

A Simple and Sensitive HPLC Method For Quantification of the Metabolin of Meclofenoxate In Human Plasma

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Abstract

A simple and sensitive high-performance liquid chromatographic method was developed for quantification of the metabolin of meclofenoxate, chlorophenoxyacetic acid, in human plasma. Ibuprofen was used as an internal standard. The present method used protein precipitation for extraction of chlorophenoxyacetic acid from human plasma. Separation was carried out on a reversed-phase C₁₈ column. The column effluent was monitored by UV detection at 254 nm. The mobile phase was a mixture of methanol and water containing 1.0% glacial acetic acid (70:30 v/v) at a flow rate of 1.0 mL/min. The column temperature was 20°C. This method was linear over the range of 0.047–28.20 µg/mL with a regression coefficient greater than 0.99. The mean recovery of chlorophenoxyacetic acid and IS were (79.54 ± 6.33)% and (78.48 ± 2.14)%, respectively, and the method was found to be precise, accurate, and specific during the study. The method was successfully applied for pharmacokinetic study of chlorophenoxyacetic acid in human.

Introduction

Meclofenoxate hydrochloride, 4-chlorophenoxy-acetic acid 2-(dimethylamino) ethyl ester hydrochloride (Figure 1), belongs to the group of psychostimulants-nootropic agents (1–7). Preliminary test results showed that meclofenoxate could not be detected in plasma at all after oral administration because meclofenoxate, the pro-drug of chlorophenoxyacetic acid (Figure 1) is absorbed from the gastrointestinal tract, then rapidly hydrolyzed to active metabolite chlorophenoxyacetic acid by nonspecific esterases in the intestinal mucosa and distributed into the blood (8–10). Chlorophenoxyacetic acid has been reported to be effective in activating action on the brain, improving the neural cell metabolism. In long-term use, chlorophenoxyacetic acid recovers the functionally injured brain cells. Meclofenoxate is used for the following conditions: senile dementia; skull and brain trauma; following a stroke;

encephalopathy; mental disorders (in combination with psychotropic agents); psychomotor retardation; brain intoxication; alcohol psychoses; neuritis, and polyneuritis. There have been no published reports on the investigation of the pharmacokinetic properties in human plasma so far. The goal of the present work was focused on developing a simple and sensitive high-performance liquid chromatographic (HPLC) method for the determination of chlorophenoxyacetic acid in human plasma. The advantages of the present method include small sample volumes, single-step extraction procedure using inexpensive chemicals, short run time, high sensitivity, and selectivity. The development of the extraction procedure was carried out by comparing protein precipitation, solid-phase extraction (SPE), and liquid–liquid extraction (LLE) for recovery and interference. Protein precipitation was selected in the present study. The pharmacokinetic study of chlorophenoxyacetic acid in human plasma has been conducted after validation of the proposed method.

Experimental

Chemicals and reagents

The meclofenoxate hydrochloride capsule (test formulation, each capsule containing 100 mg meclofenoxate), meclofenoxate hydrochloride (99.5%), chlorophenoxyacetic acid (99.3%), and ibuprofen [Figure 1, internal standard (IS), 99.3%] reference standards were procured from JianMa Pharmaceutical

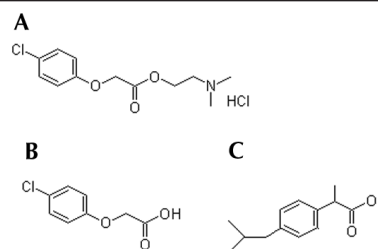


Figure 1. Chemical structure of (A) meclofenoxate hydrochloride, (B) chlorophenoxyacetic acid, and (C) ibuprofen.

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Corporation (Hang Zhou, China). HPLC-grade methanol were purchased from Tedia (Fairfield, OH). Milli-Q Water from Millipore's Milli-Q System was used throughout the pre-study validation and subject sample analysis (Billerica, MA). Other chemicals and reagents were of analytical-grade and were purchased from Nanjing Chemical Reagent Company (Nanjing, China). Drug-free and drug-containing plasma were taken from healthy volunteers. Plasma was stored at -70°C until assayed.

Instrumentation chromatographic conditions

The chromatographic system (model 1200, Agilent, Santa Clara, CA) consisted of a high-pressure pump, an autosampler, a column thermostat, and a UV detector (1200 Series Variable Wavelength Detector SL, Agilent). Separation was carried out on an Agilent Zorbax C_{18} reversed-phase column (150 mm \times 4.6 mm, 5 μm) through an Agilent Security Guard C_{18} 4 \times 3-mm precolumn cartridge with a flow rate of 1.0 mL/min. The mobile phase used was a mixture of methanol and water containing 1.0% glacial acetic acid (70:30 v/v). The mobile phase was filtered through a 0.22- μm membrane filter and degassed by ultrasonication before use. The column temperature was 20°C . The injection volume was 10.0 μL , and the UV detector was set at 254 nm.

Preparation of the calibration standards and QC samples

The stock solutions of chlorophenoxyacetic acid and ibuprofen were prepared in methanol at a concentration of 0.94 mg/mL and 0.40 mg/mL, respectively. The working solutions of 564.0, 400.0, 282.0, 94.0, 30.0, 28.2, 9.4, 2.82, 2.00, and 0.94 $\mu\text{g}/\text{mL}$ were prepared by appropriately diluting the stock solutions of chlorophenoxyacetic acid in methanol. Chlorophenoxyacetic acid working solutions were used to prepare the calibration standards for construction of seven-point calibration curve (0.047–28.2 $\mu\text{g}/\text{mL}$) and QC samples at three different levels (0.10, 1.50, 20.00 $\mu\text{g}/\text{mL}$). All the stock and working solutions were refrigerated (2 – 5°C) when not in use. Calibration standards and QC samples were prepared in bulk by spiking 25.0 μL of respective working solutions to 475.0 μL of control human plasma and then aliquoted. These were stored at -70°C until analysis.

Sample preparation

200 μL of plasma and 5.0 μL of IS (400.0 $\mu\text{g}/\text{mL}$ ibuprofen) in methanol were added and vortexed to mix. 700 μL of methanol was added for precipitation of protein in plasma, vortexed for 3 min, and centrifuged at $3500 \times g$ for 5 min at 22°C . The supernatant was transferred to a vial, and 10- μL aliquots were injected to the HPLC system for analysis.

Method validation

The method has been validated for selectivity, sensitivity, recovery, linearity, precision, accuracy, and stability.

Selectivity is the ability of the analytical method to differentiate and quantify the analyte in the presence of other components in the sample. This test was performed by analyzing the blank human plasma samples from six different batches of human plasma to test for interference at the retention time of chlorophenoxyacetic acid and IS.

Sensitivity was determined by analyzing control human plasma in replicates ($n = 5$) spiked with the analyte at the lowest level of the calibration standard, which is 0.047 $\mu\text{g}/\text{mL}$. The lower limit of quantitation (LLOQ) could be accurately (relative error $< 20\%$) and reproducibly (relative standard deviation, RSD $< 20\%$) determined.

Accuracy and precision of the QC samples were calculated using the calibration curve. The model for the calibration curve of chlorophenoxyacetic acid used the peak-area ratio of chlorophenoxyacetic acid to ibuprofen (y) and the chlorophenoxyacetic acid concentration (C), as given in the following equation $y = \text{slope} \times C + (y\text{-intercept})$. Chlorophenoxyacetic acid concentrations were estimated from y using the formula: $C = [y - (y\text{-intercept})]/\text{slope}$. Intra-day precision was evaluated by analyzing the spiked controls five times over one day in random order while inter-day precision was evaluated from the analysis of controls one each in three different days. Assay precision (RSD) was assessed by expressing the standard deviation of the measurements as a percentage of the average value. The accuracy was estimated for each spiked control by comparing the nominal concentration with the assayed concentration.

The recovery of chlorophenoxyacetic acid was determined for QC sample (at concentrations of 0.10, 1.50, 20.00 $\mu\text{g}/\text{mL}$) and for IS the recovery was determined at a concentration of 10.0 $\mu\text{g}/\text{mL}$. Three replicates of each QC sample were extracted by the previously mentioned sample preparation and injected into the HPLC system. The extraction recovery at each concentration was calculated using the following equation: Recovery = Peak area after extraction/Peak area after direct injection $\times 100$.

The stability of chlorophenoxyacetic acid in solution as well as plasma matrix was evaluated. The stock solution stability was evaluated at room temperature (22°C) for 6 h and at 2 – 5°C for 20 days and these were compared with freshly prepared stock solution. The stability of spiked human plasma stored at room temperature (22°C) was evaluated for 6 h and compared with freshly prepared extracted samples. The freeze-thaw stability was conducted by comparing the stability samples, which had been frozen and thawed three times with freshly prepared QC samples. The long-term stability was conducted by analyzing low quality control (LQC), medium quality control (MQC), and high quality control (HQC) samples stored at -70°C for 20 days and compared with freshly prepared QC samples. All stability evaluations were based on back-calculations from the calibration curves.

Pharmacokinetic evaluation

The method described earlier was applied to the pharmacokinetic study in which plasma concentrations of chlorophenoxyacetic acid in 24 healthy Chinese male volunteers were determined up to 24 h after oral administration of meclufenoxate hydrochloride capsules containing 200 mg meclufenoxate. Model-independent pharmacokinetic parameters were calculated for chlorophenoxyacetic acid. The maximum plasma concentrations (C_{max}) and the corresponding times (T_{max}) were noted directly. Blood was sampled pre-dose and at 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 14, and 24 h post-dose for determination of concentration of chlorophenoxyacetic acid. Plasma samples were obtained following centrifugation of blood at $1500 \times g$ for 10 min at 4°C and kept frozen at -70°C until analysis. The

clinical pharmacokinetic study was approved by the Ethic Committee of Nanjing First Hospital of Nanjing Medical University. All volunteers gave written informed consent to participate in the study according to the principles of the Declaration of Helsinki.

Result and Discussion

LLE was attempted using various organic solvents like diethyl ether, ethyl acetate, etc. because the chlorophenoxyacetic acid is hydrophobic in nature. It resulted in good extraction efficiency with these organic solvents. But LLE method has the chance of formation of emulsions in two immiscible phases and requires an evaporation step prior to analysis to remove excess of organic solvent, which is a time-consuming process as compared to other extraction process. SPE can be carried out to achieve the higher extraction efficiency. The extraction efficiency is reproducible and also devoid of interferences. The recovery of SPE is higher, but it is found to be expensive and time-consuming. Hence, the present study employed protein precipitation for extraction of the drug from human plasma, which had obvious advantages such as shorter processing time, less organic solvent consumption, fewer steps, good plasma sample clean up, low cost, and high recovery. Methanol was found to be the most suitable organic precipitant in the present study.

Table I. Inter-run Accuracy and Precision of Plasma Calibration Standards for Chlorophenoxyacetic acid ($n = 5$)

Standard conc. ($\mu\text{g/mL}$)	Average calculated conc. ($\mu\text{g/mL}$)	RSD (%) [*]	RE (%) [*]
0.047	0.045	7.93	5.28
0.141	0.132	7.91	6.16
0.47	0.45	5.66	3.45
1.41	1.38	2.74	2.37
4.7	4.80	3.35	-2.08
14.1	13.97	1.76	0.91
28.2	28.44	0.63	-0.85

* RSD = relative standard deviation, RE = relative error.

Table II. Intra-day and Inter-day Accuracy and Precision of Chlorophenoxyacetic Acid in Human Plasma

Added conc. ($\mu\text{g/mL}$)	Average calculated conc. ($\mu\text{g/mL}$)	RSD (%) [*]	RE (%) [*]
<i>Inter-day (n = 5)</i>			
0.10	0.105 \pm 0.0077	7.32%	-5.13
1.50	1.48 \pm 0.046	3.13%	1.56
20.0	19.59 \pm 0.39	1.98%	2.07
<i>Intra-day (n = 3)</i>			
0.10	0.095 \pm 0.0049	5.16%	5.00
1.50	1.43 \pm 0.0343	2.40%	4.67
20.00	19.65 \pm 0.34	1.73%	1.75

* RSD = relative standard deviation, RE = relative error.

Selectivity

No interfering endogenous compound peak was observed at the retention time of analytes and IS. Under chromatographic conditions described earlier, the retention time of chlorophenoxyacetic acid, meclufenoxate, and IS were 4.1, 4.5, and 5.1 min, respectively. Representative chromatograms are shown in Figure 2.

Sensitivity (LLOQ)

The sensitivity of the experiment was carried out at LLOQ level (0.047 $\mu\text{g/mL}$). The average percentage deviation from the nominal concentration was less than 8.3%, and the precision was within 2.2 % RSD.

Linearity

The calibration curves were linear over the range of 0.047–28.20 $\mu\text{g/mL}$. The correlation coefficient was 0.9969. The mean (\pm standard deviation, SD) slope of the calibration curves for chlorophenoxyacetic acid was 0.206 (\pm 0.014). The mean intercept of calibration curves for chlorophenoxyacetic acid was 0.00339 (\pm 0.00107). Calibration curve data of chlorophenoxyacetic acid are listed in Table I.

Precision and accuracy

Both intra-day and inter-day accuracy and precision of the method were determined by analysis of the control human plasma spiked with chlorophenoxyacetic acid at LQC, MQC, and HQC. All QC concentrations were calculated using the calibration curve. The accuracy and precision of the method were described as a percentage bias and the percentage RSD, respectively. The inter-day bias was \leq 5.13%, and the inter-day precision

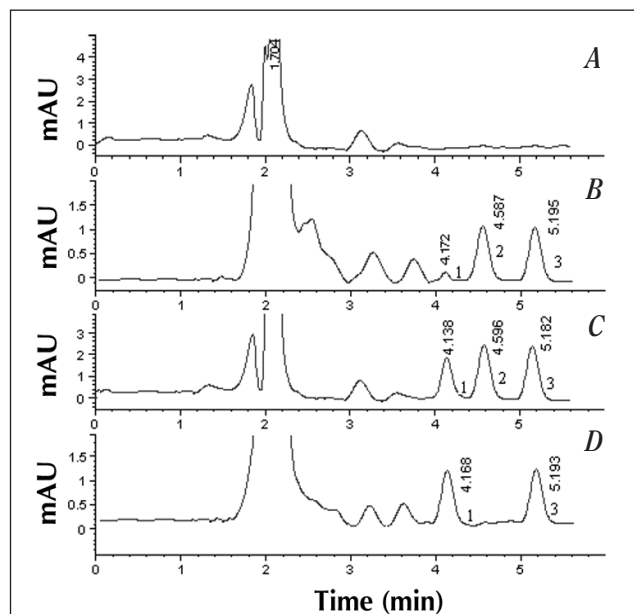


Figure 2. Typical chromatogram of blank plasma (A), LLOQ for chlorophenoxyacetic acid in plasma (0.047 $\mu\text{g/mL}$), meclufenoxate, and IS (B), plasma spiked with chlorophenoxyacetic acid (0.94 $\mu\text{g/mL}$), meclufenoxate, and IS (C), and plasma obtained from a volunteer at 0.67 h after oral administration of 200 mg meclufenoxate (D). 1, chlorophenoxyacetic acid; 2, meclufenoxate; and 3, IS.

was $\leq 7.32\%$ at all QCs. The intra-day deviation from the nominal concentration was $\leq 5.00\%$, and the intra-day precision was $\leq 5.16\%$ at all QCs. The result of accuracy and precision are shown in Table II.

Recovery

The mean absolute recovery (\pm SD) of chlorophenoxyacetic acid at LQC, MQC, and HQC was $79.54 \pm 6.33\%$. The recovery of ibuprofen was found to be $78.48 \pm 2.14\%$, and the results of recovery studies are shown in Table III.

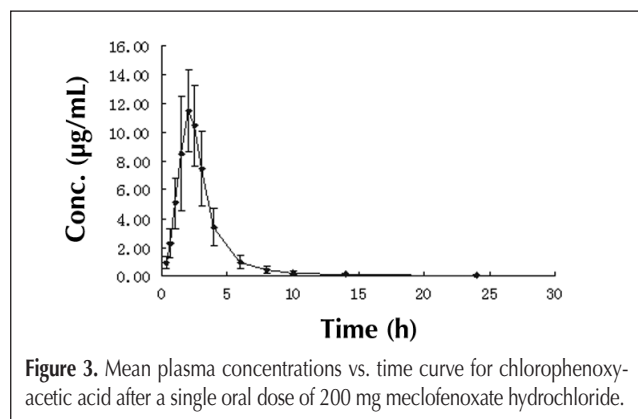


Figure 3. Mean plasma concentrations vs. time curve for chlorophenoxyacetic acid after a single oral dose of 200 mg meclofenoxate hydrochloride.

Table III. Recovery Data for Chlorophenoxyacetic Acid and IS ($n = 5$)*

Plasma conc. of CPA [†] (µg/mL)	Recovery of extraction (%) (mean \pm SD)	Plasma conc. of IS (µg/mL)	Recovery of extraction (%)
0.10	72.32 \pm 4.95		76.21
1.50	84.12 \pm 10.53		80.45
20.0	82.17 \pm 4.16	10.0	78.78
Mean \pm SD*	79.54 \pm 6.33		78.48 \pm 2.14
RSD (%)*	7.95		3.72

* SD = standard deviation, RSD = relative standard deviation.
[†] Chlorophenoxyacetic acid = CPA A

Table IV. Stability Sample Results for Chlorophenoxyacetic Acid ($n = 5$)

Stability	Spiked conc. (µg/mL)	Average calculated comparison sample conc. (µg/mL)	Average calculated stability sample conc. (µg/mL)	Average percentage (%)
Benchtop*	0.10	0.105 \pm 0.0063	0.101 \pm 0.0048	96.19
	1.50	1.47 \pm 0.064	1.43 \pm 0.087	97.28
	20.0	19.23 \pm 0.86	18.26 \pm 0.64	94.96
Freeze and thaw [†]	0.10	0.112 \pm 0.0068	0.105 \pm 0.0051	93.75
	1.50	1.55 \pm 0.074	1.53 \pm 0.089	98.71
	20.0	19.83 \pm 0.53	19.48 \pm 0.66	98.23
Long-term [‡]	0.10	0.108 \pm 0.0101	0.105 \pm 0.0049	97.22
	1.50	1.39 \pm 0.089	1.31 \pm 0.076	94.24
	20.0	19.07 \pm 0.36	18.11 \pm 0.65	94.97

* After 6 h at room temperature (22°C).
[†] After three freeze-and-thaw cycles at -70°C .
[‡] At -70°C for 20 days.

Stability

Analysis of the stock solution was performed at 28.2 µg/mL. After storage for 20 days at $2-5^\circ\text{C}$ and at room temperature (22°C) for 6 h, more than 96.56% of chlorophenoxyacetic acid remained unchanged, based on peak areas in comparison with freshly prepared solution of chlorophenoxyacetic acid (28.2 µg/mL). This suggests that the chlorophenoxyacetic acid in standard solution is stable for at least 20 days when stored at $2-5^\circ\text{C}$ and for 6 h at room temperature (22°C).

Benchtop stability of chlorophenoxyacetic acid in plasma was investigated at LQC, MQC, and HQC levels. This revealed that the chlorophenoxyacetic acid in plasma was stable for at least 6 h at room temperature (22°C) with average percentage of $\geq 94.96\%$. It was confirmed that repeated freezing and thawing (three cycles) of plasma samples spiked with chlorophenoxyacetic acid at LQC, MQC, and HQC levels did not affect the stability of chlorophenoxyacetic acid as the average percentage of $\geq 93.75\%$ was obtained. Long-term stability of the chlorophenoxyacetic acid in plasma at -70°C was also performed after 20 days of storage at LQC, MQC, and HQC levels, which showed mean percentage concentration of $\geq 94.24\%$. The results of the stability studies are shown in Table IV. The previous results indicate that the chlorophenoxyacetic acid is stable in the studied conditions.

Application

The plasma concentrations versus time profiles of chlorophenoxyacetic acid after a single oral dose of 200 mg meclofenoxate hydrochloride are shown in Figure 3. The C_{max} and T_{max} values were obtained from plasma concentration vs. time curves with interpolation. The area under the plasma concentration versus time curve (AUC_{0-24} , $AUC_{0-\infty}$) and $t_{1/2}$ were estimated by using DAS2.0 software (HeFei, China). The pharmacokinetic parameters in human were estimated based on the mean concentration versus time curve. Data from PK analysis are summarized in Table V.

Conclusion

A simple and sensitive method for the determination of chlorophenoxyacetic acid in human plasma by HPLC was developed and validated. The method consisted of sample preparation by protein precipitation followed by chromatographic separation and UV detection. No interfering peaks were observed at the elu-

Table V. Pharmacokinetic Parameters of Chlorophenoxyacetic Acid After a Single Oral Dose of 200 mg Meclofenoxate Hydrochloride*

S.R. no.	Parameters	Values
1	C_{max} (µg/mL)	12.6 \pm 2.8
2	T_{max} (h)	2.1 \pm 0.4
3	$t_{1/2}$ (h)	5.8 \pm 2.7
4	AUC_{0-24} (µg/mL \times h)	34.1 \pm 7.9
5	$AUC_{0-\infty}$ (µg/mL \times h)	34.8 \pm 7.7

* Mean \pm SD, $n = 24$.

tion times of chlorophenoxyacetic acid and IS. Adequate specificity, precision, and accuracy of the proposed method were demonstrated over the concentration range of 0.047–28.2 µg/mL. The method was accurate, reproducible, specific, and applicable to the analysis and evaluation of pharmacokinetic profiles of chlorophenoxyacetic acid in human.

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