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Article

Development and Validation of Packed Column Supercritical Fluid Chromatographic Technique for Quantification of Chlorzoxazone, Paracetamol and Aceclofenac in their Individual and Combined Dosage Forms

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A reproducible, rapid and sensitive method has been developed for the assay of chlorzoxazone (CHL), paracetamol (PCM) and aceclofenac (ACE) in their combined solid dosage forms using packed-column supercritical fluid chromatography (SFC). The analytes were resolved by elution with supercritical carbon dioxide doped with 15% v/v methanol as the modifier on an ACE 5 Phenyl column (150 × 4.6 mm, 5 μm). The detection was carried out at 215 nm using a UV-Visible detector. The densities and polarities of the mobile phase were optimized from the effects of pressure, temperature and modifier concentration on chromatographic parameters like retention time, retention factor, resolution, asymmetry and theoretical plates. Modifier concentration proved to be the most effective means for changing both retention and selectivity. The developed method was validated as per International Conference on Harmonization guidelines. The developed SFC method was compared with a reported high-performance liquid chromatography method for the estimation of CHL, PCM and ACE using Student *t*-test. With respect to the speed and use of organic solvents, SFC was found to be superior and eco-friendly. The developed SFC method was successfully used for the assay of different marketed formulations containing CHL, PCM and ACE individually and in combination.

Introduction

Supercritical fluid chromatography (SFC) is fast and environmentally friendly, especially when compared to high-performance liquid chromatography (HPLC). It is more cost-efficient and user friendly, with high throughput and short run time, and better resolution and faster analysis time than liquid chromatography (LC) and gas chromatography (GC) methods. In the recent era of science and technology, there has been a push for environmentally friendly or greener processes for the production and estimation of pharmaceutical compounds. The SFC technique minimizes the use of large amounts of organic solvents. Due to higher diffusion rates and low viscosity, SFC provides a 3–5-time increase in the speed of analysis and a decrease in cost of analysis by saving organic solvents. SFC is developing as an eco-friendly, green chromatographic technique. Thus, an attempt is made to develop a simple, eco-friendly analytical method for the estimation of widely used pharmaceutical drugs individually and in their combinations.

The combination of chlorzoxazone (CHL), paracetamol (PCM) and aceclofenac (ACE) is widely used in the treatment of musculoskeletal disorders (MSD) (Figure 1). Chemically, CHL is 5-chlorobenzoxazol-2(3H)-one, a centrally acting skeletal muscle relaxant with sedative properties. PCM, chemically *N*-(4-hydroxyphenyl) acetamide, a para-aminophenol derivative, has analgesic and antipyretic properties and weak anti-inflammatory activity. ACE, which is chemically 2-(2,6-dichloroanalino) phenylacetoxyacetic acid, a phenylacetic acid derivative, is a non-steroidal anti-inflammatory drug (NSAID). It is possible that combination therapy of CHL, PCM and ACE provides relief from muscle and joint pain (1–4).

A literature review reveals that various methods like UV-Visible spectroscopy (5, 6), HPLC (7–12) and high-performance thin-layer chromatography (HPTLC) (13) have been used for the simultaneous analysis of CHL, PCM and ACE in their combined dosage forms. However, the UV-Visible spectroscopic method is not selective and techniques like HPLC and HPTLC require large volumes of organic solvents. Hence, the present study was undertaken to develop a simple, eco-friendly SFC method for simultaneous estimation of CHL, PCM and ACE in their individual and combined dosage forms.

Experimental

Chemicals and reagents

Carbon dioxide (99.9% pure) was obtained from BOC (Mumbai, India). PCM and HPLC-grade methanol were obtained from S.D. Fine Chemicals Mumbai, India. CHL and ACE were gifted by INTAS Pharmaceuticals (Ahmadabad, India).

Marketed formulations were procured from a local market. The following formulations were used for the study: three tablet formulations containing CHL, PCM and ACE (HIFENAC MR, Intas Pharmaceuticals, India; ACECLO MR, Aristo Pharma, India; RALIWIZ MR, Wisdom Pharma, India); one tablet formulation containing CHL and PCM (DUODIL, Solvay Pharma, India); one tablet formulation containing PCM and ACE (AROFF PLUS, Unichem Pharma, India); and three tablet formulations containing CHL, PCM and ACE individually (PARAFON DSC, Jonson and Johnson, India; CALPOL, Glaxosmithkline, India; HIFENAC, Intas Pharmaceuticals, India).

Instrumentation

A Jasco-900 series SFC (Japan Spectroscopic Co.; Hachioji, Tokyo, Japan) was employed for the present study. It was

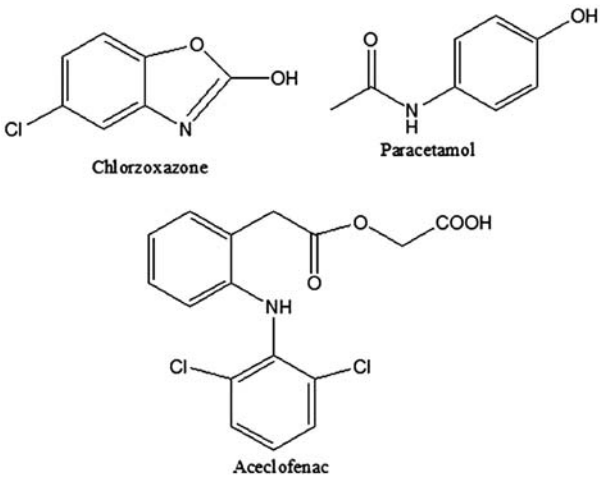

Figure 1. Structures of chlorzoxazone, paracetamol and aceclofenac.

equipped with two pumps (PU-980), which were capable of adjusting the flow rate of both liquid CO_2 and modifier. An external loop (model 7125) was equipped with a rheodyne injector that could inject 20 μL of liquid sample into the analytical column. The temperature of the column was thermostatically controlled in a column oven (Jasco-Co-965) operated in the range of 35–80°C with a cooling circulator. The outlet pressure, operated in the range of 7–45 MPa, was adjusted by a backpressure regulator (Jasco-880-81).The detection was carried using UV-Visible detector. Borwin chromatographic software was used for data integration.

A Jasco series HPLC (Japan Spectroscopic Co.) was employed for comparative study. It was equipped with binary pumps (Jasco PU-2080 plus intelligent HPLC pump) and a photodiode array (PDA) detector (Jasco MD-2015 plus multiwavelength detector). An external loop (model 7125) was equipped with a rheodyne injector that could inject 20 μL of liquid sample into the analytical column. Borwin chromatographic software was used for data integration.

Optimized chromatographic conditions

Experimentation revealed that CHL, PCM and ACE were optimally resolved on an ACE 5 Phenyl column (150 × 4.6 mm, 5 μm) with a mobile phase composed of CO_2 doped with 15% of methanol as a modifier at a flow rate of 1.21 mL/min. The temperature of the column was maintained at 45°C and the backpressure was adjusted to 10.0 MPa. Detection was carried out at 215 nm.

Preparation of standard stock solutions

Preparation of individual standard stock solutions of CHL, PCM and ACE

Each drug was weighed accurately and dissolved in methanol to get individual stock solutions containing 1,000 μg/mL of CHL, PCM and ACE, respectively. The standard stock solutions were further diluted to get working standard solutions when required.

Preparation of standard stock solution of ternary mixture (5:5:1)

To prepare the standard stock solution of ternary mixture, 50 mg of CHL, 50 mg of PCM and 10 mg of ACE were weighed accurately and transferred to a 50-mL volumetric flask and dissolved to the mark with methanol. The prepared stock solution contained 1,000 μg/mL each of CHL and PCM (200 μg/mL of ACE). The standard stock solutions of ternary mixture were further diluted to get working standard solutions when required.

Method validation

The developed SFC method was validated by evaluating linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), selectivity and ruggedness as per International Conference on Harmonization (ICH) guidelines (14).

The system suitability parameters were determined by injecting the ternary mixture containing 300 μg/mL CHL, 300 μg/mL of PCM and 60 μg/mL of ACE for six consecutive times.

To determine linearity, appropriate aliquots (0.25, 0.5, 1, 1.5, 2.5 and 4 mL) from the standard stock solution of the ternary mixture were pipetted and diluted with methanol to 10 mL. All solutions were analyzed at optimized chromatographic conditions to generate six sets of calibration curves to select the concentration range providing maximum linear relationship with the response.

Intra-day precision was determined by analyzing three concentration levels (25, 150 and 400 μg/mL) for three times in a day. The same concentration levels were further analyzed on three different days to evaluate inter-day precision. The precisions were expressed in terms of percent relative standard deviation (%RSD). Repeatability was performed by analyzing a ternary mixture with a concentration near to the assay concentration for six consecutive times (250 μg/mL of CHL and PCM and 50 μg/mL of ACE). The Accuracy was expressed as percentage recovery of each drug from pre-analyzed sample solutions. Standard addition of 80, 100 and 120% of each drug was carried out with respect to label claim. LOD and LOQ were determined using the slope of the calibration curve and the standard deviation of the response.

Analysis of marketed formulations

Marketed formulations containing CHL, PCM and ACE

The application of the developed SFC method was evaluated to determine the amount of CHL, PCM and ACE in their various marketed combined tablet dosage forms with label claims of 500 mg of CHL, 500 mg of PCM and 100 mg of ACE. Twenty tablets were crushed and an amount of tablet powder equivalent to 500 mg of CHL was accurately weighed and transferred to a 100-mL volumetric flask. The tablet powder was dissolved in 50 mL methanol, sonicated for 15 min, diluted to the mark with methanol and filtered through Whatman filter paper No. 41. This solution was diluted further with methanol to get a final solution containing 300 μg/mL of CHL (300 μg/mL of PCM and 60 μg/mL of ACE). The solution was analyzed in triplicate to determine the amount of each drug.

Marketed formulations containing CHL and PCM

Marketed tablet formulations containing CHL and PCM were evaluated in a similar way as described previously, but tablet powder equivalent to 250 mg of CHL (300 mg of PCM) was used for analysis and final dilution was prepared with a concentration of 250 μg/mL of CHL (300 μg/mL of PCM).

Marketed formulations containing PCM and ACE

For analysis of marketed formulations containing PCM and ACE, tablet powder was treated similarly as described previously. The final solution, with a concentration of 300 μg/mL of PCM (60 μg/mL of ACE), was used for analysis.

Marketed formulations containing CHL, PCM and ACE in their individual dosage forms

Formulations containing single drug component were also analyzed in the same manner as described previously.

Results and Discussion

Optimization of chromatographic conditions

In SFC, the separation of drugs is affected by various parameters like temperature, pressure, modifier concentration, flow rate of CO_2 and detection wavelength. Several trials were carried out to optimize these variables and achieve effective separation of CHL, PCM and ACE.

Selection of column

The affinity of the analyte to the stationary phase plays an important role in the selection of the stationary phase. The effective separation mechanism for NSAIDs is based on the carbon and heteroatom ratio. CHL, PCM and ACE share a six-carbon aromatic ring, which can be targeted using a phenyl-based stationary phase. As a retention mechanism, phenyl stationary phases employ π−π interactions between the phenyl groups in the stationary phase and any unsaturated bonds in the analyte. Therefore, an ACE 5 phenyl column (150 × 4.6 mm, 5 μm particle size) was chosen for separation of CHL, PCM and ACE.

Effect of modifier concentration on retention factor

Methanol in the mobile phase enhances π−π interactions between aromatic compounds and the phenyl stationary phase, leading to greater retention and superior selectivity. Methanol concentrations were changed from 12 to 20% v/v for optimization. Figure 2 gives the relation between modifier concentrations and retention factors of all three drugs at 45°C and 10.0 MPa backpressure. Retention factor decreases with increasing methanol concentrations and peaks were almost merged at a concentration of 20% v/v, whereas the problem of broader peaks was observed when methanol concentration was decreased. To strike a balance between selectivity coefficient and retention time, a modifier concentration of 15% v/v was optimized for the study.

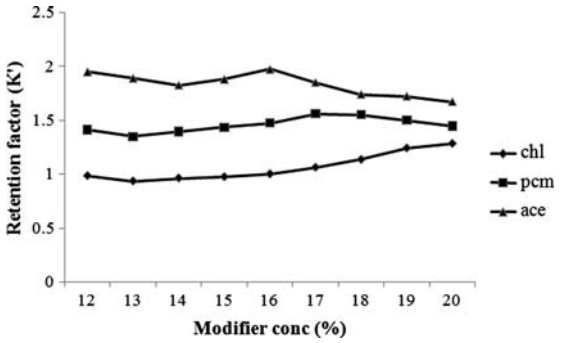

Figure 2. Effect of modifier concentration on retention factor at temperature 45°C, wavelength 215 nm, CO_2 flow rate 1.21 mL/min and pressure 10 MPa.

Effect of backpressure on retention factor

The effect of backpressure was studied in the range of 9–18 MPa. The best separation between CHL, PCM and ACE was obtained near a critical pressure of 10 MPa (Figure 3). For a given set of column and temperature, retention time gradually decreases as operating pressure is increased because fluid density increases at high pressure.

Effect of column temperature on retention factor

Figure 4 depicts the effect of column temperature on retention factors, at 10 MPa pressure and 15% v/v of methanol concentration. It was observed that 45°C could be considered as the optimum temperature because better separation was achieved

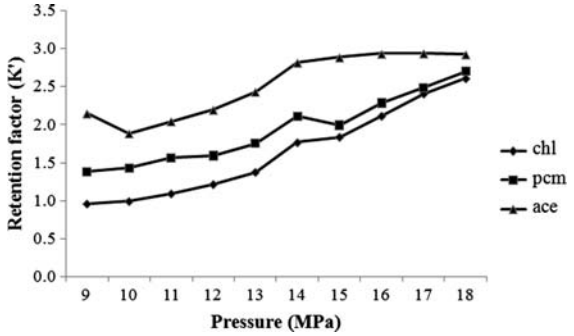

Figure 3. Effect of pressure on retention factor at temperature 45°C, wavelength 215 nm, CO_2 flow rate 1.21 mL/min and modifier 15% v/v.

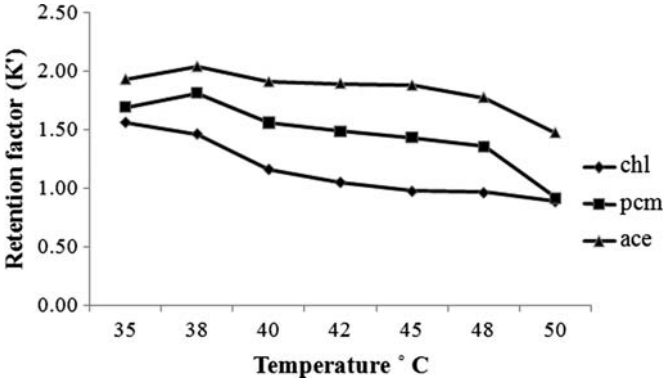

Figure 4. Effect of column temperature on retention factor at wavelength 215 nm, CO_2 flow rate 1.21 mL/min, pressure 10 MPa and modifier 15% v/v.

at this temperature. The choice of this temperature strikes a balance between selectivity and run time. At lower temperatures, CHL and PCM peaks were found to merge with each other. The peaks become broader at temperatures above 46°C.

Wavelength selection

In the combined dosage form, ACE is found at the lowest proportion, so it is necessary to select a wavelength wherein ACE gives maximum response without hampering the sensitivity of CHL and PCM. Based on this, an optimized wavelength of 215 nm was chosen for the simultaneous estimation of CHL, PCM and ACE (Figure 5).

Effect of CO_2 flow rate on retention factor

The flow rate of carbon dioxide was tuned from 0.9 to 2 mL/min. At a lower flow rate of CO_2, improper peak shapes were obtained, and at higher flow rate of CO_2, the nonpolarity of the mobile phase increased, leading to longer retention time and broader peaks. To balance retention time, peak shape and selectivity, the flow rate of CO_2 was chosen to be 1.21 mL/min (Figure 6). With all optimized parameters, a typical SFC chromatogram containing 300 μg/mL of CHL (300 μg/mL of PCM and 60 μg/mL of ACE) is shown in Figure 7.

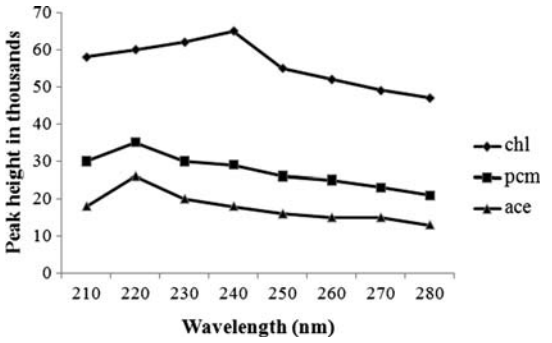

Figure 5. Wavelength selection at temperature 45°C, CO_2 flow rate 1.21 mL/min, pressure 10 MPa and modifier 15% v/v.

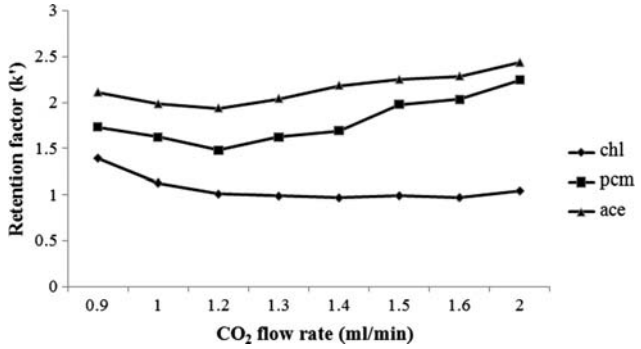

Figure 6. Effect of CO_2 flow rate on retention factor at temperature 45°C, wavelength 215 nm, pressure 10 MPa and modifier 15% v/v.

Method validation

The RSD values calculated for system suitability parameters were found to be less than 2%, which are in accordance with the acceptance criteria as per ICH guidelines (Table I). For linearity studies, six different concentrations of ternary mixtures were analyzed. A good correlation of peak area versus concentration was observed for all three drugs, as shown in Table II. Intra-day and inter-day precision and repeatability in estimation of CHL, PCM and ACE (Table III) showed that the RSD values were found to be less than 2%. These low RSD values indicate that the method is precise. The recovery data (Table IV) indicate that the accuracy of the quantification of CHL, PCM and ACE is more than 98%. The LOD values were found to be 2.57, 2.89 and 0.66 μg/mL for CHL, PCM and ACE, respectively. The LOQ values were found to be 7.79, 8.78 and 2.02 μg/mL for CHL, PCM and ACE, respectively.

Analysis of marketed formulations

The developed and validated SFC method was successfully used for the estimation of CHL, PCM and ACE from its individual and combined tablet dosage forms. The percentages of all three drugs present in each formulation are mentioned in Table V.

Comparison of developed SFC method with reported RP-HPLC assay method (10)

The developed SFC method was compared against a reported RP-HPLC method by applying a student *t*-test (paired *t*-test) to

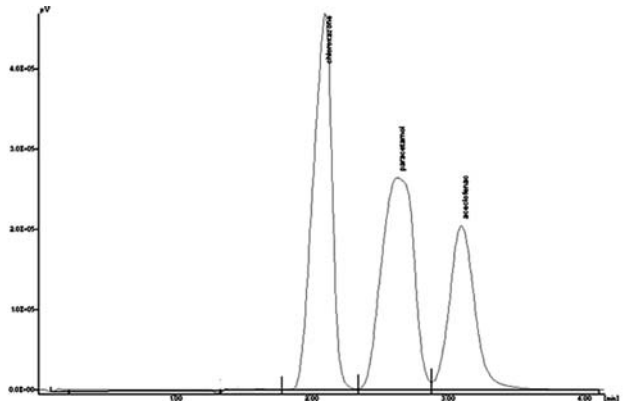

Figure 7. Typical SFC separation of drugs with retention times of CHL, PCM and ACE at 2.106, 2.605 and 3.105 min, respectively.

Table I
System Suitability Parameters of the Developed SFC Method

Parameter	CHL	%RSD*	PCM	%RSD*	ACE	%RSD*
Retention time (min)	2.033	0.56	2.608	0.23	3.105	0.44
Resolution	—	—	1.46	1.21	1.22	1.09
Asymmetry	0.87	1.23	0.96	1.57	1.08	0.98
Number of plates	2953	1.42	1386	1.66	3981	1.12
Retention factor	1.1	1.33	1.62	1.05	1.98	1.48

*RSD of six replicate injections.

Table II
Linear Regression (Least-Square Fit) Calibration Data of CHL, PCM and ACE by SFC

Parameter	CHL	PCM	ACE
Range (μg/mL)	10–500 μg/mL	25–1,000 μg/mL	10–500 μg/mL
Linearity (μg/mL) ($n = 6$)	25–400 μg/mL	25–400 μg/mL	5–80 μg/mL
Equation	$y = 13488x - 44998$	$y = 9304.2x - 39617$	$y = 19100x - 25411$
Correlation coefficient (r^2)	0.9999	0.9992	0.9991

Table III
Precision of CHL, PCM and ACE by the Developed SFC Method

Drugs	Concentration (μg/mL)	Intra-day precision %RSD*	Inter-day precision %RSD*
CHL	25	1.74	1.48
	150	0.77	0.96
	400	0.96	1.38
PCM	25	1.57	1.63
	150	1.65	0.74
	400	0.54	1.36
ACE	5	1.26	1.37
	30	0.42	1.48
	80	1.29	1.82

*RSD of triplicate determination of each concentration.

Table IV
Analytical Recoveries (Accuracy) of CHL, PCM and ACE by the Developed SFC Method

Drug	Level of recovery (%)	Amount of standard added (μg/mL)	Total amount (μg/mL)	Amount recovered (μg/mL)	% Mean recovery*	%RSD
CHL (300 μg/mL)	80	240	540	541.26	99.96	1.02
	100	300	600	597.61	100.01	1.33
	120	360	660	661.12	100.20	1.76
PCM (300 μg/mL)	80	240	540	543.93	101.40	1.26
	100	300	600	602.10	100.05	1.06
	120	360	660	659.37	100.11	1.38
ACE (60 μg/mL)	80	48	108	107.91	99.81	1.33
	100	60	120	120.78	100.27	1.25
	120	72	132	131.99	100.38	1.47

*Mean recovery of triplicate determination.

Table V
Analysis of Marketed Formulations Containing CHL, PCM and ACE by the Developed SFC Method

Brand	Drug	Label claim (mg)	%Assay ± SD*	%RSD
HIFENAC MR	CHL	500	101.76 ± 3.70	1.21
	PCM	500	99.37 ± 4.93	1.66
	ACE	100	101.97 ± 0.68	1.09
ACECLO MR	CHL	500	100.08 ± 3.21	1.08
	PCM	500	101.73 ± 4.06	1.62
	ACE	100	98.76 ± 1.21	1.82
OCTACE MR	CHL	250	99.98 ± 3.96	1.92
	PCM	325	101.34 ± 4.54	0.98
	ACE	100	98.63 ± 1.33	1.05
DUODIL	CHL	250	99.53 ± 1.42	1.23
	PCM	300	101.23 ± 1.03	0.95
AROFF	PCM	500	98.99 ± 1.55	1.44
	ACE	100	99.62 ± 1.21	1.69
PARAFON DSC	CHL	500	99.25 ± 1.21	1.36
METACIN	PCM	500	101.45 ± 0.85	1.55
HIFENAC	ACE	100	99.89 ± 2.06	1.02

*Mean of triplicate determination.

the assay results. The results of the *t*-test reveal that the t-tab values for CHL, PCM and ACE (11.8, 12.3 and 13.5) are more than the t-cal values (6.31, 5.36 and 5.96). This shows that there is no significant difference between the results obtained by the developed SFC method and the reported RP-HPLC method.

Conclusion

The present work reveals that the developed SFC method could successfully be applied for analysis of all marketed formulations containing CHL, PCM and ACE in individual and combined dosage forms. The tedious mobile phase preparation necessary for HPLC can be eliminated because selectivity can be tuned by optimizing parameters like temperature, pressure, modifier concentration and detection wavelength. SFC is an eco-friendly technique because the mobile phase used is carbon dioxide. The ability to perform faster separations without the use of organic solvents and to analyze thermolabile and nonpolar compounds are the advantages of SFC over HPLC and GC, respectively. The SFC technique, thus, could be used as an alternative method for analysis of bulk drugs and pharmaceutical dosage forms because it is relatively cheaper, faster and more cost-effective than conventional HPLC methods.

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