DEVELOPMENT OF REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD AND METHOD VALIDATION OF PARACETAMOL BY USING ECONOMICAL SINGLE MOBILE PHASE

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The computerization of method development and validation are useful in analysis of pharmaceuticals in pharmaceutical industry. In this article a simple, sensitive, and precise high performance liquid chromatographic (HPLC) method for the analysis of Paracetamol with ultraviolet detection at 257 nm has been developed, validated, and used for the determination of compounds in commercial pharmaceutical products. Paracetamol tablet dosage form (two brands) was purchased from market and was from Glaxo Smith Kline (Calpol) and from IPCA (Pacimol) respectively. The compounds were well separated on a Hypersil ODS C18 reversed-phase column by use of a mobile phase consisting of methanol and water (90:10v/v at a flow rate of 1.0 ml.min). The linearity ranges were 20- 100µg/ ml for Paracetamol. Limits of detection (LOD) obtained 3.298µg/ml limit of quantitation (LOQ) were 9.875µg/ml Paracetamol. The study showed that reversed-phase liquid chromatography is sensitive and selective for the determination of Paracetamol using single mobile phase.

Key words: RP-HPLC, ODS column, Paracetamol, Tablet

INTRODUCTION

Methods validation is the process of demonstrating the analytical procedures that are suitable for their intended use. The methods validation process for analytical procedures begins with the planned and systematic collection of the validation data to support the analytical procedures. The review chemist evaluates the analytical procedures and validation data. [1] Validation can be defined as a procedure that demonstrates a process under standard conditions capable of consistently producing a product that meets the established product specification. In pharmaceutical industry validation studies are an essential part of GMP as well as GLP and should be conducted in accordance with predefined protocols. [2] A written report summarizing recorded results and conclusions should be prepared and stored. There are guidelines given by ICH, USFDA and European Union (European Medical Evaluation Agency) for adhering the principles started for GMP and GLP requirements. [3, 4]

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Paracetamol is a pioneer antipyretic drug. Chemically it is *N*-(4-hydroxyphenyl) acetamide. The molecular formula of Paracetamol is $C_8H_9NO_2$. It is an acyclic drug, freely soluble in water, chloroform and ethanol. It is a white powder. The main mechanism of action of paracetamol is considered to be the inhibition of cyclooxygenase (COX), and recent findings suggest that it is highly selective for COX-2. While it has analgesic and antipyretic properties comparable to those of aspirin or other NSAIDs, its peripheral anti-inflammatory activity is usually limited by several factors, one of which is high level of peroxides present in inflammatory lesions.[5]

According to the literature survey, methods were developed using buffer solutions and biological fluids. Using of buffer solutions and biological fluids leads to various difficulties as, i) Only small quantity of drug or metabolite is usually present in large volume of blood, urine or tissues. ii) Endogenous pigments leads to analytical error, unless great care is taken in the analytical conditions. iii) Protein binding of the drugs may occur which may lead to poor recoveries. iv) Buffer solutions that are used should be freshly prepared.[6,7] v) Quantification of drug plasma levels

from patients is a quite complicated operation. At the patient individual level, these assays require a large volume of plasma sample. vi) TDM (Therapeutic Drug Monitoring) –Prospective clinical trials assessing the clinical usefulness of this strategy have shown contradictory results, pointing out the need to consider different issues when performing TDM. Whereas in case of methanol-water there is no need to prepare it freshly since % recovery is highly satisfactory. [8] The main objective in this research work is to develop a simple, rapid, reliable and an economical single method for Paracetamol by RP-HPLC in individual drugs and decrease the time and cost of the assays.

MATERIAL AND METHODS

HPLC instrument of LC-10AT VP series (Shimadzu Corporation, Japan) consisting of a UV-Visible detector, manual injector with 20µl loop and Hypersil ODS C18 column (250 mm X 4.6 mm i.d., 5µm particle sizes) was used. All the standards and chemicals used were of HPLC-grade. Methanol was obtained from S. d. Fine Chemicals Ltd. (Mumbai, India) and triple distilled water was received from Universal Labortories Pvt. Ltd. (Mumbai, India). Paracetamol tablets (Calpol, Glaxo Smith Kline. and Pacimol, IPCA) were purchased from market.

Standard Solution Preparation (Stock and working standard solution):

Stock and working standard solution for Paracetamol stock solution was prepared by weighing Paracetamol (100 mg) in a 100-ml volumetric flask, dissolving in 70 ml methanol and diluting to volume with the methanol, 10 ml was further diluted to 100 ml with mobile phase to obtain working standard solutions with Paracetamol. Then it was further diluting to 20µg/ml to 100µg/ml paracetamol solutions by using mobile phase as a solvent. [9]

Preparation of the sample solutions (Stock and working standard solution):

Twenty tablets (CALPOL and PACIMOL) were weighed accurately and finely powdered in a mortar and pastel. From the powder equivalent weight of 100 mg was taken in 100 ml volumetric flask. This was dissolved in 70 ml water and sonicated for 30 min with internal shaking. Then the volume was finally made to 100ml with methanol to get a clear solution of 1mg/ml. The above solution was centrifuged at 3000 rpm for 5 min. 10 ml was further diluted to 100 ml with mobile phase to obtain working standard solutions with Paracetamol. Further it was diluted to 20µg/ml to 100µg/ml solutions by using mobile phase as solvent[10]

Chromatographic condition

Chromatography was performed on Hypersil C18 reversed phase column (250 mm x 4.6 mm i.d., 5µl) base deactivated silyl bonded amorphous silica. The mobile phase was methanol and water, 90:10 (v/v). Flow-rate was maintained at 1ml/min. The column was kept at 25.0 ± 0.1^0C during the analysis. The detection wavelength was set at 257 nm and the injection volume at 20µl.

RESULTS

Method Validation

To optimize the HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and peak symmetry for Paracetamol was obtained with mobile phase consisting of methanol and water (90:10v/v) so as to obtain a better reproducibility and repeatability. Quantification was achieved with UV detection at 257nm based on the peak area. Better resolution of the peaks with clear base line separation was found (Fig. 1). Limits of detection (LOD) obtained a value of 3.298 µg/ml and the limit of quantitation (LOQ) obtained a value of 9.875µg/ml paracetamol. The study showed that reversed-phase liquid chromatography is sensitive and selective for the determination of Paracetamol using single mobile phase. [11]

Specificity (Selectivity)

The selectivity of the RP-HPLC method was checked by comparison of chromatograms obtained from samples and the corresponding placebo. Additives in tablets are practically insoluble in methanol or the mobile phase whereas the active constituents are freely

soluble. Hence, no interference from additives of the tablets was obtained.

Linearity

The linearity of the method was determined by analysis of standard plots associated with six point standard calibration curve. The concentration was calculated from the simple linear equation using regression analysis of calibration standard with the reciprocal of the drug concentration as a weighing factor. The peak area ratio values of calibration standards were proportional to the drugs. The plot of peak area of each sample against respective concentration of Paracetamol was found to be linear in the range of 20-100µg/ml. Using regression analysis the linear equation was obtained Y= 43.45x - 3.101 with correlation coefficient of R^2= 0.9999. Linear regression least square fit data obtained from the measurements are given in table. [12] The linearity of the method was determined by analysis of standard plots associated with a six -point standard calibration curve.

Accuracy

The recovery experiments were carried out by the standard addition method. The percentage of the recoveries obtained were 99.713% and 99.72% respectively for Paracetamol (Brand1 and Brand 2) and the results are shown in the table below. The recovery of the method was highly satisfactory.

Fig I: Peak area and peak height Paracetamol 40µg

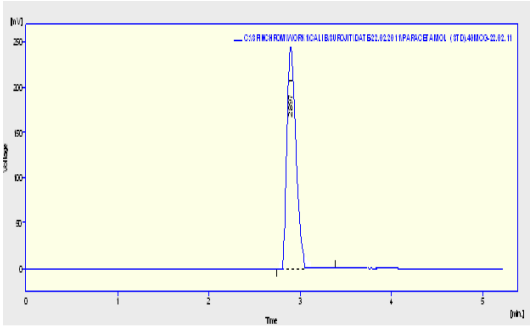

Table I: Condition applied

Concentration	Regression Equation	R^2
20-80	Y=43.44X +12.44	0.999
20-80	Y=43.32X-6.190	0.999

Fig II: Standard calibration curve of Paracetamol

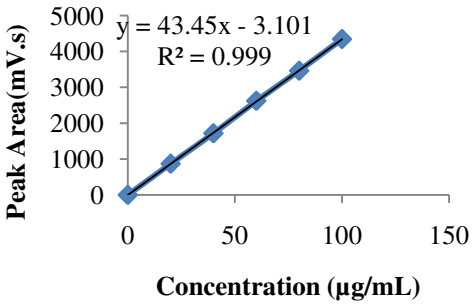

Table II: Paracetamol regression statistics

Column Mode	: C18 RP-HPLC
Detector	: UV – Visible
Type of Analysis	: Peak area and peak height
Detection Limit	: 257 nm
Flow Rate	: 1.0 ml/min
Run Time	: 10 min
Injection volume	: 20 µl

Table III: Recovery studies

Labeled claim 75mg	Amount added (mg)	% Recovery
Brand 1 (Calpol, Glaxo Smith Kline)	10	99.67
	20	100.04
	30	99.43
Brand 2 (Pacimol, IPCA)	10	99.85
	20	99.23
	30	100.08

*Mean determination of five is 99.713 & 99.72

Precision

System precision

The precision of an analytical method is the degree of agreement among the individual test results when the method is applied repeatedly to multiple sampling of a homogenous sample. The precision was calculated as the percentage coefficient of variation (% CV) or Relative standard deviation (% RSD). Repeatability refers to the use of the analytical procedure within a laboratory over a short period of time (Intra-day) using the same analytes with the same equipment. Repeatability was studied by injecting 3 concentrations

of standard drug three times during the same day. The method passed test for repeatability, as the % RSD ≤2%.

Table IV: Intraday precision of Paracetamol by the proposed RP- HPLC method

Concentration Paracetamol (µg/ml)	Mean area (n= 3)	Standard deviation	%RSD (n= 3)
20	865.004	12.265	1.417
40	1799.36	24.457	1.359
80	3467.13	47.031	1.356

Intermediate precision

Involve in the estimation of variations in analysis when method is used within a laboratory, as on different days (Inter-day). Intermediate precision was studied by injecting 3 concentration of standard in triplicates on 3 different days. The method was passed the test for intermediate precision, as the % RSD ≤1.5%.

Ruggedness

A different analyst, using a different batch of chemicals, tried the method graph. There was not much variation in results. These studies showed that the method is rugged.

Table V: Inter- Day precision of Paracetamol by the proposed RP- HPLC Method

Concentration Paracetamol (µg/ml)	Mean area (n= 9)	Standard deviation	%RSD (n= 9)
20	864.371	11.093	1.283
40	1708.29	21.991	1.287
80	3467.99	43.302	1.248

Robustness

Robustness of the method was tested by small but deliberate variations of flow rate mobile phase and temperature. Effects of variation in the flow rate (±1 ml/min) were studied at three different concentrations and temperatures (±1˚C to ±5˚C). Effect of variation in the different mobile phase ratio was also studied at three different concentrations. The results are given in the following table.

Fig III: Interday graph of Paracetamol

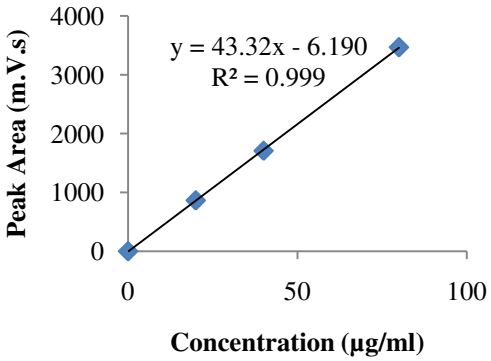

Fig IV: Intraday- graph of Paracetamol

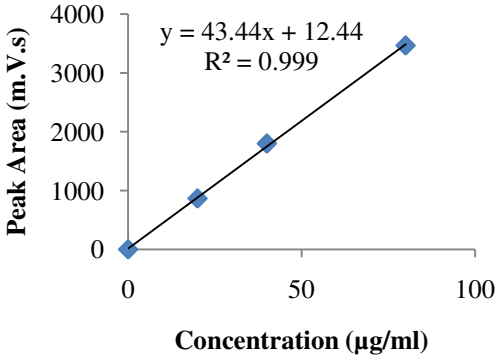

Fig V: Analyst-1 Paracetamol

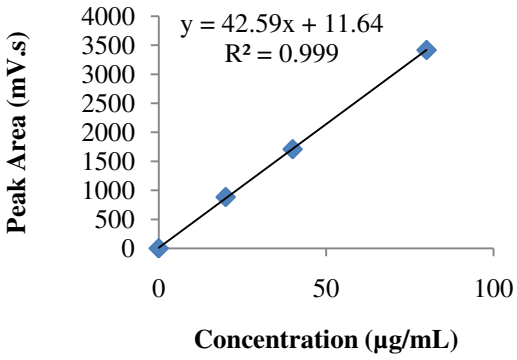

Fig. VI: Analyst -2 Paracetamol

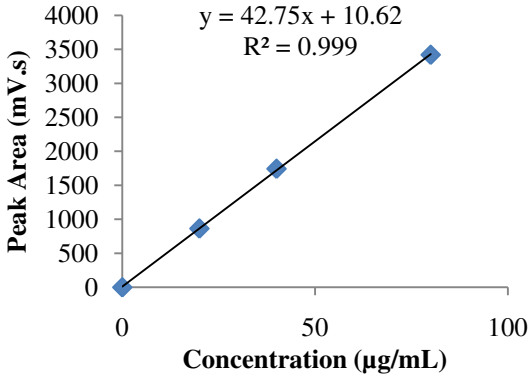

Table V: Analyst-1 for Paracetamol

Concentration	Peak area (mV.s)*	SD	%RSD*
20	883.659	10.006	1.132
40	1708.514	19.512	1.142
80	3417.634	29.182	0.853

*Average of three values

System suitability test

The percentage of relative standard deviation (% RSD) for Paracetamol was found to be 1.144, using this method. All the results were within the acceptable range. System suitability tests are an integral part of chromatographic methods and are used to verify that the resolution and reproducibility of the system are adequate for analysis to be performed. All the values for system suitability parameters were within acceptable range. [13]

Table VI: Analyst-2 for Paracetamol

Concentration	Peak area (mV.s)*	SD	%RSD*
20	864.563	9.895	1.144
40	1743.614	17.574	1.007
80	3419.619	32.938	0.963

*Average of three values

Table II describes Paracetamol regression statistics for intraday and interday. The linearity of the method was observed in the concentration range of 50-200µg/ml, with a regression coefficient of 0.9999, demonstrating its suitability for analysis.

Table VII: Effect of variations in the flow rate of (1.0 ml/min) for Paracetamol

Concentration	Retention Time*	Peak area (mV.s)*	SD	%RSD
20	2.897	864.465	10.01	1.158
40	2.898	1729.17	17.98	1.039
80	2.900	3457.60	24.87	0.719

*Average of three values

Table VIII: System suitability test for Paracetamol Regression analysis

Parameters	Values
Retention Time	2.898
HETP(mm)	0.009
Tailing factor	1.011
Theoretical plates/m	110103.84
LOD(µg/ml)	3.298
LOQ(µg/ml)	9.875

DISCUSSION

Apropos of the above, liquid chromatographic method was described and the results were given. According to the result, limits of detection (LOD) was obtained 3.298µg/ml, limit of quantitation (LOQ) was 9.875µg/ml and % recovery was 99.713% and 99.72% for CALPOL and PACIMOL respectively, with a regression coefficient of 0.9999 (Intraday &Interday). So the method was proven to be validated. From the standard calibration curve the regression coefficient (r^2) value was found 0.999 by using the concentration of 20-100µg/ml (Y=43.44X +12.44, Y=43.32X-6.190).

CONCLUSION

Developed high performance liquid chromatographic method is simple, reliable, reproducible and economical for the analysis of paracetamol in pharmaceutical formulations. The reported method can be used successfully for the effective qualitative and

quantitative analysis of Paracetamol in tablets or other pharmaceutical formulations. Limits of detection (LOD) obtained was 3.298µg/ml, limit of quantitation (LOQ) was 9.875µg/ml.

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