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# Determination of mephenoxalone in human plasma sample by high-performance liquid chromatography-fluorescence detection

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#### Abstract

A simple and sensitive high-performance liquid chromatographic method involving fluorescence detection was developed for the determination of mephenoxalone in human plasma. A Cosmosil 5C18-MS column (250 mm×4.6 mm I.D., 5  $\mu$ m) was used as stationary phase and the mobile phase consisted of water–acetic acid–acetonitrile (200:1:300) at a flow-rate of 1.0 ml/min. The fluorescence absorbance was monitored at 280 nm for excitation wavelength and 310 nm for emission wavelength. Temperature control was kept at 40°C for the column. The limit of quantitation achieved was 10 ng/ml, and the standard curve was found to be linear in the concentration range of 10–10 000 ng/ml. Under these analytical conditions, a sufficient mephenoxalone plasma concentration profile could be obtained for pharmacokinetic study. © 2001 Elsevier Science B.V. All rights reserved.

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#### 1. Introduction

Mephenoxalone, as Fig. 1 shows, is a tranquilizer of the propanediol type. It has neuropharmacologic activity in laboratory animals. Clinical studies indicate that mephenoxalone has a therapeutic effect on anxiety disorders and serves as a muscle relaxant in the treatment of muscle spasm in human subjects [1].

There are several assay methods for the determination of mephenoxalone concentration in tablets. These methods involve direct measurement of mephenoxalone in solution by UV spectrophoto-

metric or fluorimetric analysis, measurement of mephenoxalone substance by differential scanning calorimetry, and measurement of the hydrolytic product of mephenoxalone by a potentiometric method

Mephenoxalone

Estradiol

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Fig. 1. Structures of mephenoxalone and internal standard (estradiol).

[2–4]. None of these methods contain a separation procedure. Only the method described by Jancik et al. was applied to the determination of mephenoxalone in biological samples [3]. For pharmacokinetic research, it is important to have both sufficient assay specificity and sensitivity for mephenoxalone in biological samples. However, these reported methods are considered to have insufficient specificity and sensitivity for the determination of mephenoxalone concentration in biological samples due to an inherent interference by endogenous substances or metabolites.

Therefore, the purpose of this study is to report a simple, sensitive and reproducible high-performance liquid chromatography (HPLC) method for the determination of mephenoxalone concentration in human plasma.

## 2. Experimental

#### 2.1. Chemicals and reagents

Mephenoxalone and estradiol were obtained from Chemagis (Ramat-Hovav, Israel) and Sigma (St. Louis, MO, USA), respectively. HPLC-grade acetonitrile and analytical-grade acetic acid were purchased from BDH (Poole, UK) and E. Merck (Darmstadt, Germany), respectively. Hexane and dichloromethane were GR grade and purchased from E. Merck. All other chemicals were analytically graded and used without further purification.

#### 2.2. Apparatus and chromatographic conditions

The HPLC system consisted of a Hitachi Model L-7100 pump, a Model L-7200 autosampler, a Model L-7480 fluorescence detector, and a Model L-7000 HPLC system manager as data processor (Hitachi, Japan), and a Model Super CO-150 column oven (Enshine Scientific, Taiwan). Separation was achieved on a Cosmosil 5C18-MS column (250 mm $\times$ 4.6 mm I.D., 5  $\mu$ m; Nacalai Tesque, Japan). The column eluent was monitored with a fluorescence detector (280 nm for excitation wavelength and 310 nm for emission wavelength). The mobile phase was water–acetic acid–acetonitrile (200:1:300) at a flow-rate of 1.0 ml/min which was

filtered and degassed before use. The column temperature was set at  $40^{\circ}$ C.

## 2.3. Solution preparation

A stock solution (1 mg/ml) of mephenoxalone was prepared by dissolving 10 mg of mephenoxalone in 10 ml of acetonitrile—water (1:1, v/v) solution and stored at  $-80^{\circ}$ C. Working solutions were also prepared in the acetonitrile—water (1:1, v/v) solution at concentrations of 0.1, 1, 10 and 100  $\mu$ g/ml and stored at  $4^{\circ}$ C.

#### 2.4. Sample preparation

The sample preparation consisted of a single liquid–liquid extraction procedure. A 40- $\mu$ l volume of estradiol solution [2 ng/ml in acetonitrile–water (3:1, v/v) solution] as an internal standard was added to 0.2 ml of plasma sample. A 3-ml volume of hexane–dichloromethane mixing solution (7:3, v/v) as extraction solvent was added to the sample and vortex-mixed for 30 s. After centrifugation for 10 min at 1945 g, the supernatant was transferred to a clean tube and evaporated to dryness under a stream of nitrogen at ambient temperature. Then, 200  $\mu$ l of mobile phase was added and vortex-mixed for 30 s. Finally, 20  $\mu$ l was injected onto the HPLC system.

## 2.5. Freeze and thaw stability

Freeze and thaw stability for mephenoxalone in plasma samples was studied in three cycles with two concentrations (40 and 8000 ng/ml) in six batch plasmas. Samples were frozen at  $-80^{\circ}$ C for at least 2 h and then thawed to room temperature for another at least 2 h.

### 2.6. Standard curve

A standard curve was prepared by adding 20  $\mu$ l of 0.1  $\mu$ g/ml, and 4, 10 and 20  $\mu$ l of 1, 10 and 100  $\mu$ g/ml of mephenoxalone working solution, respectively, to 0.2 ml of blank plasma and prepared according to the aforementioned sample preparation procedure. The concentrations used were 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 and 10 000 ng/ml. The peak height ratio of mephenoxalone to estradiol

was plotted against the concentration of mephenoxalone added. Linearity was determined by weighted linear regression analysis  $(1/x^2)$ . The concentration of mephenoxalone in the test samples was calculated using the regression parameters obtained from the standard curve.

#### 2.7. Accuracy and precision

Four different concentrations of mephenoxalone (10, 40, 600 and 8000 ng/ml) were added to drug-free plasma and the concentrations were determined using the corresponding standard curves. The accuracy of the method was shown as relative error and calculated based on the difference between the mean calculated and added concentrations, while precision was evaluated by calculating the within- and between-run relative standard deviations (RSDs).

#### 2.8. Recovery

Triplicate human plasma samples at five concentrations (10, 40, 600, 8000 and 10 000 ng/ml) were prepared. The internal standard solution was added to reconstitute the residues of plasma samples after sample preparation. Solutions of mephenoxalone were spiked into the internal standard solution, representing 100% recovery, at the same volume as those spiked into plasma samples and prepared in triplicate. After analyzing by HPLC, the absolute recovery for mephenoxalone in human plasma after preparation procedure was determined by comparing the mean peak height ratio of mephenoxalone in human plasma (called plasma sample) to that of mephenoxalone in internal standard solution (called solvent sample).

## 2.9. Pharmacokinetic study

Four healthy volunteers were selected to enroll in the study. Complete physical examinations, blood chemistry, urinalysis and hematological evaluations, and medical history were obtained from the subjects prior to and after to the study, together with chest X-rays and EKGs. Written informed consent were also obtained from each prospective subject prior to the study. No smoking, alcohol and caffeine consumption was permitted for at least 48 h before and

during the study. No strenuous activity was allowed. Water was limited from 1 h pre-dose and until 2 h post-dose of each study phase. The subjects were required to fast for 12 h prior to each study session until 4 h post-dose.

After oral ingestion of one 200 mg of Dorsiflex tablet (Syntex Pharm, Allschwil/Basel, Switzerland), blood samples (10 ml each) were collected with coded vacutainers (green top, containing heparin as anticoagulant) at the following times: 0 (pre-drug), 0.33, 0.67, 1, 1.33, 1.67, 2, 2.5, 3, 4, 6, 9, 12, 14 and 23 h. The blood samples were centrifuged for 10 min at 1945 g, immediately. The plasma for each sample was separated and aliquotted into polypropylene tubes stored at  $-80^{\circ}$ C in a freezer until analysis.

#### 3. Results and discussion

Mephenoxalone contains a 2-methoxyphenoxy functional group in its molecule as shown in Fig. 1. This functional group makes it fluorescence-absorbing. For analysis of biological samples, applying fluorescence detection would be a good choice for its specificity. In the development of our analytical method, HPLC involving fluorescence detection was used. The monitoring wavelengths were set at 280 nm for excitation and 310 nm for emission. Typical chromatograms are shown in Fig. 2. No significant endogenous peak co-eluted with the mephenoxalone as shown in the corresponding chromatogram of drug-free plasma. In addition, the baseline is very smooth and no interference peak is shown in the chromatogram except for the front peak. The retention times for mephenoxalone and internal standard are 3.1 and 4.7 min, respectively. The analytical time was only 8 min for a sample analysis.

The standard curve for mephenoxalone was made using 10 spiking plasma samples over a concentration range of 10-10~000~ng/ml. In order to cover the concentration range and to increase the sensitivity without sacrificing precision and accuracy, it was necessary and advisable to use weighted linear regression analysis. A linear relationship was obtained in the concentration range. Corresponding correlation coefficients  $(r^2)$  were over 0.99 from each of six different standard curves in one run (within-run) and from each standard curve on six

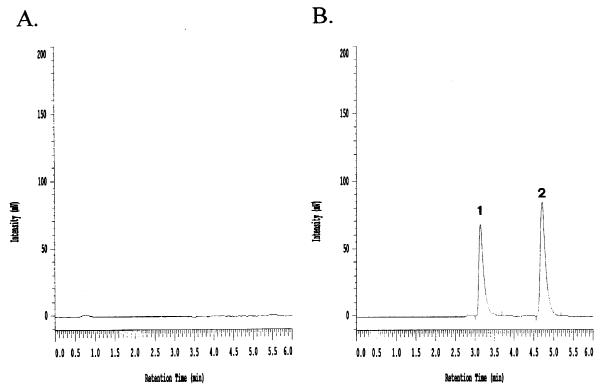


Fig. 2. Typical chromatograms of (A) drug-free plasma, and (B) a plasma sample taken 2 h after oral administration of mephenoxalone to a healthy volunteer. Peaks: 1=mephenoxalone; 2=internal standard.

separate runs (between-run). The regression parameters (n=6) were y=0.00100x-0.00024 for withinrun and y = 0.00097x - 0.00060 (y: peak height ratio; x: spiking concentration). The relative standard deviations of the slope for six different standard curves were 0.9% for within-run and 4.4% for between-run. The within-run precision and accuracy of standard curve were evaluated with each of standard curve concentration point after back-calculation. As Table 1 shows, RSDs ranged from 2.8 to 6.6% and relative error from -3.1 to 6.0%. The between-run precision and accuracy of standard curve were also evaluated with each standard curve concentration point after back-calculation. As Table 2 shows, RSDs ranged from 1.6 to 5.9% and relative error from -1.4 to 2.5%. These results indicate that the standard curve had a good linearity after weighted linear regression analysis and was reproducible. It also shows that the method permits the determination of mephenoxalone in plasma over a relatively wide range of concentrations.

The criteria for the determination of the limit of quantitation in plasma is based on signal-to-noise, the reproducibility of the response and the variability of the back-calculated concentration. Spiked plasma samples with a final concentration of 10 ng/ml were prepared and analyzed and were found to have a signal-to-noise ratio of ≥5 for both within-run and between-run validation (n=6). The mean peak height ratio and standard deviation were 0.009453 and 0.000771 for within-run validation, and 0.009045 and 0.000581 for between-run validation, respectively. The mean peak height ratio was greater than three standard deviations and the relative standard deviations of the peak height ratio were only 8.2 and 6.4% for within-run and between-run validations, respectively. The relative standard deviation and relative error of the back-calculated concentration were 4.6

Table 1 Reproducibility of the standard curve (n=6)

Spiking plasma concentration (ng/ml)	Within-run			Between-run		
	Concentration calculated (mean±SD) (ng/ml)	RSD <sup>a</sup> (%)	Relative error <sup>b</sup> (%)	Concentration calculated (mean±SD) (ng/ml)	RSD <sup>a</sup> (%)	Relative error <sup>b</sup> (%)
10	9.69±0.45	4.6	-3.1	9.95±0.31	3.1	-0.5
20	$21.2 \pm 1.4$	6.6	6	$20.5 \pm 1.2$	5.9	2.5
50	$50.4 \pm 1.8$	3.6	0.8	$49.8 \pm 1.3$	2.6	-0.4
100	$97.7 \pm 3.8$	3.9	-2.3	$99.0\pm2.5$	2.5	-1
200	199±12	6	-0.5	$203 \pm 6$	3	1.5
500	$498 \pm 21$	4.2	-0.4	$493 \pm 8$	1.6	-1.4
1000	$1004 \pm 28$	2.8	0.4	$1006 \pm 21$	2.1	0.6
2000	$2005 \pm 74$	3.7	0.3	$2030 \pm 56$	2.8	1.5
5000	$4964 \pm 192$	3.9	-0.7	$4954 \pm 139$	2.8	-0.9
10 000	$9882 \pm 304$	3.1	-1.2	$10.039\pm245$	2.4	0.4

<sup>&</sup>lt;sup>a</sup> RSD= $100\% \times (SD/mean)$ .

and -3.1% for within-run validation, and 3.1 and -0.5% for between-run validation, respectively, as Table 1 shows. These results indicate that the limit of quantitation is 10 ng/ml and that the variability is less than 20%, which shows that the limit of quantitation has acceptable accuracy, precision and reproducibility.

Within- and between-run accuracy and precision were examined by performing replicate analyses of plasma samples (n=6) to which four different known concentrations of mephenoxalone had been added. As Table 2 shows, the within-run precision was between 1.5 and 4.0% over the concentrations examined. In addition, between-run precision was

between 1.8 and 5.8%. The accuracy for within-run and between-run was from -1.5 to 1.5% and from -0.6 to 3.0%, respectively, over the concentrations examined. These results show that the method has both good reproducibility and accuracy.

To obtain good extraction efficiency and remove interfering peak, a hexane-dichloromethane (7:3, v/v) mixture was used as extraction solvent. The result indicated that no significant endogenous peak was coeluted with the mephenoxalone as shown in the corresponding chromatogram of drug-free plasma (Fig. 2A). The absolute recovery for mephenoxalone in plasma after the extraction procedure was found to be 68.6%, as shown in Table 3. It indicates that the

Table 2
Precision and accuracy (analysis with spiking plasma samples at four different concentrations)

Spiking plasma	Within-run			Between-run		
concentration (ng/ml)	Concentration measured (mean±SD) (ng/ml)	RSD <sup>a</sup> (%) (n=6)	Relative error <sup>b</sup> (%)	Concentration measured (mean±SD) (ng/ml)	RSD <sup>a</sup> (%) (n=12)	Relative error <sup>b</sup> (%)
10	10.1±0.4	4.0	1.0	10.3±0.6	5.8	3.0
40	$39.4 \pm 1.0$	2.5	-1.5	$39.8 \pm 1.1$	2.8	-0.5
600	$609 \pm 13$	2.1	1.5	$601 \pm 11$	1.8	0.2
8000	8113±119	1.5	1.4	$7949 \pm 141$	1.8	-0.6

<sup>&</sup>lt;sup>b</sup> Relative error=100%×(concentration calculated-spiking plasma concentration)/spiking plasma concentration.

Table 3 Recovery

Spiking plasma concentration	Peak height ratio (mean±SD)	Recovery <sup>a</sup> (%)	
(ng/ml)	Water sample $(n=3)$	Plasma sample $(n=3)$	(/0)
10	0.00412±0.00019	$0.00275 \pm 0.00002$	66.9
40	$0.01605 \pm 0.00030$	$0.01114 \pm 0.00021$	69.4
600	$0.25962 \pm 0.00216$	$0.18018 \pm 0.00119$	69.4
8000	$3.51670\pm0.01455$	$2.41018 \pm 0.01360$	68.5
10 000	$4.47977 \pm 0.00735$	$3.08390\pm0.03153$	68.8

<sup>&</sup>lt;sup>a</sup> Recovery=100%×(peak height ratio of plasma sample/peak height ratio of water sample).

hexane-dichloromethane (7:3, v/v) mixture is a good medium for extraction of mephenoxalone from plasma sample.

The freeze and thaw stability study proposes a short-term stability of compound in plasma sample in the thaw procedure. Furthermore, some plasma protein could coagulate after freezing and precipitating after thaw. Owing to the fact that this compound could bind to plasma protein, the phenomenon of plasma protein coagulation in frozen condition and precipitation at room temperature after thaw would result in concentration loss of the compound. As Table 4 shows, the differences of mephenoxalone concentrations between initial and each cycle were less than 10% for both concentrations (40 and 8000 ng/ml). In addition, there are no statistical significant differences between cycles by two-way analysis of variance (ANOVA) analysis for both concentrations. It indicates that mephenoxalone has a good

Table 4
Freeze and thaw stability

Cycle	Concentration (ng/ml)		
	40	8000	
Initial	35.5±2.1	7882±119	
1	37.4±2.2 (5.4%) <sup>a</sup>	8084±163 (2.6%)	
2	38.0±2.2 (7.0%)	$8081 \pm 115$ (2.5%)	
3	38.1±2.5 (7.3%)	8014±251 (1.7%)	

<sup>&</sup>lt;sup>a</sup> Data in parentheses mean % difference to initial concentration.

stability in plasma sample after three freeze and thaw cycles.

The procedure was applied to a pharmacokinetic study in which mephenoxalone was orally administered to four healthy volunteers. Typical plasma concentration-time profiles are shown in Fig. 3. The plasma concentrations of mephenoxalone were in the standard curve range and remained above the 10 ng/ml quantitation limit for the entire sampling period. The pharmacokinetic parameters obtained were described as follows. The value of area under the plasma concentration-time curve from time 0 to the last sampling time (AUC<sub>0-t</sub>) was 15 942 $\pm$ 7096 (ng/ml×h), and area under the plasma concentration-time curve from time 0 to time infinite  $(AUC_{0-\infty})$  was 16 274±7340 (ng/ml×h). The observed maximum plasma concentration  $(C_{max})$  was 2849±535 (ng/ml), time to observed maximum plasma concentration ( $T_{\rm max}$ ) was 2.17 $\pm$ 0.58 h, and elimination half-life was 2.77±1.21 h. In addition, the plasma concentration-time profile of mephenoxalone for the entire sampling period can depict about 90% of absorption according to the ratio of  $AUC_{0-t}$ divided by  $AUC_{0-\infty}$ .

These results demonstrate that this method is simple, sensitive, reproducible and accurate and meets the requirement of the report of the conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic studies [5]. As shown by the data obtained during a pharmacokinetic study in which this particular method was applied, it is concluded that the method described here offers the opportunity to derive pharmacokinetic parameters with an acceptable accuracy.

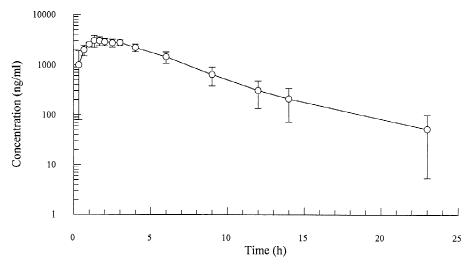


Fig. 3. Plasma concentration—time profile of mephenoxalone after oral administration of Dorsiflex tablet to four healthy volunteers. Data are shown as mean ±SD.

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