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Full Length Research Paper

A fast and simple HPLC-UV method for simultaneous determination of three anti-cancer agents in plasma of breast cancer patients and its application to clinical pharmacokinetics

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A rapid, sensitive and reproducible high performance liquid chromatographic (HPLC) method was developed and validated in plasma for simultaneous quantification of 5-fluorouracil, adriamycin and cyclophosphamide (FAC) which are being prescribed in cancer chemotherapy. Isocratic RP-HPLC system (Agilent technologies USA) with C_{18} column (4.6 mm × 250 mm i.d., 5 µ particle size) and a detector UV-VWD was employed. The mobile phase of 0.05 M disodium hydrogen phosphate and acetonitrile (65:35 v/v) containing 0.5 mL/L triethylamine (pH 3.7) was pumped at 0.650 mL/min. UV detection of FAC was set at 266, 254 and 198 nm, respectively. Total run time was 15 min and retention times for 5-fluorouracil, cyclophosphamide and adriamycin were 4.1, 7.7 and 10.9 min, respectively. Parameters of validation (LOD, LOQ, Linearity, accuracy, precision and sensitivity) were established. The method provides specific quantification for FAC with high resolution. A simple, reproducible and inexpensive HPLC method was developed and applied for pharmacokinetic evaluation in breast cancer patients.

Key words: Simultaneous, 5-fluorouracil, adriamycin, cyclophosphamide, HPLC.

INTRODUCTION

5-Fluorouracil, adriamycin (also known doxorubicin) and cyclophosphamide (FAC) are widely used antineoplastic agents which could be prescribed in combination or separately in most of malignancies (Casale et al., 2002; Kristein et al., 2009). Chemical structures have been shown in Figure 1. The 5-fluorouracil, a pyrimidine analogue, is cytotoxic agent which is extensively used in different solid tumors such as breast, lung and gastrointestinal tract cancers (Rossella et al., 2005; Mark et al., 2002; Compagnon et al., 1996; Ciccolini et al., 2004). Adriamycin, an anthracycline glycoside, has been used as common chemotherapeutic agent in various malignant disorders but adverse effect of irreversible cardiomyopathy limits its use (Zhou and Chowbay, 2002). Clinical uses of adriamycin require therapeutic drug monitoring and individualization of therapy which can be accomplished with its analytical evaluation in biological fluids. The cyclophosphamide, an alkylating agent, is often administered with other antineoplastic agents. This regimen can be intolerable due to severe and life-threatening toxicities, therefore maximum clinical assessment will lead to safer administration in different situations (Milly et al., 2004). The fast monitoring of these agents is only possible with rapid, simple and sensitive

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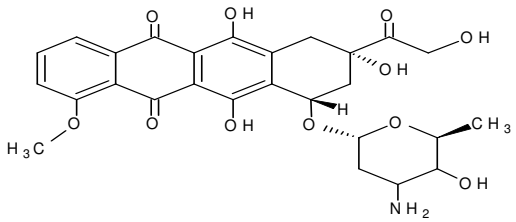
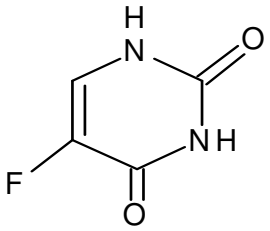
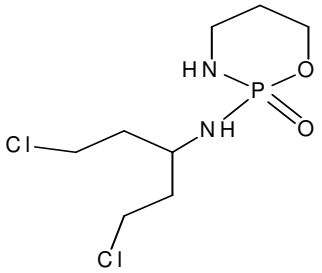
Abbreviations: FAC, Fluorouracil, adriamycin and cyclophosphamide; **FU,** fluorouracil; **AD,** adriamycin; **CP,** cyclophophamide; **LOQ,** limit of quantitation; **LOD,** limit of detection; **VWD,** variable wavelength detector.



Adriamycin



Fluorouracil



Cyclophosphamide

Figure 1. Chemical structures of adriamycin, fluorouracil and cyclophosphamide.

analytical methods through commonly available techniques such as HPLC with UV detections.

Among different types of chromatographic methods, high performance liquid chromatography (HPLC) is found to be more effective to achieve separation, identification, purification, and quantification of various compounds (Larson et al., 2003). Applicability of HPLC in pharmaceutical and biomedical analysis has increased during the last three decades and also for simultaneous determination in biological fluids (Arayne et al., 2010).

Previous analytical methods have not been developed and validated for simultaneous determination of FAC in human plasma samples using simple HPLC-UV analytical technique. Several analytical methods have been reported for FAC but with various limitations of time consuming, low extraction recovery, lengthy process for sample preparation, use of expensive techniques and in different combinations (Casale et al., 2002; Rossella et al., 2005; Mark et al., 2002; Compagnon et al., 1996; Ciccolini et al., 2004; Zhou and Chowbay, 2002; Milly et al., 2004; Wattanatorn et al., 1997; Eiji et al., 1997; Stephen et al., 1997; Dafeng et al., 2003; Alsarra and Alarifi, 2004; Ping and Alekha, 1999; Loreto et al., 1999). Larson et al. (2003) conducted a comprehensive evaluation of five anticancer drugs within single analysis but not validated in biological fluids, for example plasma and urine. Therefore, the present study was designed to develop and optimize a selective, accurate and reproducible HPLC method for quantification of FAC simultaneously in human plasma.

EXPERIMENTAL

Chemicals and reagents

5-Fluorouracil, adriamycin and cyclophosphamide were kind contribution from Pharmedic Laboratories Pvt. Limited, Pakistan. Disodium hydrogen phosphate, acetonitrile, phosphoric acid and tricholoroacetic acid were obtained from Merck, Germany and triethylamine by Fluka, Switzerland.

Instrumentation and chromatographic conditions

An isocratic HPLC system of Agilent technologies 1200 series consisted of a pump with a column of Thermo Electron Corporation USA (ODS hypersil C_{18} 4.6 mm × 250 mm), a UV-detector (VWD) with data processing Chem station software employed to assay the prepared plasma samples. The UV detection of FAC was set at 266, 254 and 198 nm, respectively. The mobile phase of 0.05 M disodium hydrogen phosphate and acetonitrile (65:35 v/v) containing 0.5 mL/L triethylamine at pH 3.7 (adjusted with 2 M phosphoric acid) was delivered at flow rate of 0.650 mL/min at ambient temperature.

Stock solutions and standards

The stock solutions of FAC were prepared in triplicate by dissolving

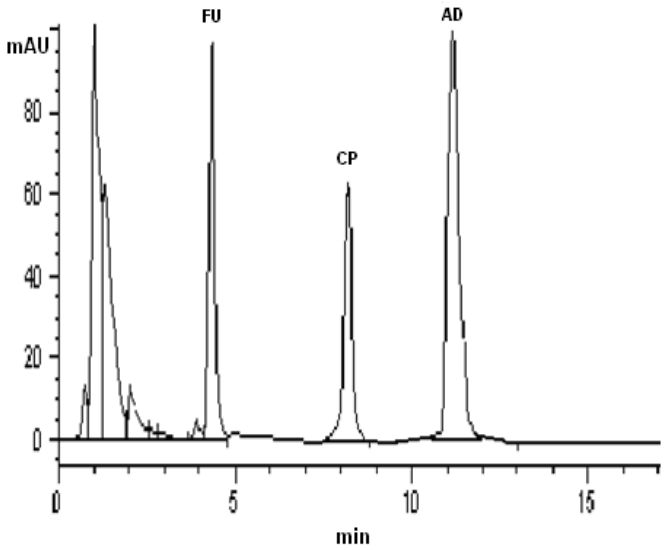


Figure 2. Representative chromatogram of 5-Fluorouracil cyclophosphamide and adriamycin in human plasma.

100 mg in 100 mL of mobile phase separately. Further dilutions were made from these stock solutions. A combined standard solution in range of 0.098 to 50 µg/mL, 0.150 to 80 µg/mL and 0.150 to 40 µg/mL for FAC was prepared from three stock solutions.

Sample preparation

Combined standard solution (100 µl) was added to 1 mL of blank plasma (to prepare spiked plasma samples) and trichloroacetic acid (60 µl) was added to precipitate plasma proteins, vortexed for 5 min and centrifuged (with centrifuge of Hettich-Germany) at 5000 rpm for 10 min. The supernatant layer was transferred to polypropylene tube (1.5 mL) and 20 µl injected into HPLC system. Blank plasma samples (drugs free) were processed similarly as control samples.

Quantification

Plasma samples were collected from healthy human volunteers and spiked with combined standard solution of FAC. These spiked plasma samples were extracted for separation and assayed by HPLC. Peak areas were calculated and a standard curve for each drug was constructed by plotting the peak areas versus plasma concentrations of drugs. Drug-free plasma samples (as controls) were also measured for comparative assessment with spiked samples.

Patients

Plasma samples were collected from breast cancer patients with prospect of pharmacokinetic study. A total of 18 breast patients were enrolled with age ≥ 18 and screened in Bahawalpur Institute of Nuclear Medicine and Oncology (BINO) Hospital. Written informed consent was obtained from each patient. This study was approved after collaborative review of Board of Advanced Studies and Research (BASR), the Islamia University of Bahawalpur and committee of experts, Bahawalpur Institute of Nuclear Medicine and Oncology Hospital, Pakistan. Health assessment including vital signs, physical examination and clinical laboratory testing was performed before administration of anticancer regimen. All the patients were included with Oncology Group Performance Status ≤ 1; having hemoglobin level 10 mg/dl, total bilirubin level ≤ 2.0 mg /dl, serum creatinine ≤ 1.5 mg /dl, leukocyte count ≥ 3000 /µl, absolute neutrophil count ≥ 1500 /µl and platelet count ≥ 100000 /µl.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed using one compartment open model of analysis by Kinetica 4.4® PK/PD software. Maximum concentration of FAC in plasma (C_{max}), time to these concentrations (T_{max}) and area under concentration time curve ($AUC_{0-\infty}$) were calculated in plasma samples of breast cancer patients.

Statistical analysis

Statistical analysis was performed to determine variability level between the samples. The data was analyzed statistically using Med-Calculator software at 95% confidence level.

RESULTS

Assay development

In method development process, a mobile phase was optimized with various combinations of buffer solutions and organic solvents. Acetonitrile, methanol and buffer (acetate, phosphate buffers) in different percentages were investigated and finally a mobile phase of disodium hydrogen phosphate-acetonitrile in the ratio of 65:35% (v/v) containing triethylamine 0.5 mL/L showed higher elution and resolution with sharp peaks for FAC. The pH and molarity of mobile phase was also selected after various trials. The pH of 3.7 was found suitable for the desired objectives of no interference at low wavelength and was sufficient in concentration to avoid peak tailing because silica-based particles are unstable at low pH (pH < 2). An optimal flow rate of 0.650 mL/min was found appropriate for peak resolution with short run time of only 15 min. The retention times for fluorouracil, cyclophosphamide and adriamycin were 4.1, 7.7 and 10.9 min, respectively. A representative chromatogram has been shown in Figure 2.

Method validation

Linearity

The standard curves of FAC were produced using known plasma concentrations within ranges of 0.098 µg/mL to 50 µg/mL (FU), 0.150 to 80 µg/mL (AD) and 0.150 to 40 µg/mL (CP). Linear regression was applied to fit straight line. Mean r^2 values for FAC were determined as 0.9978, 0.9988 and 0.9989, respectively and values of slope,

Table 1. Standard curve parameters (mean ± SD) for *FAC in human plasma.

Drug	Slope	Intercept	r^2
FU**	22.256 ± 0.4	5.499 ± 0.5	0.9978 ± 0.003
AD**	45.146 ± 0.7	63.384 ± 0.4	0.9988 ± 0.004
CP**	71.604 ± 0.9	58.315 ± 0.4	0.9989 ± 0.004

*FAC, fluorouracil adriamycin cyclophosphamide.
** FU, fluorouracil; AD, adriamycin; CP, cyclophosphamide.

Table 2. Intra-day and Inter-day precision and accuracy of FAC in human plasma.

Parameter	5-Fluorouracil			Adriamycin			Cyclophosphamide		
	LQC (µg/mL)	MQC (µg/mL)	HQC (µg/mL)	LQC (µg/mL)	MQC (µg/mL)	HQC (µg/mL)	LQC (µg/mL)	MQC (µg/mL)	HQC (µg/mL)
Intra-day									
Nominal Conc.	0.098	10.0	50.0	0.150	15.0	80.0	0.150	8.0	40.0
Mean	0.0977	9.934	49.527	0.1493	14.852	79.478	0.1482	7.947	39.614
S.D.	0.00067	0.068	0.645	0.0025	0.158	1.229	0.0018	0.0846	0.699
Precision CV (%)	0.0690	0.685	1.302	1.674	1.064	1.546	1.215	1.065	1.765
Accuracy (%)	99.69	99.34	99.05	99.53	99.01	99.34	98.80	99.33	99.04
Inter-day									
Nominal Conc.	0.098	10.0	50.0	0.150	15.0	80.0	0.150	8.0	40.0
Mean	0.0967	9.841	49.257	0.1471	14.801	78.898	0.1478	7.899	39.497
S.D.	0.0011	0.126	0.742	0.0028	0.163	1.408	0.0021	0.153	0.648
Precision CV (%)	1.138	1.280	1.506	1.904	1.101	1.783	1.421	1.937	1.641
Accuracy (%)	98.67	98.41	98.51	98.06	98.67	98.62	98.53	98.73	98.74

intercept and r^2 are shown in Table 1.

Precision and accuracy

Percent coefficient of variation (%CV) was calculated to find out intra-day precision, inter-day precision and accuracy of the present method for FAC in plasma. The findings are shown in Table 2. The validation run consist of calibration curve and three replicates of each; low, medium and high quantification concentrations. For inter-day, analysis of three batches of each of the drug of FAC samples was performed on three different days.

Quantification limits

Limit of detection (LOD) and Limit of quantitation (LOQ) of FAC as mean ± SD were 0.078 ± 0.0003 µg/mL and 0.098 ± 0.0005 µg/mL (FU), 0.096 ± 0.0004 µg/mL and 0.150 ± 0.006 µg/mL (AD) and 0.123 ± 0.0043 µg/mL and 0.150 ± 0.0052 µg/mL (CP). Lower quantification limits showed the higher sensitivity of present method for three drugs in single run.

Extraction yields

Percent extraction yield was calculated by comparing mean drug concentration from spiked plasma samples (extracted) with mean concentration from standard solutions (true solutions of FAC), determined from response (peak areas). Mean extraction recoveries were determined by analyzing four replicates of plasma samples at three concentration levels of each drug of FAC combination. The values of extraction yields are presented in Table 3 for FAC.

Freeze and thaw stability

The stability of FAC in human plasma was assessed by analyzing replicates (n = 9) of low, medium and high dilution of plasma samples during the sample storage and processing procedures. All samples were processed

Table 3. Percent extraction yield of FAC in human plasma.

	Conc. found in spiked samples	Conc. found in standard solutions	Conc. found in spiked samples	Conc. found in standard solutions
Fluorouracil				
Parameters	**Conc. Added (0.098 µg/ml)**		**Conc. Added (50 µg/ml)**	
Mean± S.D.	0.0973±0.00046	0.0979±0.00031	49.240±0.566	49.673±0.597
CV (%)	0.473	0.317	1.149	1.202
PEY (%)	99.30		99.13	
Adriamycin				
Parameters	**Conc. Added (0.150 µg/ml)**		**Conc. Added (80 µg/ml)**	
Mean± S.D.	0.1483±0.0022	0.1492±0.0027	78.897±1.079	79.563±1.541
CV (%)	1.483	1.809	1.368	1.937
PEY (%)	99.40		99.16	
Cyclophosphamide				
Parameters	**Conc. Added (0.150 µg/ml)**		**Conc. Added (40 µg/ml)**	
Mean± S.D.	0.1472±0.0019	0.1492±0.0024	39.570±0.499	39.913±0.604
CV (%)	1.291	1.609	1.261	1.513
PEY (%)	98.66		99.14	

in dark to protect from photo-oxidation and stored at -20°C in ultra low freezer (Sanyo, Japan). From zero to three freeze–thaw cycles (cycle 0, cycle 1, cycle 2 and cycle 3), stability studies were performed and illustrated in Table 4.

Application of method

This method was applied for pharmacokinetic evaluation of the studied drugs in breast cancer patients. The values of $AUC_{0-\infty}$ for FAC (64.792 ± 5.19 µg.h/mL, 155.37 ± 19.749 µg.h/mL, 544.29 ± 29.73 µg.h/mL); C_{max} (27.3 ± 4.1 µg/mL, 9.8 ± 0.71 µg/mL, 106.242 ± 3.742 µg/mL); T_{max} (0.083 ± 0.0 h, 1.057 ± 2.1h, 2.033 ± 0.045 h) and $t_{1/2}$ (0.37 ± 0.01h, 23.697 ± 2.959 h, 4.548 ± 0.214 h) were determined in patient's plasma samples.

DISCUSSION

HPLC is a convenient, economical, universal, easily accessible and reliable technology for the analysis of number of chemical agents in various mediums (Nama et al., 2011). Operating cost of HPLC is also comparatively less than advanced analytical instruments. High analytical cost indirectly increases the cost of product for patients. Previously these anticancer agents have been quantified individually or simultaneously by other analytical techniques (Vainchtein et al., 2010; Bhaskar et al., 2010).

The short retention time, simple mobile phase composition and high resolution characterized a fast, accurate, selective and cost effective method for quantification of FAC in plasma samples.

The r^2 values (0.9978, 0.9988 and 0.9989) and %CV < 2% were found for FAC which confirmed a linear response between concentration of analyte (studied drugs) and response (peak area). The good linearity and minimum variability substantiated the precision and reproducibility. Wattanatorn et al. (1997) analyzed the only 5-FU in biological, the present study showed comparable

Table 4. Freeze thaw stability in plasma for FAC.

Parameters	Freeze-Thaw cycles							
	Cycle 0 (µg/ml)		Cycle 1 (µg/ml)		Cycle 2 (µg/ml)		Cycle 3 (µg/ml)	
Fluorouracil								
Nominal conc.	0.098	50	0.098	50	0.098	50	0.098	50
Mean±SD	0.0977±0.00048	49.920±0.557	0.0796±0.00038	49.716±0.517	0.0776±0.00091	49.559±0.642	0.0765±0.00099	48.424±0.621
CV (%)	0.491	1.116	0.477	1.04	1.173	1.295	1.294	1.282
Difference	-	-	-0.0181	-0.2040	-0.0201	-0.361	-0.0212	-1.496
Adriamycin								
Nominal conc.	0.150	80	0.150	80	0.150	80	0.150	80
Mean±SD	0.1496±0.0027	79.873±1.441	0.1486±0.0028	79.253±1.561	0.1478±0.0021	79.120±1.379	0.1482±0.0023	78.167±1.448
CV (%)	1.805	1.804	1.884	1.969	1.421	1.743	1.552	1.852
Difference	-	-	-0.0010	-0.620	-0.0018	-0.753	-0.0024	-1.705
Cyclophosphamide								
Nominal conc.	0.150	40	0.150	40	0.150	40	0.150	40
Mean±SD	0.1493±0.0017	39.72±0.511	0.1486±0.0018	39.30±0.530	0.1483±0.0021	38.717±0.601	0.1475±0.0022	38.643±0.611
CV (%)	1.139	1.287	1.211	1.349	1.416	1.552	1.492	1.581
Difference	-	-	-0.0007	-0.420	-0.0010	-1.003	-0.0018	-1.077

results with advantage of simultaneous determination of three important anticancer agents.

The accuracy of an analytical method describes the closeness of mean test results with true value (concentration) of the analyte. Accuracy was determined by triplicate analysis of low, medium and high concentrations of samples containing known concentration of the analyte. Accuracy in present method is measured by using a minimum of three concentrations in the range of expected concentrations (intra-day). The deviation of mean from true value serves as the measure of accuracy. The mean value of intra-day and inter-day accuracy was above 99 and 98% at low, medium and high concentrations for FAC.

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological fluid. Precision was measured in triplicates with three concentrations in the range of expected concentrations. The percent coefficient of variation (%CV) in both intra-day and inter-day precision was less than 2% which is well within the range of 15% for lowest and 20% for highest concentration of FDA criteria for biological fluids (US FDA, 2009).

The stability results from the current method demonstrated that spiked plasma samples were found stable without significant degradability of drugs after three freeze-thaw cycles.

The Extraction yield of an analyte (drug) during analysis is a detector response obtained from nominal concentration added to and extracted from the plasma matrix which is compared to detector response obtained for known concentration of standard samples.

Extraction efficiency found to be consistent, precise, and reproducible. Recovery experiments were performed by comparing analytical results of spiked plasma samples at two concentration levels (low and high) with standard solutions of FAC. The percent yield for fluorouracil and adriamycin at low and high concentration levels was 99.30, 99.13% and 99.40, 99.16%, respectively.

Similarly, this was 98.66 and 99.14% at low and high concentrations for cyclophosphamide. The extraction efficiency of the present method was found better than previously published methods, which have stated the extraction recovery values from 93 to 97% in biological fluids (Alsarra and Alarifi, 2004; Ping and Alekha, 1999; Loreto et al., 1999; Mahdadi et al., 1987). Wei et al. (2008) analyzed the 5-FU in rat plasma with extraction recoveries from 94 to 105%. di Paolo et al. (2005) stated the 81 to 85% recoveries from human blood. Yuan et al. (2010) described the 81% recovered concentration of 5-FU from plasma.

CONCLUSION

The present chromatographic method provides a reproducible, accurate, selective and simultaneous quantification of three commonly prescribed anticancer drugs in human plasma. The method was applied successfully for pharmacokinetic analysis of FAC in breast cancer patients this could be applied for therapeutic drug monitoring and individualization of therapy in single sample processing step which make this method a rapid and cost effective analysis of FAC in highly expensive cancer therapy.

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