solvent with methanol & water yields the better selectivity on comparing with other solvents, possible because of the preference for polar interaction i.e. protic Vs aprotic solvents.

On imparting solvent containing the solution mixture of methanol & water as diluent, molecules containing the impurities of same functional groups scatter through the substantially thinner solvent layer, resulting in stronger and additional interactions. Furthermore, the formation of labile methanolates could facilitate polar interactions leading to good polar selectivity in methanol. In a thermostatically controlled water bath at, 60, 70◦C, 80◦C & 90◦C for 10 minutes. Take 5.0 mL samples at 20, 40 60, 80, 100 & 120 minutes and then complete as directed under sec. For studying the kinetic Order of the Reaction.

Plot the log of % of acetaminophen remaining against at different temperatures. Also Plot the Arrhenius plot for the effect of temperature on the rate of hydrolysis.

RESULTS AND DISCUSSION

Degradation of Acetaminophen proposed scheme for preparing the degradation product:



Acetaminophen and its degradation product have more or less max. High end response at same UV spectrum. Since both the parental molecule & Impurity structure represents the benzene ring with same functional group at its Ortho- position. Acetaminophen and its impurities are stable for 24 hrs. This has been confirmed by HPLC analytical methodology. The retention of acetaminophen and its impurities strongly depends on the ionization of the functional groups. The degree of ionization is determined by the pH of the mobile phase & solvent used in sample preparation i.e. diluent. The retention of an ionizableanalyte depends on degree of ionization, in turn the degree of ionization of analyte depends on the pH of the solution and pKa of ionization stages of analyte. The ionized always has low retention in Reverse phase chromatography. Therefore, the retention lowest under acidic condition for a basic analyte and under basic conditions for an acidic analyte. High retention is observed when the analyte is in its neutral form i.e. under acidic conditions for acidic analytes and basic conditions for basic analytes, the retention of analyte depend strongly on the pH of the mobile phase. In order to maintain the reproducible pH values, use of buffers in all pH ranges expect at very acidic or strongly alkaline pH values have been chosen. For this reason new HPLC method is developed with gradient elution programmer, instead of double salts mixed with organic solvents. Acetaminophen is the compound of benzene ring with hydroxyl group in ortho position and other groups of Keto & amide are in Para-position. In the connectivity of its electronegative nature hydroxyl group will donate the electron in the form of lone pair present on oxygen atom. Apart from this nitrogen atom consisting of lone pair electrons will readily donate to the double bond oxygen atom. Ethanamide is an extensively conjugated system, as the lone pair on the hydroxyl oxygen, the benzene π- cloud, the nitrogen lone pair and the P-orbital on the carboxyl group are all conjugated. The presence of two active groups also makes the benzene highly reactive towards electrophilic aromatic substitution. Impurity-k is delocalized with the presence of hydroxyl group at its Ortho position and Para position is electron releasing group made easier to elute at earlier retention time when comparing with

any other impurity. Imp-B refers with propanamide side chain at the Para- position, as well as hydroxyl group at Ortho-position makes little bit steric hindrance and elution of this peak is followed by the parental molecule due to the increase in its carbon chain. Impurity–A is the moiety consisting of two delocalizing active groups present at the position of Ortho & Meta of the benzene ring. Analytical method gradient is stepped up its organic phase contains in pump-B by about 5% at the run time of the fifteenth minute. As the two high electron donating groups placed at side by side of π- cloud benzene molecule, makes elution faster due to the organic content.

For Imp-C all three positions of benzene ring i., e Ortho, Para & Meta are with high electronegative nature which possess the delocalized character and elution made in the order followed by Imp-A due to its steric hindrance.

With respect to the Imp-F, Imp-E, Imp-D, Imp-G & Imp-H possess more number of resonating structures. As per the rule "If number of resonating structures increases stability of molecule also increases". Therefore elution pattern follows with greater retention time.

Impurity-I consists of two oxygen atoms at each side at each side of adjacent carbon atom of the benzene ring leads in the formation of phenoxide with the π-clouded benzene ring of P-orbital. So the elution's of impurity evolutes at high end of gradient time programme.

Impurity –J consists of functional groups with Chloro&ethanamide at Ortho & Para position which in turn stability shows due to the exhibition of N-number of resonating structures. Therefore elution of this impurity makes at the last high end of the gradient programme of analytical method.

Initially experimental procedures were made using a mixed 375 volumes of a 17.9gm/L solution of Disodium Hydrogen Phosphate, 375 volumes of a 17.8gm/L solution of sodium Dihydrogen Phosphate and 250 volumes of methanol containing 4.6gm/L of a 400gm/L of solution Tetrabutyl Ammonium Hydroxide. First graphs were chromatographed separately by spiking of all eleven impurities in the test sample. The eluted peak of Impurity-J is not identified and found to be merging with Impurity-I. The peaks of Impurity-C & Impurity-A are found to be merging with each other at the retention times of 7.52 & 7.69. Impurity-G, Impurity-F and Impurity-D are not well separated from each other at the retention times of 11.97, 12.41 & 13.90 respectively as represented in the chromatogram of Figure-I

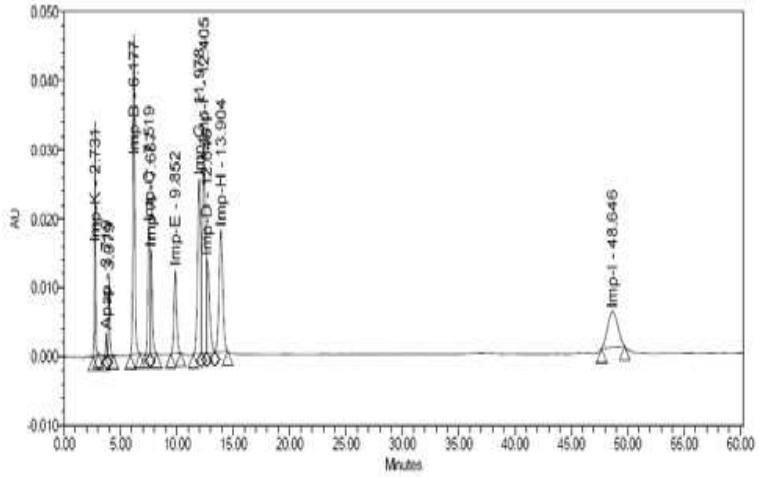


Fig.I: A typical Chromatogram representing the elution pattern of Impurities with double salts

Further attempts were made with the mobile phase containing mixed salts and its organic solvents at higher ratio as 375:375:300and remaining procedure as per above initial trail. Only due to the organic phase solvent gives the identification of two separate impurities as A & C. Besides this it was found that Impurity-E, Impurity-D, Impurity-G are not well separated as represented in the chromatogram of figure-II.

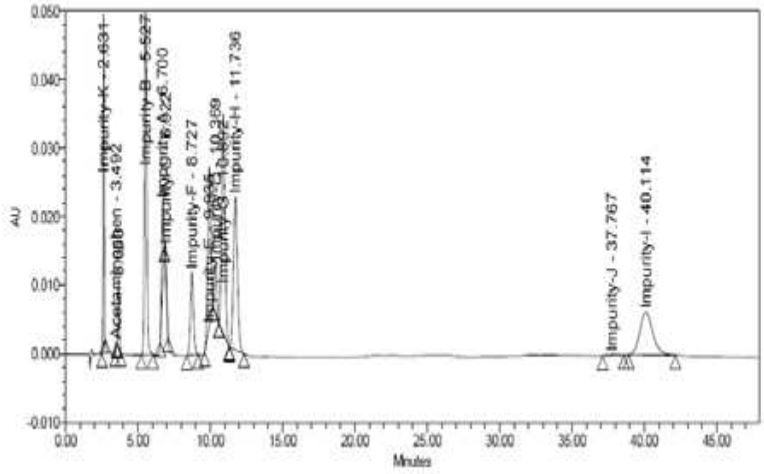


Fig.II: A typical Chromatogram representing the elution pattern of Impurities with double salts with increasing the Organic Solvent ratio.

Analytical methodology were designed by choosing the mobile phase containing the monobasic hydrogen phosphate buffer which plays a vital role in eluting the neutral and weakly alkaline compounds i.e. pH of the impurities and analyte will be eluted in the range 6.2 to 8.2

Two main properties of the monoacid that may affect the solvation of the basic analytes are as follows:

1. The hydrogen bonding ability of the acids.
2. The pKa of the acid and its ionization at a particular pH.

In case of phosphoric acid, the phosphate counter ion is known to exhibit strong hydrogen bonding properties. It may acts as a hydrogen donor through its hydrogen atoms and as an acceptor through phosphone group. Therefore it is highly solvated counter ion. The lowest and highest pKa of phosphoric acid are 2.1 & 12.3 respectively and therefore at a pH of 2.1 it is only 50% ionized. The negative charge is dispersed over two oxygen atoms. Further decreasing the pH reduces the ionization of the phosphoric acid. Here with respect to acetaminophen and its impurities almost exhibits the similar ionization constant.

A Di potassium hydrogen phosphate buffer at a pH of 7.0 with diluted ortho-phosphoric acid for mobile phase-A and Mobile Phase-B was chosen with 800 mL of methanol and 200 mL of water with gradient elution programme and diluent as carbonated free water by keeping column temperature 30°C at the flow rate of 1.0 mL/min. Good separation obtained between Impurities and principle analyte in the chromatogram peak shapes are exhibiting front tailing as represented in Fig.3

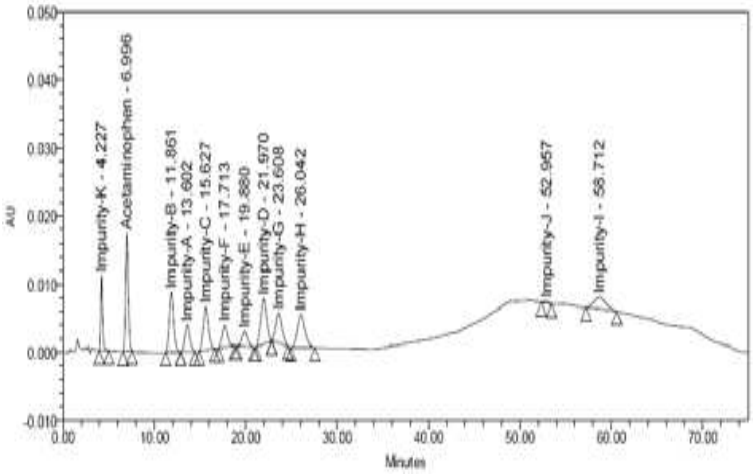
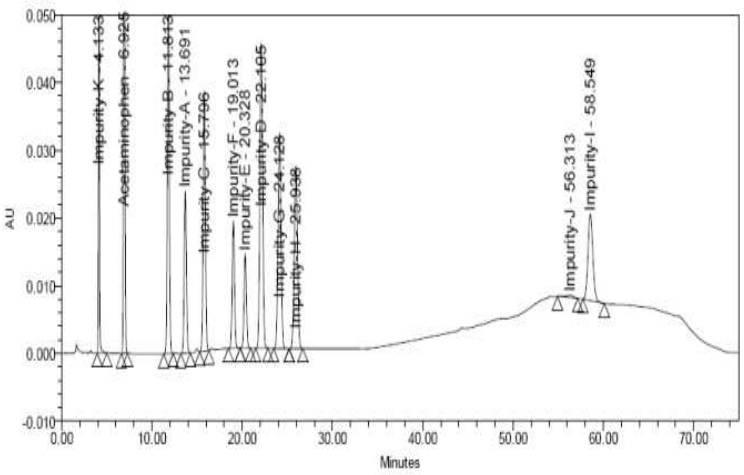


Fig.III: A Typical chromatogram representing the elution pattern of all impurities with mobile phase as monobasic buffer imparting the diluent as Water.

Therefore further attempts were made without changing gradient time programme for 75 minutes using Mobile phase-B containing 800mL methanol and 200 mL of Dipotassium hydrogen phosphate buffer at a pH of 7.0 with diluted ortho phosphoric acid by altering the diluent with mixture of methanol & water in the ratio of 70::30 respectively. Reference chromatograms with all spiked Impurities have been presented in Fig.IV.

Fig.IV a typical chromatogram representing the elution pattern of all impurities with mobile phase as monobasic buffer imparting the diluent as Methanol::Water.

Method validation:



Analytical method which is developed as described in text was validated by the parameter of specificity, linearity, Accuracy & Relative retention factor and Limits of Detection and Limit of Quantification.

Specificity:

Specificity /Selectivity are the parameter by which we can know the interference of other impurities with respect to the main principle analyte. Interference study can be plotted by its peak purity.

Condition of peak purity through Water's system is **"Peak angle is less than the purity threshold"**

Retention Times, Purity Angle & Purity Threshold for Principle peaks and Known Impurity peaks in Test Spiked solution is illustrated in Table.1

Table.1:

Component	Retention Time	Purity Angle	Purity Threshold
Acetaminophen	6.86	11.25	19.00
Impurity A [N-(2-hydroxyphenyl) acetamide	13.76	0.18	0.33
Impurity B [N-(4-hydroxyphenyl) propanamide]	11.95	0.15	0.29
Impurity C [N-(3-chloro-4-hydroxyphenyl) acetamide]	15.65	0.15	0.29
Impurity D [N-phenylacetamide]	22.33	0.21	0.31
Impurity E [1-(4-hydroxyphenyl) ethanone]	19.85	0.17	0.27
Impurity F [4-nitrophenol]	17.19	0.26	0.29
Impurity G [1-(4-hydroxyphenyl) ethanoneoxime]	24.44	0.59	15.12
Impurity H [4-(acetyl amino) phenyl acetate]	26.39	0.51	15.22
Impurity I [1-(2-hydroxyphenyl) ethanone	59.31	16.93	25.65
Impurity J [N-(4-chlorophenyl)acetamide (chloroacetanilide)]	56.97	0.41	15.38
Impurity K [4-aminophenol]	4.10	0.15	0.25

From the above the table it is evident peak purity of each individual impurities are under the Acceptance criteria.

Linearity: Linearity has been plotted by dilution of standard stock solutions. The slope and Y-Intercept were provided as equation together with the correlation coefficient in order to demonstrate the linearity of the developed method.

Linearity was established for working solutions containing concentrations between 2.00µg/mL to 16.00µg/mL for Acetaminophen, Imp-A, Imp-B, Imp-C, Imp-D, Imp- E, Imp-F, Imp-G, Imp-H, Imp-I, Imp-K and concentration of Imp-J at 0.05µg /mL to 0.35µg/mL. The equations obtained are Y=95164 X -19965 (R^2=0.9992) for APAP, Y=41393X+564.6 (R^2 = 0.999) for Imp Y=75990X +17581 (R^2=0.9985) for Imp–BY=68529X+4963.8 (R^2=0.9998) for Imp C, Y=94180X 17041 (R^2=0.9989) for Imp-D, Y=25771X+18085 (R^2=0.9999) for Imp-E, Y=33674X+1392.3 (R^2=0.9996) for Imp-F, Y=730366X+12858 (R^2=0.9972) for Imp G, Y=64927X+ 22552 (R^2=0.9993) for Imp-H, Y=51324X +8342.6 (R^2=0.9977) for Imp-I, Y=101335X-102.45 (R^2=0.9995) for Imp-J, Y=36543+10367 (R^2=0.9996) for Imp-K,

Limits of detection and Quantification:

Limit of Detection and Limit of Quantification for Acetaminophen and its Impurities were calculated by Slope–Intercept form method. The obtained values are illustrated in Table.2.

Name of the known impurity	LOD in ppm	LOQ in ppm
Acetaminophen	0.4687	1.4204
Impurity A [N-(2-hydroxyphenyl) acetamide]	0.1086	0.3290
Impurity B [N-(4-hydroxyphenyl) propanamide]	0.0632	0.1914
Impurity C [N-(3-chloro-4-hydroxyphenyl) acetamide]	0.0428	0.1297
Impurity D [N-phenylacetamide]	0.0641	0.1942
Impurity E [1-(4-hydroxyphenyl) ethanone]	0.2018	0.6116
Impurity F [4-nitrophenol]	0.2390	0.7241
Impurity G [1-(4-hydroxyphenyl) ethanoneoxime]	0.1529	0.4634
Impurity H [4-(acetyl amino) phenyl acetate]	0.1857	0.5627
Impurity I [1-(2-hydroxyphenyl) ethanone	0.2116	0.6411
Impurity J [N-(4-chlorophenyl)acetamide (chloroacetanilide)]	0.0024	0.0074
Impurity K [4-aminophenol]	0.2982	0.9038

Table.2: Accuracy & Relative Retention Factor

Name of the known impurity	%Purity	Relative Retention Time	Relative Response Factor	% Recovery Calculated using RRF
Impurity A [N-(2-hydroxyphenyl) acetamide]	99.8	2.01	0.47	104.1
Impurity B [N-(4-hydroxyphenyl) propanamide]	99.9	1.74	0.92	94.7
Impurity C [N-(3-chloro-4-hydroxyphenyl) acetamide]	99.6	2.28	0.82	94.6
Impurity D [N-phenylacetamide]	100.0	3.25	1.12	92.5
Impurity E [1-(4-hydroxyphenyl) ethanone]	100.0	2.89	0.32	97.3
Impurity F [4-nitrophenol]	99.5	2.51	0.38	109.9
Impurity G [1-(4-hydroxyphenyl) ethanoneoxime]	99.6	3.56	0.87	105.1
Impurity H [4-(acetyl amino) phenyl acetate]	99.7	3.85	0.88	101.7
Impurity I [1-(2-hydroxyphenyl) ethanone	99.9	8.64	0.61	106.4
Impurity J [N-(4-chlorophenyl)acetamide (chloroacetanilide)]	99.5	8.30	1.17	106.4
Impurity K [4-aminophenol]	98.2	0.60	0.42	108.8

Accuracy was determined by calculating the recovery of each analyte with the estimating of its each Relative Retention Factor. The obtained values for the recoveries were in the range 90.0% to 110.0%. The results are presented as perTable.3.

Kinetics of the Degradation:

A linear relation- ship was obtained by plotting the log concentrations of the remaining Acetaminophen against time (Fig. v). Since the hydrolysis was performed in a large excess of Hydrochloride acid (1.0 N), it follows a pseudo-first order reaction rate which is the term used when two reactants are involved in the reaction but one of them is in such a large excess (HcL) that any change in its concentration is negligible compared with the change in concentration of the other reactant (Drug).

Different parameters that affect the rate of the reaction were studied. The effect of temperature was studied by conducting the reaction at different temperatures using different concentrations of the acidic solution (Figs. VI, VII and VIII). At each temperature the rate constant and $t_{1/2}$were calculated and then the log of the rate constant was plotted

against the reciprocal of the temperature in Kelvin units (Arrhenius plot (Fig. IX)) to demonstrate the effect of temperature on the rate constant. It was concluded that as the temperature increased the rate of hydrolysis increased with a decrease in the $t_{1/2}$(Table 4). Also, the energy of activation was determined by calculating the rate constant from the following equation.

$$\log \frac{K_2}{K_1} = \frac{E_a}{2.303R}\left(\frac{T_2 - T_1}{T_1 T_2}\right)$$

Where **"E_a"** is the activation energy, **" T"** and**"T_2"**are the two temperatures degrees in Kelvin, **"R"** is the gas constant, and **" K "** and **"K_2"**are the rate constants at the two temperatures used.

The calculated **"E_a"** was found to be 88.74 kilo joule mol^{-1}which was a comparatively low value for amine substituted benzene rings, suggesting the instability of acetaminophen in acidic medium.

Another factor that affects the rate of the reaction is the acidic strength of Hydrochloric acid, thus different normality's of HcL solutions were used to study the hydrolysis reaction. The rate of hydrolysis increased with an increasing HcL concentration, although the effect was minor compared to the effect temperature (Figs. VI, VII and VIII) and (Table 4).

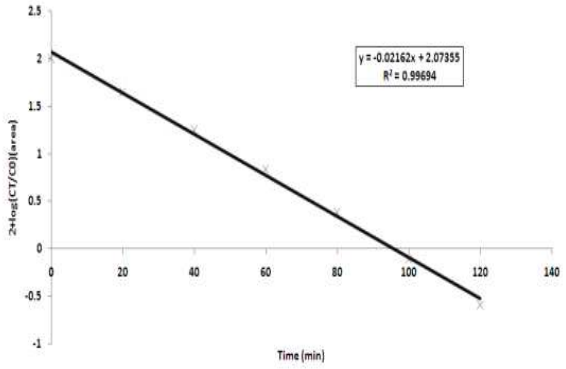


Fig.V: First Order Plot of the Hydrolysis of Acetaminophen with 1.0 N HCl at 90°C

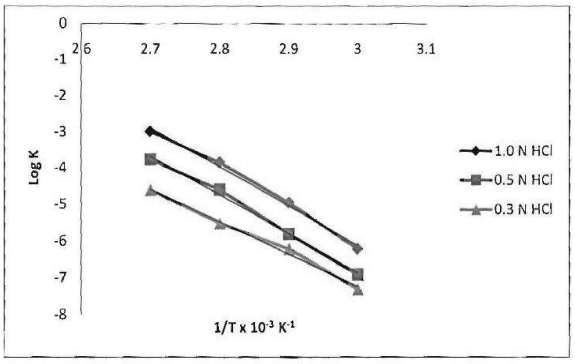


Fig.VI: First Order Plot of the Hydrolysis of Acetaminophen with 0.3 N HCl at Different Temperatures

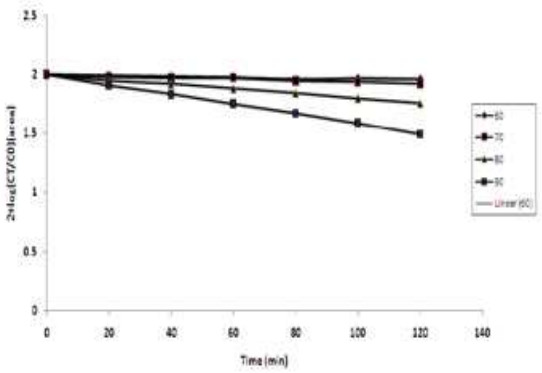


Fig.VII: First Order Plot of the Hydrolysis of Acetaminophen with 0.5 N HCl at Different Temperatures

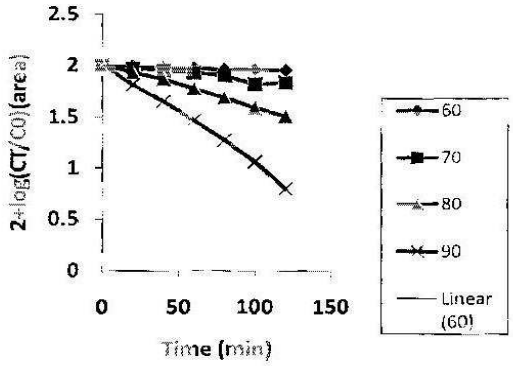


Fig.VIII: First Order Plot of the Hydrolysis of Acetaminophen with 1.0 N HCl at Different Temperatures

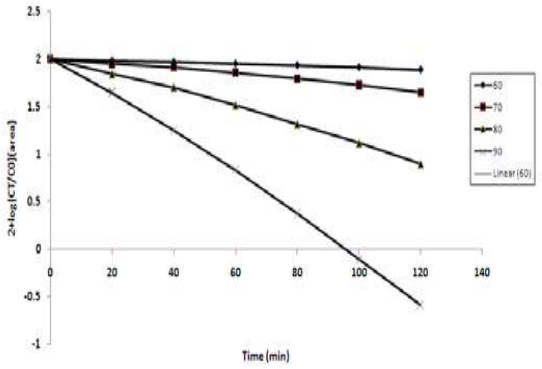


Fig.IX: Arrhenius Plot for the Hydrolysis of Acetaminophen with 0.3, 0.5 and 1.0 N HcL

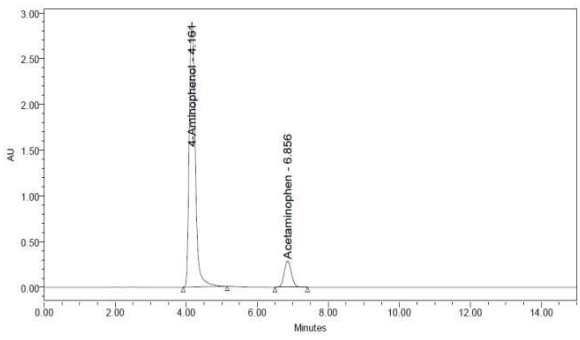


Fig.x: A typical Chromtogram representing the degradation study for 0.3 N at 90° for 120 minutes.

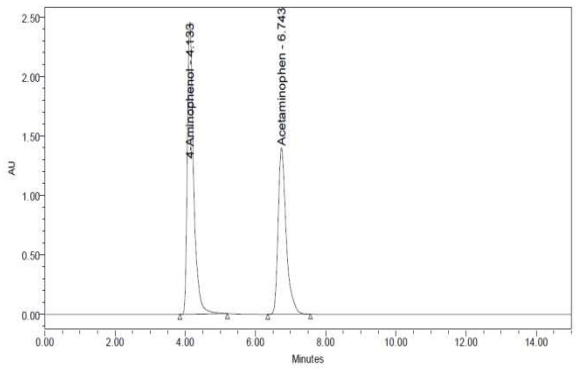


Fig.XI: A typical Chromtogram representing the degradation study for 0.5 N HcL at 90° for 120 minutes.

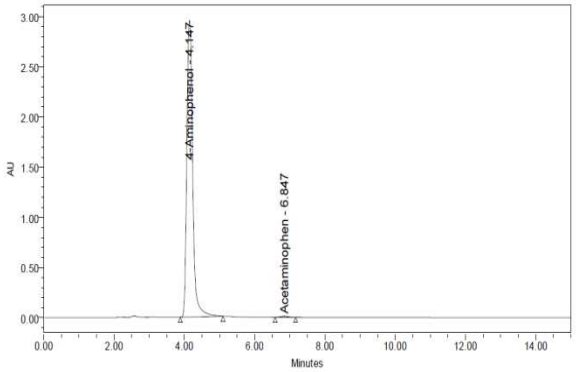


Fig.XII: A typical Chromtogram representing the degradation study for 1.0 N HcL at 90° for 120 minutes.

In conclusion the acidic hydrolysis of Acetaminophen was found to follow a pseudo first order reaction rate. Also the reaction rate increases with increase in the temperature and the strength of the acidic solution.

Table.4: Kinetic Data of AcetaminophenHydrolysis

Normality of HcL	Temperature	K in min^{-1}	$t_{½}$ in min
1.0 N HcL	90°C	0.050	14.0
	80°C	0.021	32.8
	70°C	0.007	103.0
	60°C	0.002	315.0
0.5 N HcL	90°C	0.023	29.74
	80°C	0.010	71.4
	70°C	0.003	198.0
	60°C	0.001	533.0
0.3 N HcL	90°C	0.010	71.4
	80°C	0.004	147.4
	70°C	0.002	462.0
	60°C	0.001	999.0

CONCLUSION

A new HPLC method for the determination of acetaminophen and its impurities [N-(2-hydroxyphenyl) acetamide], [N-(4-hydroxyphenyl) propanamide], [N-(3- chloro-4-hydroxyphenyl) acetamide], [N-phenylacetamide], [1-(4-hydroxyphenyl) ethanone], [4-nitrophenol], [1-(4- hydroxyphenyl) ethanoneoxime], [4-(acetyl amino) phenyl acetate], [1-(2-hydroxyphenyl) ethanone, [N-(4-chlorophenyl)acetamide (chloroacetanilide)], [4-aminophenol] was developed by the gradient elution pattern with Mobile phase-A and Mobile phase-B. Both elution and flow rate gradients contribute to the total time of analysis for 75 minutes. The selectivity and the efficiency of the separation are very good. A kinetic investigation of the acidic hydrolysis of acetaminophen was carried out in Hydrochloric acid solutions of 0.3N, 0.5N and 1.0N by monitoring the parent compound itself. The reaction order of acetaminophen followed pseudo-first order reaction kinetics. The activation energy could be estimated from the Arrhenius plot and it was found to be 21.2kcal/mol.

ACKNOWLEDGMENTS

Authors thank Cheminnova remedies pvt ltd Hyderabad for supporting with impurities and instruments to complete this journal publication.

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