Validated Stability Indicating LC Method for Carprofen: Characterization of Degradation Products by MS

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Abstract

A simple, sensitive, and selective stability indicating high-performance liquid chromatographic method has been developed and validated for quantitative analysis of carprofen (CPF) in presence of its degradation products. All degradation products in acid hydrolysis and photolysis were separated, identified by mass spectroscopic method and probable structures were elucidated. The forced degradation studies were performed on a bulk sample of CPF by using various methods like 0.1 M hydrochloric acid, 0.1 M sodium hydroxide, 0.33% hydrogen peroxide (H_2O_2), heating at 60°C and exposure to UV light at 254 nm. A 5 µm particle octa desyl silane (ODS) column (150 mm × 4.6 mm) was used with acetonitrile–ammonium acetate (100 mM, pH-6.7) 40:60 (v/v) as a mobile phase at flow rate of 1.2 mL/min. Column oven temperature was maintained at 30°C and quantitation was achieved at 239 nm on the basis of peak area. The linear range and correlation coefficient (r^2) was found 0.5–60 µg/mL and 0.9999 respectively. The limit of detection (LOD) and limit of quantitation (LOQ) were obtained 0.066 µg/mL and 0.20 µg/mL respectively. The proposed method was found to be suitable and accurate for quantitative analysis, stability study and characterisation of degradation product of CPF.

Introduction

Carprofen (CPF) [2-(6-chloro-9H-carbazol-2-yl) propanoic acid] is a nonsteroidal anti-inflammatory drug (NSAID) that is used by veterinarians as a supportive treatment for the relief of arthritic symptoms in geriatric dogs (1–3). However incidences of gastric irritation caused by these NSAIDs are frequently reported (4). CPF was previously used in human medicine for over 10 years as 1985–1995. It was generally well tolerated with the majority of adverse effects being mild such as gastro-intestinal pain and nausea similar to those recorded with aspirin and other NSAIDs. It is no longer marketed for human uses after being withdrawn on commercial grounds.

Many researchers have developed the stability indicating high-performance liquid chromatography (HPLC) method for Pipenzolate bromide (5), Rimonabant (6), and Lumefantrine (7). And especially the work focussed on CPF (i.e., the phototoxicity and photosensitivity disorders induced) (8–10), determination of chemical structure of photodegradents based on the mass to charge ratio of quasimolecular ions and molecular weight changes by comparison with the parent drug (11,12) have been reported earlier. The reported stability indicating LC method for CPF (13) has failed to explain the identification of degradation products and also takes very long time for analysis. Therefore it was proposed to carry out the detailed and insightful study on stability indicating assay method for CPF which is suitable for quantitative analysis in very short period and identify the degradation products in acid hydrolysis and photolysis by mass spectroscopy.

In this manuscript the stability indicating LC method for the analysis of CPF in presence of its degradation products has been developed. This paper deals with the separation and identification of degradation product of CPF under stress conditions like acid hydrolysis, base hydrolysis, oxidation, heating, and UV light. All degradation products in acid condition and photolysis were characterized by mass spectroscopy according to their *m/z* ratio. And the probable structures of the degradation products were assigned by checking the molecular weight compared to parent ion molecule. The stability indicating method is direct, sensitive, accurate and rapid which can be used for analysis of CPF in bulk drug sample and to identify the degradation products in stress conditions.

Experimental

Material and reagents

Acetonitrile (HPLC grade), AR grade sodium hydroxide, ortho phosphoric acid, methanol, and ammonium acetate buffer were procured from Qualigens Fine Chemicals (Mumbai, India). Hydrochloric acid and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). Milli-Q water was used throughout the experiment (Billerica, MA).

Extraction of carprofen from tablets

A CPF tablet (100 mg) was crushed to powder and sonicated in 100 mL methanol for 10 min. The solution was filtered through 0.45-µm membrane filter then lyophilized for 12 h. The solid CPF obtained was 99.8% pure.

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Equipment

The HPLC system (LC2010, Shimadzu Corporation, Kyoto, Japan) consisted of low-pressure gradient quaternary pump, Auto sampler, column oven and photo diode array detector (SPD M20A). LCsolution workstation software was used for data acquisition. The analysis was performed on C18, 150 mm length, 4.6 mm i.d. and 5 µm particle size column (YMC ODS A, Japan).

Mass spectroscopic analysis was performed using LC–MS-2010 equipped with electrospray ionization interface (Shimadzu Corporation, Kyoto, Japan). The data were collected and processed using LC–MS solution software.

Chromatographic conditions and LC–MS parameters

Chromatographic separation was achieved using YMC C18 column (150 mm × 4.6 mm) with acetonitrile–ammonium acetate (100 mM, pH 6.7) 40:60 (v/v) as a mobile phase at flow rate of 1.2 mL/min. Mobile phase was filtered through 0.45-µm filter and degassed for 10 min. Column oven temperature was maintained at 30°C and quantitation was achieved at 239 nm on the basis of peak area. Injection volume was 10 µL. Standard and test solutions were prepared with mobile phase.

An LC–MS-2010 single quadrupole mass spectrometer was interfaced with electrospray ionization (ESI) probe. The temperatures were maintained at 250, 250, and 200°C for the probe, CDL, and block, respectively. The voltages were set at 4.5 kV, –30 V, 25 V, 150 V, and 1.6 kV for the probe, CDL, Q-array 1, 2, 3 bias, Q-array radio frequency (RF), and detector, respectively. The flow rate of nebulizer gas and dried gas were set at 1.5 L/min.

Sample preparation

Stock solution (1000 µg/mL) was prepared by dissolving 25 mg CPF in minimum amount of methanol, kept for sonication up to 15 min and diluted to 25 mL using volumetric flask. Then standard solutions were prepared by dilution of stock solutions using mobile phase within the range 0.5–60 µg/mL.

Triplicate 10 µL injection of each solution were chromatographed. Average peak areas were plotted against concentration to obtain the calibration plot.

Validation of the Method

Linearity

Linearity test solutions for developed method were prepared from stock solutions at seven concentrations levels of 0.5, 1, 5, 10, 20, 40, and 60 µg/mL. Standard curve was obtained by plotting peak area against concentrations for evaluation of linearity by linear regression analysis using least square method. An excellent correlation existed between the peak area and concentration of CPF.

Limit of detection and limit of quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) for CPF were estimated at a signal to noise ratio of 3:1 and 10:1 respectively by injecting a series of dilute solutions with known concentrations. The precision study also carried out at LOQ level by injecting six individual injections of sample solution.

Specificity

Specificity is the ability of a method to measure analyte response in the presence of its potential impurities. Specificity of developed HPLC method was carried out in presence of its degradation products formed on hydrolysis and photolysis. Stress studies were performed for CPF bulk drug to provide an indication of stability indicating property and specificity of proposed method. Peak purity test was carried out for CPF peak by using photo diode array (PDA) detector in stress samples.

Robustness

To determine the robustness of the developed method, experimental conditions were purposely altered and resolution of CPF from its degradation products was evaluated. The flow rate of the mobile phase was 1.2 mL/min. To study the effect of flow rate on the resolution, it was changed by 0.2 units from 1 to 1.4 mL/min while the other mobile phase composition was kept constant. The effect of the percent organic strength on resolution was studied by varying acetonitrile from ±3% while other mobile phase components were held constants. The effect of temperature on the resolution was studied at 25°C and 35°C while the other mobile phase components were held constant.

Solution stability and mobile phase stability

Solutions of the sample prepared from stock solution and diluted by mobile phase were kept in tightly capped volumetric flask for 48 h at room temperature and analyzed by preparing the fresh mobile phase. Mobile phase stability was checked by analyzing the freshly prepared sample solutions at an interval of 2, 4, 6, 8, 12, 16, 24, and 48 h by keeping the same mobile phase throughout analysis.

Accuracy

Accuracy of developed method was evaluated in triplicate at three concentration levels (i.e., 40, 50, and 60 µg/mL in bulk drug sample). The percentage recoveries were calculated from slope and y intercept on the calibration curve.

Results and Discussion

Optimization of chromatographic condition

The chromatographic conditions were optimised with a view to consider a symmetrical peak shape of CPF and resolution of degradation products. To develop a rugged and specific LC method various trials have been taken in different buffers (Phosphate, formate, and carbonate) and using columns (Silica, Cyano, and Phenyl) but peak shape observed was broad and the

Table I. System-Suitability Report*

Compound (n = 3)	t_R	*RS*	*N*	*T*
CPF	2.9 ± 0.2	3.62	25429	1.1

* n = 3 determinations; t_R = retention time in minutes; *Rs* = USP Resolution; *N* = Number of theoretical plates; and T = USP tailing factor.

resolution was poor. The results obtained were good by using 100 mM ammonium acetate–acetonitrile (60:40) and 150 × 4.6 mm id, 5 µm particle ODS column (YMC) as the stationary phase. In the developed LC method all the degradation products in alkaline hydrolysis and photolysis were well separated and resolution achieved was more than two. The system suitability results were entitled in the Table I. The typical retention time of CPF was 2.9 min.

Forced degradation study

Degradation of CPF was performed with various stress conditions like 0.1 N HCl, 0.1 N NaOH, 0.33 % H_2O_2, heating at 60°C and photolytic degradation. CPF has been dissolved in mobile phase, 0.1 N NaOH was added in the sample and kept at room temperature up to 8 h. Then the sample is directly injected to the HPLC analysis and for LC–MS analysis. The same procedure is applied for acid hydrolysis using 0.1 N HCl and for oxidation using 0.3% H_2O_2. In case of photolysis, the sample is dissolved in mobile phase, kept in UV chamber at 254 nm up to 8 h, and injected directly to HPLC and LC–MS. For the thermal degradation study solid CPF was kept at 60°C in the oven up to 8 h. Then sample was dissolved in mobile phase and injected for the analysis. The summary of forced degradation studies is given in the Table II. The CPF was found to be sensitive in acid hydrolysis and photolysis while stable in other stress conditions like alkaline hydrolysis, oxidation, and thermally. In acid hydrolysis only one impurity was observed (Figure 1) and found to be stable in acetonitrile solution while in photolytic degradation seven impurities were formed in methanolic solution (Figure 2). CPF is soluble in methanol and at the time of reaction or work up procedure methanol can incorporate the impurities like methyl ester of CPF. Even at the time of analysis, if methanol was used instead of acetonitrile as an organic solvent in the mobile phase, there were chances of introduction of impurities. To avoid all these chances of formation of methyl ester of CPF the formation, identification of methyl ester and use of acetonitrile in mobile phase besides methanol have been highlighted. All impurities formed in photolytic degradation and acid hydrolysis were successfully separated and characterised by mass spectroscopy to elucidate probable structures of the degradation products. The noninterference of forced degradation product with CPF confirms specificity of developed method.

LOD and LOQ

In accordance with International Conference on Harmonisation (ICH) recommendations, the approach based on the standard deviations (SD) of the response and the slope of the calibration plot was used for determinations of limit of detection and limit of quantification. The calculated values of LOD and LOQ are 0.066 µg/mL and 0.20 µg/mL, respectively.

Table II. Summary of Forced Degradation Study of CPF

Stress condition	Time	Assay (%)	Mass balance*	Remarks
Acid hydrolysis (0.1 N HCl)	48 h	78.2%	99.92	CPF degraded to Imp 1
Base hydrolysis (0.1 N NaOH)	48 h	99.99	99.99	No degradation product formed
Oxidation (0.33% H_2O_2)	48 h	99.99	99.99	No degradation product formed
Thermal (60°C)	7 days	99.99	99.99	No degradation product formed
UV (254 nm)	8 h	88.16	99.93	CPF degraded to Seven Imp

* % assay + % impurity

Linearity

The linearity calibration plot was obtained on seven points over the calibration ranges tested (i.e., 0.5–60 µg/mL). The values of correlation coefficient, slope, and intercept were 0.9999, 79943, and 13248, respectively.

Accuracy

To obtain the accuracy of the method, recovery experiments were carried out at three concentration levels (i.e., 40, 50, and 60

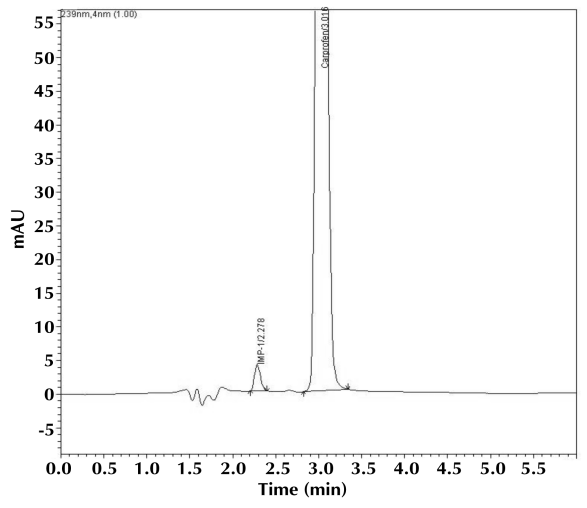


Figure 1. Representative chromatogram obtained from acid hydrolysis solution of CPF. Peaks: 1 = hydrolysis product, 2 = carprofen.

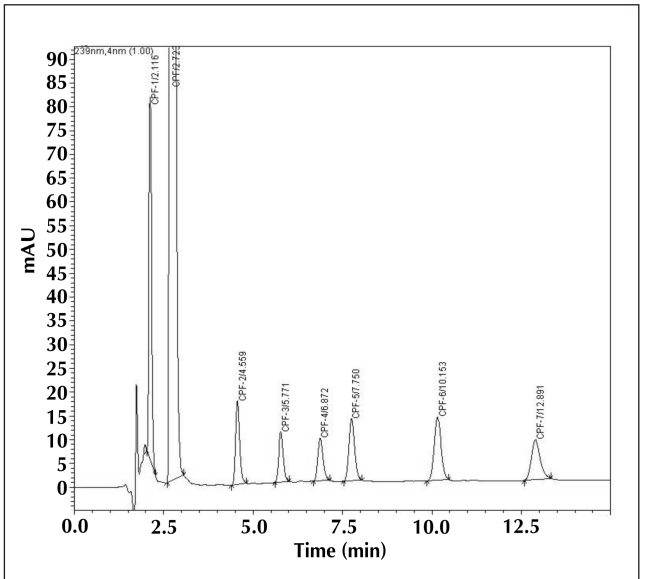


Figure 2. Representative chromatogram obtained on photolysis of CPF.

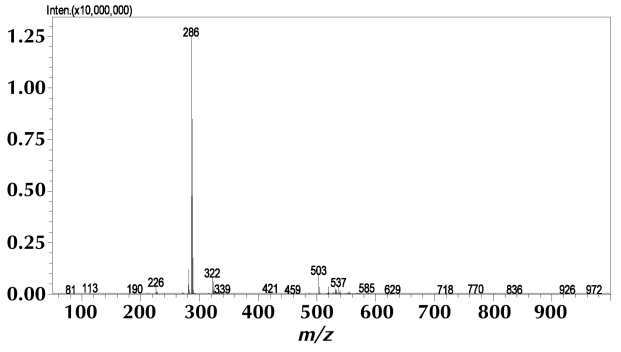


Figure 3. Representative MS of methyl ester formed on acid hydrolysis.

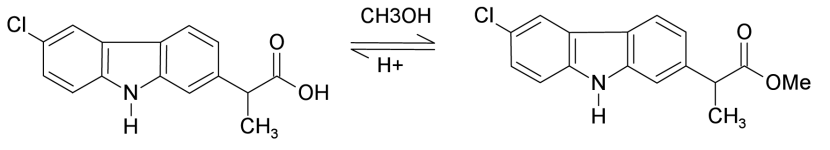
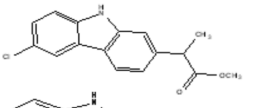
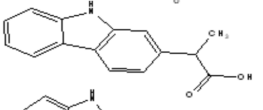
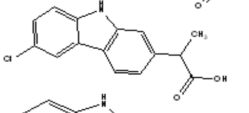
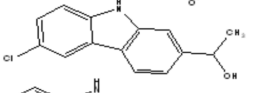
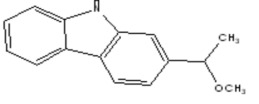
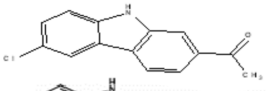
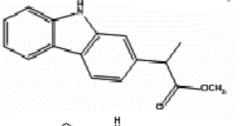
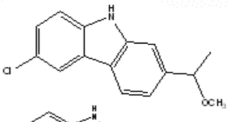
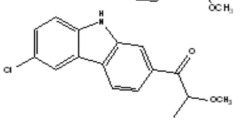


Figure 4. Scheme of esterification reaction of CPF on acid hydrolysis.

Table III. Probable Structures of Degradation Products of CPF

Stress condition	t_R (min)	Resolution	Observed Mole. Weight	Structure
Acid hydrolysis	2.27	4.63	286	
Photolysis	2.12	–	238	
Photolysis	2.72	3.63	272	
Photolysis	4.56	9.23	244	
Photolysis	5.77	5.20	224	
Photolysis	6.87	4.22	242	
Photolysis	7.75	3.06	252	
Photolysis	10.15	7.21	258	
Photolysis	12.89	6.66	286	

µg/mL). The percentage recovery of CPF in bulk drug samples was ranged from 99.2 to 100.7. From this result it was confirmed that the method is remarkably accurate.

Precision

The relative standard deviation (RSD) of CPF during intra-day study was found to be 0.3 and inter-day study was within 0.5. The results confirm the repeatability of the method.

Robustness

In all the deliberate varied chromatographic conditions (flow rate, percentage organic strength, column temperature), good resolution was observed between CPF and its degradation products, illustrating the robustness of method.

Identification of degradation product

CPF contains carboxylic group which indicates that ionisation of sample occurs in the negative polarity. The ionisation of the drug has been done using electrospray ionisation and single quadrupole mass analyser. In acidic condition drug was found to be stable in acetonitrile solution but one impurity was formed as methyl ester in the methanolic solution. Methyl ester formed in methanolic solution shows 286 (M-1) molecular weight confirming esterification of the CPF in acidic condition. The typical mass spectrum is shown in Figure 3. The esterification reaction to form methyl ester in methanolic solution was shown in Figure 4. In photo induced reactions dechlorination was confirmed as impurity shows 238 (M-1) molecular weight and absence of chloro pattern in mass spectrum as compared to parent ion drug. CPF has a molecular weight 273.7 Daltons and it has a chloro group in position 6. The characteristic feature of chloro pattern exhibits evidence for C-Cl cleavage (dechlorination) or for a presence of chlorine with degradents by only careful observation of mass spectrum. The degradents with the retention times 2.12 min, 5.77 min, and 7.75 min have a missing chloro pattern. The structures of remaining degradents having chloro pattern were finally assigned with their mass to charge ratio differences related to the probable variations in the propionic acid side chains. All the degradents retained an intact carbazole ring during the photolytic process. In acid condition esterification and in photolytic degradation dechlorination, decarboxylation, and esterification reactions were confirmed by MS detection. The probable structures of degradation products were elucidated according to their *m/z* ratio as compared to parent ion molecular weight and described in Table III.

Conclusion

A developed and validated stability indicating LC method was carried out for analysis of CPF in presence of its degradation products. This method also provides the information about photo induced reaction on irradiation of UV light and elucidate the probable structures of degradation products by mass spectroscopy. The method is simple, rapid, accurate and can be applicable for qualitative as well as quantitative analysis of CPF in bulk drug and for the characterisation of degradation products of CPF.

References

1. T.F. Yu and J. Perel. Pharmacokinetic and clinical studies of carprofen in gout. *J. Clin. Pharmocol.* **20:** 447–451 (1980).
2. R.L. Williams, D.E. Furst, H.G. Mandel, D. Nicol, J.J. KoniKoff, and L.Z. Benet. Effects of indomethacin and carprofen on renal homeostasis in rheumatoid arthritis patients and in healthy individuals. *J. Clin. Pharmacol.* **21:** 493–500 (1981).
3. W.M. O'Brien and G.F. Bagby. Carprofen: a new nonsteroidal anti-inflammatory drug. Pharmacology, clinical efficacy and adverse effect. *Pharmacothearapy* **7:** 16–24 (1987).
4. L.B. Katz, R.J. Capetla, D.C. Argentieri, L.E. Moore, T. Gena, M.C. Porter, V. Justy, R.E. Hartnagel, D. Abrutyn, and D.A. Shriver. Rioprostil prevents gastric bleeding induced by non steroidal anti-inflammatory drugs in dogs and arthritis rats. *J. Rheumatol.* **13(5):** 887–894 (1986).
5. G. Hadad. Validated stability indicating LC method for analysis of Pipenzolate bromide its hydrolysis product. *J. Chromatographia* **68:** 207–214 (2008).
6. R.T. Satyanarayana, M. Ravikumar, K.K. Vijay, K.S.V. Raghavachary, V.M. Satish, R.C.S.N. Malleswara, and S.P. Yadagiri. A stability indicating LC method for Rimonabant. *J. Chromatographia* **69:** 97–102 (2009).
7. K.R. Patil, V.P. Rane, J.N. Sangshetti, and D.B. Shinde. A. stability indicating LC method for Lumefantrine. *J. Chromatographia* **69:** 375–379 (2009).
8. B. Liunggren. Propionic acid derived non-steroidal anti-inflammatory drugs are phototoxic in vitro. *Photodermatology* **2:** 3–9 (1985).
9. B. Liunggren and K. Lundberg. In vivo phototoxicity of non steroidal anti-inflammatory drugs evaluated by mouse tail technique. *Photodermatology* **2:** 372–377 (1985).
10. B. Scheuer and H. Kietzmann. Photoallergic reaction to carprfen. *Allergologie* **9:** 131–136 (1986).
11. M.-T. Sheu, H.-O. Ho, P.-Y. Wang, Y.-B. Liou, and A.-B Wu. Photolysis of NSAIDs. I. Photodegradation product of carprofen determined by LC-ESI-MS. *J. Chromatogr. Sci.* **41:** 200–204 (2003).
12. F.-A. Chen, P.-Y. Wang, K.-C. Wen, C.-Y. Chen, and A.-B. Wu. Photolysis of NSAIDs. II. Online LC-MS determination of photodegradants from carprofen. *J. Food Drug Anal.* **11:** 186–190 (2003).
13. A.-B. Wu, C.-Y Cheng, S.-D. Chen, Y.-C. Tsai, and F.-A. Chen. Stability-indicating high performance liquid chromatographic assay method and photostability of carprofen. *J. Chromatogr. Sci.* **39:** 7–11 (2001).

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