EXPERIMENTAL-METHOD

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Quantitative Estimation and Method Validation of Acetaminophen by RP-HPLC in Biological samples.

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ABSTRACT

Suicidal and accidental poisoning with drug overdose is one of the common causes of morbidity and mortality in India. Considering such fact, quantification of drug becomes imperative to distinguish narrow margin of therapeutic dose and toxic dose. Acetaminophen (Paracetamol) is extensively used as antipyretic and analgesic drug. Generally it is used in headaches, slight pains and fever. Overdose of Paracetamol causes hepatotoxicity. It can be fatal in large dose (>10g).It can potentiate the harmful effect of other drugs if taken in combination. A quick, simple and precise method is developed for quantitative estimation of acetaminophen using RP-HPLC in biological samples viz. Blood, Vitreous Humor and CSF. The separation was done using RPC-18 HPLC column and Methanol: water as mobile phase. On the basis of ICH guidelines validation parameters such as Linearity, Accuracy, Precision, Recovery, Limit of Quantification and Limit of Detection were done.

INTRODUCTION

Suicidal and accidental poisoning with drug overdose is one of the common causes of morbidity and mortality in India[1] as well as globally[2]. Increased incidences of deaths due to suicidal poisoning including drug overdose were found in teenagers' also[3]. Acetaminophen (Paracetamol) is extensively used as an antipyretic and non–opioid analgesic agent which acts centrally and peripherally[4]. It belongs to the class of "aniline analgesic" drugs and is available easily as over the counter (OTC) drug[5,6]. Chemically paracetamol is 4-hydroxy acetanilide which is a derivative of aniline . It is chiefly used for treatment of mild pain, fever, headache and in cases of severe pains[7]. Maximum therapeutic dose of paracetamol is 4 gm/day[8], overdose causes hepato-toxicity[9] and may lead to fatality in doses of >10gm[10]. The overall mechanism of action of paracetamol is inhibition of cyclooxygenase[11]. It can also potentiate the harmful effects of other drugs and may contribute to fatality. Considering this, quantification of it becomes imperative to distinguish between therapeutic dose and toxic dose. An attempt has been made to develop a quick, simple and precise method for quantitative estimation of acetaminophen using Reverse Phase High Performance Liquid Chromatography (RP-HPLC) from biological materials viz. Blood, Cerebrospinal fluid & Vitreous humor.

Table 1: Showing Precision study of paracetamol using concentration of 1µg/ml

Test (at different time intervals)	Intra-day Precision		Inter-day Precision	
	Mean ±SD	RSD	Mean± SD	RSD
Test 1	1.003±0.011	1.11%	1.028±0.0135	1.31%
Test 2	1.002±0.017.	1.76%	1.003±0.011	1.11%
Test 3	1.031±0.021	2.09%	1.019±0.021	2.08%

Table 2: Showing accuracy study of paracetamol using concentration of 1µg/ml

Test (at different time intervals)	Intra-day Accuracy		Inter-day Accuracy	
	Mean ±SD	Accuracy	Mean ±SD	Accuracy
Test 1	1.003±0.011	0.3	1.028±0.0135	2.83
Test 2	1.002±0.017	0.2	1.003±0.011	0.3
Test 3	1.031±0.021	3.1	1.019±0.021	1.99

MATERIALS & METHODS

Preparation of stock solution: 1000µg/ml solution was prepared using Paracetamol standard in mobile phase as stock solution. Stock solution was filtered and then sonicated for 3minutes. Serial dilution of concentration 1µg/ml,5µg/ml,10µg/ml,20µg/ml, 30µg/ml, 50µg/ml & 100µg/ml were prepared from the stock solution with mobile phase. A. Preparation of Sample: Biological matrix viz. blood, cerebrospinal fluid and vitreous humor were spiked with various concentration of standard paracetamol and kept for overnight in incubator. Then these matrices were subjected to liquid –liquid extraction[12] using di-ethyl ether after necessary pre-sample preparation. After that organic layer was collected, air-dried and concentrated. Concentrated samples were again reconstituted using mobile phase before applied in HPLC analysis. B. Method Validation: Experimental conditions for analysis of paracetamol by RP-HPLC were validated on the basis of ICH guidelines.

a. Specificity: It is an ability to distinguish indisputable presence of eluent in matrix and in impurities. For establishing specificity of the analytical method, a blank biological matrix, a standard and spiked biological matrix (Blood, CSF & VH) were injected in the system and further analyzed.

b. Range and Linearity: For determining linearity, seven solutions of concentration 1µg/ml, 5µg/ml, 10µg/ml, 20µg/ml, 30µg/ml, 50µg/ml, and 100µg/ml were prepared by serial dilution from stock solution and analyzed by RP-HPLC. The calibration curve was obtained by plotting peak area versus known concentration. The linearity of the developed method was calculated in terms of coefficient correlation and intercept.

c. Precision: For any analytical method precision is a degree of closeness between the measured values of repeated samples of same concentration. In the proposed method, it was calculated by intra and inter-day response of five replicates of concentration 1µg/ml in terms of relative standard deviation.

d. Limit of detection(LOD) and Limit of quantification(LOQ): The LOD is the minimum concentration of the eluent which can be detected but not quantified by the proposed analytical method. The LOQ is the minimum concentration of eluent which can be quantified by the proposed method. The LOD and The LOQ were calculated by using signal to noise ratio of 3 and 10 in this stated method.

e. Accuracy and recovery: Accuracy in any analytical method is defined as degree of closeness between amount of concentration recovered and the true value. It is calculated as percentage recovery. In this stated method recovery was calculated by spiking the various biological samples viz. blood, CSF, Vitreous Humor with 10µg/ml concentration of paracetamol in triplicate. Other concentrations viz 20µg/ml and 50µg/ml were also spiked in various biological samples as described before.

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Fig. 1: Showing Rt 2.823 for paracetamol.

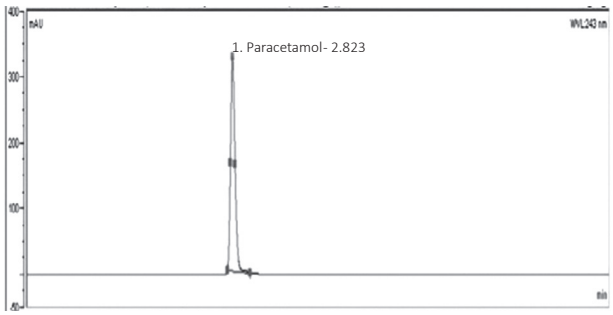
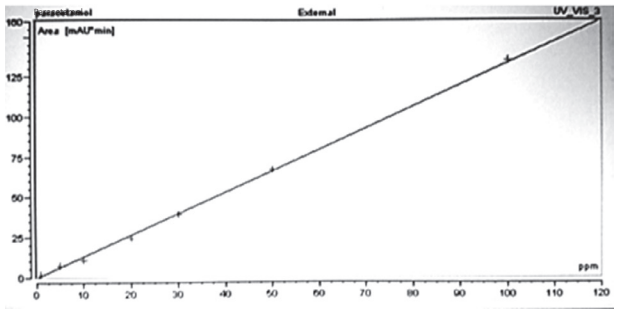


Fig. 2: Linearity of calibrartion curve using peak area against concentration of paracetamol.



EXPERIMENTAL CONDITIONS

A. CHEMICALS: Paracetamol standard 99.50% pure was procured from Sigma Aldrich. Sodium tungstate, Conc Sulphuric acid, Diethyl-ether were of analytical grade procured from Merck India. HPLC grade Methanol was procured from Merck India. Ultra-pure water of Rions India pvt Ltd.

B. CHROMATOGRAPHIC SYSTEM: The Reverse Phase High Performance chromatographic system (Dionex Ultimate 3000) was composed of mobile phase reservoirs, pump compartment with Vacuum degasser, column compartment with 250mm,5µ ,120Å shodex C-18 column. Manual injector of 20µl loop, Photo-Diode-Array detector and Chromeleon software- 6.80SR13.

C. CHROMATOGRAPHIC CONDITION: The eluent was analyzed using solvent system, Methanol and Ultra-pure water in ratio of 45:55(by volume) with flow rate of 1.2ml/min. 20µl Injection volume was used and the detection was done at wavelength 243nm. Total run time was 8 min. The analysis was achieved at ambient temperature (~25º C). All solvents were filtered using 0.22µm membrane filter, sonicated before use.

RESULTS

For analysis of paracetamol using RP-HPLC, Mobile phase was selected by trying various combinations of methanol and water. Best separation response of eluent was observed with methanol and water at ratio of 45:55(v/v) at a flow rate of 1.2ml/min. In stated method retention time for paracetamol was 2.82 minutes (Fig.1). There was no intrusive peak observed as confirmed by blank run of mobile phase. Presence of no peaks of other impurities other than standard analyte, showed that method was specific for paracetamol. Total run time for this analysis was 8 min. Correlation coefficient (r^2) of calibration curve was attained as 0.999 (y=0.7465x-4E-05) when peak area was plotted against concentration range of 1-100µg/ml. Obtained correlation coefficient shows good linearity of stated method (Figure2).The relative standard deviation for intra-day precision and inter-day precision of the method was calculated at different time interval which is explained in (Table.1). Intraday precision and inter-day precision were in acceptable range. Accuracy was calculated as intraday accuracy and inter-day accuracy which is described in the table (Table 2). The LOD and LOQ were found as 0.04µg/ml and 0.09µg/ml respectively. Recovery was calculated by spiking the various biological samples viz. blood, CSF, Vitreous Humor with 10µg/ml concentration of paracetamol in triplicate. Other concentrations viz 20µg/ml and 50µg/ml were also spiked in various biological samples as described before. For blood, recovery was 85%, for CSF it was 90% and for Vitreous Humor recovery was 90%.

DISCUSSION

As increasing incidence of suicidal as well as accidental poisoning are observed both in adults and children due to drug overdose, quantification of drugs becomes imperative to distinguish the narrow margin of therapeutic dose and toxic dose. Paracetamol is an over the counter drug and easily available in houses. Its easy availability increases risk of its use as suicidal agent. Considering this a modest & quick method for quantification of paracetamol from biological samples viz. Blood, CSF, vitreous humor was developed and validated. The stated method possesses good linearity with 0.99 correlation coefficient, precision and accuracy in acceptable range with LOD and LOQ as 0.04µg/ml and 0.09µg/ml respectively. However recovery

from whole blood, CSF and Vitreous humor was low i.e. 85%, 90% and 90% respectively. Previously various investigators had published methods for estimation of paracetamol in dosage form, in body fluid such as plasma, serum and urine but not in CSF, Vitreous humor and whole blood.

CONCLUSION

The above developed isocratic method is simple, rapid, sensitive, precise and accurate and can be used for accurate quantitative analysis of paracetamol in biological samples such as blood, vitreous Humor, CFS etc.

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