HIGHLY SENSITIVE ANALYSIS OF THE CHALCONE IN HUMAN PLASMA AND URINE BY RP-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the quantitation of chalcone in human urine and plasma. The assay of the drug was performed on a CLC C_{18} (5 μ, 25 cm x 4.6 mm i.d.) with UV detection at 310 nm. The mobile phase consisted of methanol-water mixture in the ratio of 90:10, and a flow rate of 1 ml/min was maintained. The standard curve was linear over the range of 25-150 μg/ml (r^2=0.9961). Between and within-day precision and accuracy were acceptable down to the limit of quantification of 0.89 μg/ml in plasma. The proposed method was validated for accuracy and precission. Statistical analysis proves that the method was found to be simple, precise, accurate, rapid and reproducible and can be used for the routine determination of chalcone. The proposed method was successfully applied to the determination of chalcone in spiked human plasma and urine samples.

Keywords: Chalcone; Human plasma; RP-HPLC.

INTRODUCTION

Chalcone (and related compounds "chalconoids") is an aromatic ketone that forms the central core for a variety of important biological compounds, which are known collectively as chalcones (Fig. 1.). They show antibacterial, antifungal, antitumor and anti-inflammatory properties [1]. Some chalcones demonstrated the ability to block voltage-dependent potassium channels [2]. They are also intermediates in the biosynthesis of flavonoids, which are substances widespread in plants and with an array of biological activities. Chalcones are also intermediates in the Auwers synthesis of flavones.

Due to ineffective drugs, cancer is the secondmost leading cause of death after heart attack. Therefore, the researchers have accelerated their efforts for the generation of new anticancer drugs with high therapeutic index. In this connection, a clinically effective antitumor derivative of chalcone (calicheamicin) has stimulated the investigatiors to concentrate their studies on chalcones and related compounds [3].

Until now, several research teams have reported the interaction of substituted chalcone with DNA [4]. However, literature survey revealed that no work has been carried on the parent chalcone. In view of pharmaceutical importance of chalcone and the lack of literature on its analytical work, the determination of chalcone for a wider range is necessitates a method to be developed. Hence RP-HPLC method was used for its analysis. The proposed method was also applied for the determination of chalcone in spiked human plasma and urine samples.

EXPERIMENTAL

Instrumentation

All HPLC measurements were made on a Shimadzu Corporation system (Analytical Instruments division, Kyoto, Japan) consisting of a LC10AT solvent pump, SPD10AVP detector and a data station with win-chrome software version 3.1. The separation was performed on a CLC C_{18} column (5 μ, 25 cm x 4.6 mm i.d.). A CLC ODS (4 cm x 4.6 mm, i.d.) was used as a guard column to protect analytical column. Hamilton 702 μR injector with a 25 μl loop was used for the injection of the samples.

Chemicals and reagents

Pure drug sample of Chalcone was purchased from Sigma Aldrich and was used as such. The water and methanol used were of HPLC grade.

Plasma sample preparation

Blood Plasma: Human blood samples were collected in dry and evacuated tubes (which contained saline and sodium citrate solution) from same healthy volunteers. The samples were handled at room temperature and were centrifuged for 10 min at 1500 rpm for the separation of plasma within 1 hour of collection. The samples were then transferred to polypropylene tubes and stored at 20^0C until analysis. The plasma samples, 0.2 mL, were deproteinized with 2 mL of methanol and water mixture (90:10 v/v), vortexed for 15 minutes centrifuged at 6000 RPM for 15 min, and supernatants were collected. The supernatants were spiked with an appropriate volume of suitably diluted stock solutions of chalcone, giving final concentrations of 25 – 150 μg/mL. Each sample containing 20 μL was injected through a Rheodyne injector and the effluent was monitored at 310 nm. The above procedure was repeated five times and the plot of peak area of chalcone and concentration of chalcone was plotted in the range of 25 – 150 μg/mL in –plasma.

Urine sample preparation

Urine concentrations of chalcone were found to be extremely high in the first few hours following adminstration. For this reason, urine concentrations were assayed by direct injection of 1/100 dilutions of urine samples in mobile phase. Standards were prepared in 1/100 diluted blank urine with final concentrations of 25 – 150 μg/mL.

Chromatographic conditions

Chromatographic separation was achived at ambient temperature (25^0 C) on a RP- HPLC by using a mobile phase consisiting of methanol and water in the ratio of 90:10 (v/v) by 10 minutes. The mobile phase was pumped at a rate of 1.0 ml/min. The detector wavelentgh was set at 310 nm.

Through out the study, the suitability of the chromatographic system was monitored by calculating the capacity factor (k^1), the resolution (R), the selectivity (α) and the peak asymmetry (T).

Working standard of drug solution

A stock solution (100 μg/ml) was prepared by dissolving 10 mg of chalcone in 10 ml of methanol and then volume was made up to 100 ml with mobile phase in 100 ml volumetric flask. The working solution of chalcone was prepared by diluting the stock solution with mobile phase. Studies on the stability of analytes in working solution showed that there were no decomposition products in the chromatogram and also no difference in area-ratio during analytical procedure and even after storing for two days at 4^0 C.

Assay procedure

Composition and flow rate of the mobile phase methanol: water 90:10 was passed through 0.45μm membrane filter. It was delivered at 1.0 ml/min for column stabilization. During this period, the base line was continuously monitored. The wave length selected for the detection was 310 nm. The prepared dilutions containing

concentrations of chalcone in the range of 25 to 150 µg/ml were injected into the column. The peak areas were recorded for all the chromatograms (Table 1).

The chromatogram of chalcone is shown in Fig.2. Calibration curve was constructed by plotting peak areas [y-axis] vs concentration [x-axis] and the linear relationship was evaluated by calculation of regression line by the method of least squares. The calibration curve of chalcone is shown in Fig. 3.

RESULTS AND DISCUSSION

The development of an analytical method for the determination of drugs by HPLC has received considerable attention in recent years because of their importance in quality control of drugs and drug products. The run time was set at 10 min and the retention time for Chalcone was 4.42 min. Each sample was injected 3 times and the retention times were same.

The peak area of the drug was reproducible as indicated by low coefficient of variance [below 3.0%]. When the concentrations of Chalcone and its respective peak areas were subjected to regression analysis by least squares method, a good linear relationship [r^2 = 0.9961] was observed between the concentration of Chalcone and the respective peak areas in the range 25 to 150 µg/ml above which a deviation from linearity was observed. The regression of Chalcone concentration over its peak area was found to be Y = 506907X, where Y is the mean peak area and X is the concentration of Chalcone. The regression equation was used to be estimate the amount of Chalcone, either in plasma and urine samples or in validation study [precision and accuracy].

Recovery

To study the accuracy and reproducibility of the proposed method, recovery experiments were carried out. The recovery of the added standard was studied at five times. To aliquots of the previously analyzed preparations, a known concentration of standard solution of chalcone was added. The content of chalcone was once again determined by the proposed method. From the amount of drug present, percentage recovery was calculated using the following formula.

$$\% \text{ Recovery} = \frac{N\left(\sum XY\right) - \left(\sum Y\right)\left(\sum X\right)}{N\left(\sum X^2\right)\left(\sum X\right)^2}$$

Where,

X = Amount of standard drug added

Y = Amount of drug found by proposed method

N = number of observations

The extraction recoveries for chalcone were satisfactory. The results were found to be in the range of 98% to 104%. The results from the recovery experiments are summarized in Table 2 and 3.

Calibration

Calibration plots were constructed using six standard solutions of different concentrations in pure, plasma and urine samples daily for three days, by plotting the peak area against corresponding concentrations of chalcone. Equations of the calibration plots were generated by linear regression analysis.

The regression equations for chalcone were Y = 506907X (r^2 = 0.9961) for pure drug, Y = 953367X (r^2 = 0.9967) for plasma and Y = 1E+06X (r^2 = 0.9926) for urine, X is the concentration of the drug (µg/ml) and Y is the peak area of chalcone (Fig. 3).

Accuracy and precission

The accuracy and precission of the assay were determined by replicate analysis of plasma and urine samples. The method showed good precission and accuracy. Table 2 and 3 summarizes the intra- and inter-assay precission and accuracy for chalcone from plasma and urine samples. The results demonstrate the acceptable accuracy and precission of the developed method.

Ruggedness

The ruggedness of the proposed method was evaluated by carrying out the analysis of the working solution using the same chromatographic system and the same column on different days [5]. Small differences in area-ratios and good constancy in retention times were observed after 48 h time period. The RSD values of less than 1.0% for both areas and retention times were obtained. The comparable detector responses obtained on different days indicated that the method is capable of producing results with high precision on different days.

Limit of Detection and Limit of Quantification

The Limit of Detection (LOD) is the smallest concentration of the analyte that gives the measurable response. LOD was calculated using the following formula.

LOD = (3.3 x standard deviation)/ slope of calibration curve

The LOD for chalcone was found to be 0.29 µg/ml and 0.24 µg/ml in plasma and urine samples respectively.

The Limit of Quantification (LOQ) is the smallest concentration of the analyte, which response that can be accurately quantified. LOQ was calculated using the following formula.

LOQ = (10 x standard deviation)/ slope of calibration curve

The LOQ for chalcone was found to be 0.89 µg/ml and 0.72 µg/ml in plasma and urine samples respectively.

Suitability of the method

The suitability of the method was checked by determining the chromatographic parameters viz., resolution, selectivity and peak asymmetry and the results are shown in the Table 4. The observed values of resolution (more than 10), selectivity (is equal 1) and peak asymmetry (less than 1.5) revealed ideal chromatographic conditions for the quantification of chalcone.

Selectivity and Specificity

The selectivity was checked by injecting the solution of drug into the system and it was observed that one sharp peak of chalcone having retension time of 4.42 min. Specificity of the method was assessed by comparing the chromotographs obtained from standard drug, with the chromotogram obtained from plasma and urine samples was same, so the method was specific. The developed method was found specific and selective, as there was no interference of excipients (6-mercaptopurine, ciprofloxacin and diclofenac sodium) found.

CONCLUSION

A new, RP-HPLC method has been developed for the analysis of chalcone in pure, plasma and urine samples. The methanol: water mobile phase was used throughout the experimental work and no interference from any excipient was observed. It was shown that, the method was highly sensitive, linear, accurate, reproducible, repeatable, precise, selective and specific proving the reliability of the method. The run time relatively short, i.e, 6 min, which enable rapid determination of many samples in routine analysis of chalcone in spiked human plasma and urine samples. The proposed chromatographic method can be used to analyse a large number of plasma samples from patients each day in clinical and analytical laboratories.

Table 1: Linearity of chalcone

Concentrationsµg/ml	Peak area for Pure drug samples
25	11713992
50	24971790
75	36591406
100	48492192
125	64080124
150	77901964

Table 2: Within-day and between-day precision (C.V.) and overall accuracy (%) of the determination of chalcone in human plasma during a 3-day validation (n=3 each day).

Concentrations µg/ml	C.V. (%)		Recovery (%)
	Within–day	Between-day	
25	1.17	1.36	101.7
50	0.17	0.36	100.2
75	0.18	0.66	99.7
100	0.42	0.58	100.6
125	1.13	0.64	98.4
150	2.21	2.60	103.2

Table 3: Within-day and between-day precision (C.V.) and overall accuracy (%) of the determination of chalcone in human urine during a 3-day validation (n=3 each day).

Concentrations µg/ml	C.V. (%)		Recovery (%)
	Within–day	Between-day	
25	2.70	3.02	103.9
50	0.13	0.16	100.2
75	0.25	0.20	99.6
100	1.04	0.93	98.5
125	1.44	0.53	102.0
150	1.10	1.83	101.6

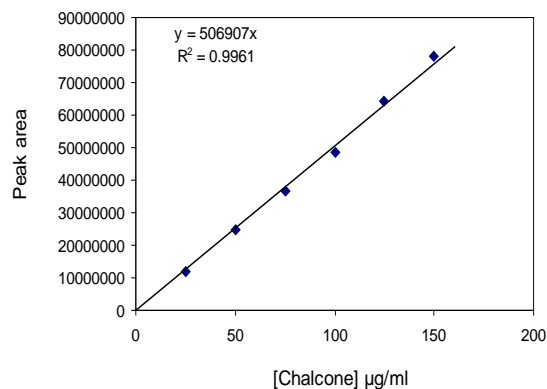**Table 4: System performance parameters of chalcone**

Retention time (t_R) in min	4.42
Capacity factor (k^1)	1.18
Selectivity factor (α)	1.00
Resolution (R)	10.94
Peak asymmetry (A_S)	1.01
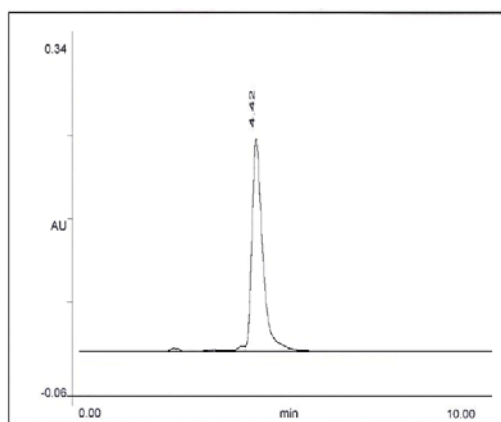
Fig. 2: Representative chromatograms of plasma spiked with chalcone (25 µg/ml).
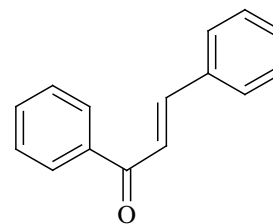
Fig. 1: Chemical structure of chalcone (1, 3-Diphenyl-2-propen-1-one).

Fig. 3: Calibration curve for chalcone in pure drug samples

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