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A New and Simple HPLC Method for Determination of Etamsylate in Human Plasma and its Application to Pharmacokinetic Study in healthy adult male volunteers

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Abstract

A new and simple HPLC assay method was developed and validated for the determination of etamsylate in human plasma. After protein precipitation with 6% perchloric acid, satisfactory separation was achieved on a HyPURITY C18 column (250 mm X 4.6 mm, 5µm) using a mobile phase comprised 20 mM sodium dihydrogen phosphate-2 hydrate (pH was adjusted to 3.5 by phosphoric acid) and acetonitrile at a ratio of 95:5 v/v. The elution was isocratic at ambient temperature with a flow rate of 0.75 ml/min. Allopurinol was used as internal standard. The calibration curve was linear over range from 0.25 to 20 µg/ml \( (r^2 = 0.999) \). The limit of quantification for etamsylate in plasma was 0.25 µg/ml. The day-to-day coefficient of variance (%CV) ranged from 3.9% to 10.2 %, whereas the between-days %CV ranged from 3.1% to 8.7%. The assay method has been successfully used to estimate the pharmacokinetics of etamsylate after oral administration of a 500 mg tablet under fasting conditions to 24 healthy Egyptian human male volunteers. Various pharmacokinetic parameters including \( \text{AUC}_{0-\infty} \), \( \text{AUC}_{0-t} \), \( \text{C}_{\text{max}} \), \( \text{T}_{\text{max}} \), \( \text{t}_{1/2} \), MRT, CI/F, and \( \text{V}_{d}/F \) were determined from plasma concentration-time profile of etamsylate.

Keywords
Etamsylate; HPLC; validation; plasma; pharmacokinetics

1. Introduction

Etamsylate is an orally administered haemostatic drug. It is used in the treatment of capillary hemorrhage, hematemesis, hemoptyisis, malena, hematuria, epistaxis, menorrhagia and post partum hemorrhage (Sweetmann, 2002). It works by increasing capillary endothelial resistance and promoting platelet adhesion. It reduces capillary bleeding in the presence of normal number of platelets. In
addition, etamsylate is effective in the prevention and treatment of pre-, per-, or postsurgical capillary hemorrhages in all delicate operations and in those affecting highly vascularized tissues such as E.N.T., gynecology, obstetrics, urology, ophthalmology, plastic and reconstructive surgery (Symes, 1975; Harrison and Campbell; 1976). Etamsylate is chemically 2,5-dihydroxy benzene sulfonic acid with N-ethyl ethanamine (Fig. 1). The molecular formula of etamsylate is C_{10}H_{17}NO_{5}S and the molecular weight is 263.33 g/mol. It is completely soluble in water, methanol, and ethanol but partially soluble in methylene chloride (Merck Index, 1989).

Several analytical methods were developed for the assay of etamsylate in pharmaceutical formulations. These methods include; Spectrophotometry (Xu et al., 1994; El-Shabrawy et al., 2004; El-Enany et al., 2007; Goyal and Singhvi, 2008; Zhang et al., 2010), HPTLC (Jaiswal et al., 2005a,b), HPLC (Ma and Liu, 1984; Xu et al., 1994; El-Shabrawy et al., 2004; Kaul et al., 2005; Jaiswal et al., 2005a,b; El-Enany et al., 2007; Nagaraju et al., 2008; Goyal and Singhvi, 2008; Zhang et al., 2010; Chandrshekhara et al., 2010; Vamshikrishna and Sathish Kumar, 2011), electrochemical methods (Wang et al., 2001; Wang et al., 2005; Wang and Xu, 2007; Zhang and Wang, 2005), chemiluminescence (Zhang et al., 1998; Yang et al., 2002; Li et al., 2002; Du et al., 2002) and capillary electrophoresis (Li and Ju, 2006). To the best of our knowledge, no HPLC method has been yet reported for the determination of etamsylate in human plasma. Also, no pharmacokinetic studies have been published before. Owing to its great therapeutically importance and widespread use, studying the pharmacokinetic parameters of the drug is a prerequisite for the current study. Therefore, a new and simple validated HPLC assay method for the determination of etamsylate in human plasma was developed.

2. Materials and methods

2.1. Chemicals

Etamsylate and allopurinol were purchased from Sigma-Aldrich Co., St. Louis, MO, USA; Dicynone® tablets (500 mg Etamsylate) were purchased from OM Pharma, Geneva, Switzerland; All chemicals and reagents used were of LC grade and were purchased from Merck Chemicals, Germany; Milli-Q
(Millipore, Bedford, MA, USA) water was used in all cases. Serum was prepared from normal human blood, procured freshly from healthy volunteers through a local blood bank and pooled for spiking and using as control blank samples.

2.2. Instruments

The analysis of etamsylate was performed in HPLC system equipped with a variable wavelength ultraviolet (UV) detector and an automatic injector (LC-10 VP, Shimadzu Scientific Instrument, Kyoto, Japan). Data analysis program Class-VP (Shimadzu Scientific Instruments, Kyoto, Japan) was used for data acquisition and processing. Other instruments used in this study include Bath sonicator (Ultrasonic 57X, Elmsford, U.S.A); Balance (Sartorius, Germany); Magnetic stirrer (Stuart, Stone, Staffordshire, U.K.); Centrifuge (Hettich, Germany); pH meter (520 laboratory instrument, U.S.A.); Vortex tube mixer (Heidolph, Germany); Brand auto micro-pipettes (Labnet, USA); and micro liter syringes from Hamilton (Bonaduz, Switzerland).

2.3. HPLC system and operating conditions

Separation was accomplished with a HyPURITY C\textsubscript{18} column (250 mm x 4.6 mm, 5\textmu m; Thermo Fisher Scientific, Waltham, MA). The mobile phase comprised 20 mM sodium dihydrogen phosphate-2 hydrate (pH was adjusted to 3.5 by phosphoric acid) and acetonitrile at a ratio of 95:5 v/v; the elution was isocratic at ambient temperature with a flow rate of 0.75 ml/min. The UV detector was set at 309 nm for etamsylate and 254 for the internal standard (IS; allopurinol). The detector was programmed at 254 for 6.8 minutes and at 309 nm for the subsequent 1.2 minutes to measure etamsylate and IS, respectively, and the peak areas were calculated using the data analysis program Class-VP (Shimadzu Scientific Instruments, Kyoto, Japan).

2.4. Standard solutions

For construction of calibration curves, drug stock solution (500 \mu g/ml) was prepared by dissolving etamsylate powder in methanol. The stock solution was further diluted in methanol to prepare seven working solutions of etamsylate with concentrations of (200, 100, 50, 20, 10, 5 and 25 \mu g/ml) for spiking human plasma samples. Standard solutions were stored at +4 \degree C. The internal standard stock
solution was prepared by dissolving allopurinol powder in methanol to give concentration of 30 µg/ml which is used as the IS for etamsylate analysis in human plasma.

2.5. Plasma sample preparation

The calibration standards of etamsylate were prepared by transferring 25 µl from each working solution of etamsylate and IS to a set of test tubes. The solvent was evaporated, and 0.25 ml of blank plasma was added to each tube to form a set of calibration standards with concentrations of 0.25, 0.5, 1, 2, 5, 10 and 20 µg/ml. The sample was vortexed, and then 0.25 ml of 6% trichloroacetic acid was added, shaken for one minutes, and centrifuged at 3000 rpm for 7 minutes. The supernatant was separated and 25 µl was injected into the HPLC.

2.6. Assay Validation

2.6.1. Linearity

The linearity of the calibration curve in human plasma was confirmed by plotting the peak-area ratios of (drug/IS) versus the corresponding etamsylate concentration with least-squares linear regression analysis.

2.6.2. Selectivity

The retention time of the drug in the standard curve of spiked plasma blanks and the study samples were compared to define any endogenous materials and/or degradation peaks appeared at the same retention time of etamsylate and IS in the HPLC chromatograms.
2.6.1 Precision and accuracy

The precision of the assay was measured by the day-to-day and between-days percent coefficient of variation (%CV) over the concentration range of calibration curve of etamsylate in plasma during the course of validation. Day-to-day precision of the assay was determined by the analysis of three replicates of the calibration curve in the same day. The between-days precision was evaluated by the analysis of six different calibration curves in six different days during the study period. The %CV of the measured concentrations was used to determine the precision of the assay. The accuracy of the assay was defined as the absolute mean value of the ratio of the back calculated mean values of the unknown samples and their nominal values, expressed as a percentage. The measured concentrations of the day-to-day and between-days analysis were compared to the nominal concentrations to determine the accuracy of the assay.

2.6.2 Specificity

Three quality control samples (QC) at concentrations of 0.5, 2.0 and 10.0 μg/ml, (LLOQ, MQC and HQC) were prepared as unknown samples. The concentration in each QC sample was determined from the calibration curve and the calculated concentration was compared with the nominal concentration. Intra-day precision and accuracy were evaluated by analyzing each QC sample six times on the same day, while inter-day precision and accuracy were evaluated by analyzing each QC sample in 6 consecutive days.

2.6.3 Stability

The stability of the drug at room temperature was determined by preparing three different plasma samples for each drug concentration then the samples were injected immediately into the HPLC. The samples were kept at room temperature and were injected again after 24 hrs. The concentrations measured at time zero and after 24 hrs were compared to determine any changes in the concentrations with time. The 24 hrs period was selected because it is the maximum time between the preparation of samples and injection into the HPLC. The stability of the drug in frozen plasma was investigated by the
analysis of the study samples obtained from three volunteers twice. The first analysis was performed at the beginning of the study and the second analysis was performed at the end of the study. The samples were stored at -80°C between the analyses.

2.7. Clinical pharmacokinetic study

2.7.1. Study design

This study was monitored in accordance with the International Conference of Harmonisation (ICH) guidance on general considerations for clinical trials.

2.7.2. Study Population

Twenty-four male Egyptian volunteers with a mean ± SD age of 22.70 ± 4.090 years (range, 18-32 years) and mean ± SD weight of 69.37 ± 7.037 kg (range, 58-82 kg) were chosen to participate in the study. All subjects completed an acceptable medical history, medication history, physical examination, an electrocardiogram, blood pressure, screens for HIV-1 and -2 antibodies and hepatitis B surface antigen, and a urine drug screen prior to study initiation. Selected routine clinical laboratory measurements were performed during screening. Exclusion criteria were: (1) treatment with any known enzyme-inducing/inhibiting agents within 30 days prior to the start of the study and throughout the study; (2) susceptibility to allergic reactions to drugs; (3) any prior surgery of the gastrointestinal tract that may interfere with drug absorption. Upon completion of study, the physical examination and clinical laboratory measurements were repeated. The subjects were instructed to abstain from taking any medication for 1 week prior to and during the study period. All enrolled volunteers were healthy, and none of the participants showed any signs of adverse drug reactions during or after completion of the study. Informed consent was obtained from the subjects after explaining the nature and purpose of the study. The study protocol was approved by the ethical committee of Tanta University Hospital, Egypt.
2.7.1 Dosing schedule

In the morning of study, after an overnight fasting (10 h), volunteers received a single dose of one Dicyone® tablet (500 mg etamesylate) followed by 200 ml of water. The volunteers were continuously monitored by a qualified staff throughout the confinement period of study. Food and drinks were withheld for at least 2 hours after dosing. Water intake was allowed after 2 h of dose; water, lunch and dinner were given to all volunteers according to a time schedule. All subjects were asked to abstain from the consumption of fruit juices during the study period.

2.7.4 Collection of blood samples

Venous blood (5 ml) was taken from the forearm by vein puncture prior to (0 hr) and at 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, 12, 24 and 48 hrs after each drug intake. The blood samples were drawn into 14 ml heparinized tubes and plasma was directly separated by centrifugation at 3000 rpm for 10 minutes, transferred into polypropylene plastic tubes, and stored at -80°C until assay.

2.7.5 Monitoring of adverse effects

Throughout the study, the subjects were questioned and examined for the presence of adverse drug reactions. All the participated volunteers did not show any sign of adverse drug reactions during or after completing the study.

2.7.6 Determination of pharmacokinetic parameters

The developed HPLC method was used to investigate the plasma concentration–time profile of etamesylate after administration of 500 mg tablet. Study samples were treated as the calibration standards. A non-compartmental model was used to determine the pharmacokinetic parameters of etamesylate. The elimination rate constant (Kₑ) was estimated by least squares regression of plasma concentration–time data points in the terminal log-linear region of the curves. Half-life (t_{1/2}) was calculated as 0.693 divided by Kₑ. The AUC from zero to the last quantifiable plasma concentration (AUC₀ₜ) was calculated using the linear trapezoidal rule. The AUC from zero to infinity (AUC₀₋∞) was
calculated as \( \text{AUC}_{0-\infty} (\text{AUC}_{0-t} + C / K_e) \), where \( C \) is the last measured concentration (Gibaldi, 1982; Hedaya, 2007). \( C_{\text{max}} \) and \( t_{\text{max}} \) were obtained directly from the individual plasma concentration-time curve. The Apparent total clearance (\( \text{Cl/F} \)) was calculated as oral clearance (\( \text{CL/F} = \text{Dose} / \text{AUC}_{0-\infty} \)). While the apparent volume of distribution (\( \text{Vd/F} \)) was estimated as Oral Clearance/\( K_e \) relative to the bioavailability (\( F \)) of etamsylate. Area under the first moment curve (AUMC) was calculated by trapezoidal integration and extrapolation to infinity. Mean residence time (MRT) was calculated as the ratio \( (\text{AUMC})/\text{AUC}_{0-\infty} \) (Gibaldi, 1982; Hedaya, 2007).

3. Results and discussions

3.1. Conditions of chromatography

Method development was focused on the development and optimization of suitable sample preparation and chromatographic separation. Several tests were performed for optimizing the components of mobile phase in order to achieve good chromatographic peak shape and resolution. Good separation of etamsylate and IS with short run times were obtained using a mobile phase system of 20 mM sodium dihydrogen phosphate-2 hydrate (pH was adjusted to 3.5 by phosphoric acid) and acetonitrile at a ratio of 95:5 v/v; at 0.75 ml/min flow rate. The UV detector was programmed at 309 nm for 6.8 minutes and at 254 nm for the subsequent 1.2 minutes to measure etamsylate and IS, respectively at retention times were quite short for IS and etamsylate at 6.3 ± 0.09 and 7.2 ± 0.1 minutes, respectively. For plasma samples, deproteinization with 6% trichloroacetic acid was adopted, which was simple and fast resulting in sharp and symmetrical peaks.

3.2. Validation of HPLC method

3.2.1. Linearity

Calibration curves were linear with a correlation coefficient \( R^2 > 0.999 \) throughout the course of the assay (0-0.25 \( \mu \)g/ml) for etamsylate as shown in Fig.2.
3.2.3 Selectivity

Typical chromatograms obtained from blank human plasma and the plasma spiked with etamsylate and IS are shown in Fig. 3, which indicates that no peaks for any endogenous compound appeared at the same retention time for etamsylate and IS.

3.2.4 Precision and accuracy

Precision and accuracy results demonstrated good precision and accuracy over the concentration range selected. Intra- and inter-day precision and accuracy for etamsylate from plasma data are listed in Table 1. The day-to-day and between-days %CV were always within ±10% in the entire range of the calibration curve. The day-to-day %CV ranged from 3.9% to 10.2%, whereas, the between-days %CV ranged from 3.1% to 8.7%. The intra-day accuracy ranged between 99.0% and 109.4%, whereas the inter-day accuracy ranged between 98.4% and 108.2%.

3.2.5 Specificity

Intra- and inter-day precision and accuracy for the three quality control samples in human plasma were evaluated and depicted in Table 2.

3.2.6 Stability

Short-term stability showed that etamsylate is stable in plasma at least 24 h at room temperature. The difference in the drug concentration in all samples obtained from volunteers in the two analyses performed at the beginning and at the end of the study was always less than 10% in each sample in frozen plasma stored at -80°C during the study period, indicating the stability of etamsylate in frozen plasma.

The described analytical method was proven to be sensitive and accurate for the determination of etamsylate in plasma where, all validation parameters were within the acceptable limits which indicate the suitability of the developed analytical method for pharmacokinetic study.
3.2. Pharmacokinetic results

The developed method was used for the pharmacokinetic study of etamsylate in 24 healthy Egyptian volunteers. After oral administration of 500 mg etamsylate, the concentration versus time profile of etamsylate was constructed. The mean plasma concentration–time curve of etamsylate is shown in Fig. 4. The results showed that the method was reliable and adequate to provide pharmacokinetic concentration–time profile for a dose of etamsylate of 500 mg tablet. Etamsylate was readily absorbed from the gastrointestinal tract and it was measurable at the first sampling time (0.25 h) in almost all volunteers. The major pharmacokinetic parameters of etamsylate were listed in Table 3.

4. Conclusions

A new and simple HPLC method for the determination of etamsylate in human plasma was developed and validated over the concentration range from 0.25-20 μg/ml. The method has been successfully applied to the pharmacokinetic study of etamsylate in Egyptian healthy male volunteers and satisfactory results were obtained, which demonstrates that the method is reproducible, sensitive and reliable. Owing to great therapeutically importance of etamsylate and widespread use; this work is considered as a primary step in preparing controlled release preparations in the future.

References


Table 1

The day-to-day (intra-day) and between-days (inter days) precision and accuracy of the assay

<table>
<thead>
<tr>
<th>Nominal Concentration (µg/ml)</th>
<th>day-to-day (intra-day)(^a)</th>
<th></th>
<th>between-days (inter-days)(^b)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration found (µg/ml) (mean±S.D)</td>
<td>Precision (%)</td>
<td>Accuracy (%)</td>
<td>Concentration found (µg/ml) (mean±S.D)</td>
</tr>
<tr>
<td>20</td>
<td>20.9 ± 1.21</td>
<td>5.77 %</td>
<td>104.8 %</td>
<td>20.09 ± 1.27</td>
</tr>
<tr>
<td>10</td>
<td>10.2 ± 0.40</td>
<td>3.91 %</td>
<td>102.3 %</td>
<td>10.14 ± 0.32</td>
</tr>
<tr>
<td>5</td>
<td>5.10 ± 0.32</td>
<td>6.38 %</td>
<td>102.1 %</td>
<td>5.185 ± 0.40</td>
</tr>
<tr>
<td>2</td>
<td>1.98 ± 0.20</td>
<td>10.2 %</td>
<td>99.00 %</td>
<td>1.97 ± 0.08</td>
</tr>
<tr>
<td>1</td>
<td>1.08 ± 0.07</td>
<td>6.67 %</td>
<td>109.4 %</td>
<td>0.984 ± 0.04</td>
</tr>
<tr>
<td>0.5</td>
<td>0.50 ± 0.03</td>
<td>6.09 %</td>
<td>101.8 %</td>
<td>0.541 ± 0.04</td>
</tr>
<tr>
<td>0.25</td>
<td>0.27 ± 0.01</td>
<td>4.44 %</td>
<td>108.0 %</td>
<td>0.251 ± 0.02</td>
</tr>
</tbody>
</table>

\(^a\)n = 3 for each concentration  \(^b\)n = 6 for each concentration
Table 2
Quality control samples

<table>
<thead>
<tr>
<th>Nominal concentration (µg/ml)</th>
<th>Concentration found (µg/ml; mean±S.D.)</th>
<th>Precision (%)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>9.84 ± 0.99</td>
<td>10.0 %</td>
<td>98.4 %</td>
</tr>
<tr>
<td>2.0</td>
<td>1.89 ± 0.13</td>
<td>6.87 %</td>
<td>94.5 %</td>
</tr>
<tr>
<td>0.5</td>
<td>0.53 ± 0.05</td>
<td>9.39 %</td>
<td>106.4 %</td>
</tr>
</tbody>
</table>
Table 3
Pharmacokinetic parameters of etamsylate after a single oral dose administration of one
Dicynone® (500 mg etamsylte) to 24 healthy Egyptian male volunteers.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Etamesylate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/ml)</td>
<td>8.05</td>
</tr>
<tr>
<td>$t_{\text{max}}$ (hrs)</td>
<td>5.02</td>
</tr>
<tr>
<td>AUC$_{0\rightarrow t}$ (µg. hr/ml)</td>
<td>78.7</td>
</tr>
<tr>
<td>AUC$_{0\rightarrow \infty}$ (µg. hr/ml)</td>
<td>84.8</td>
</tr>
<tr>
<td>$t_1/2$ (hr)</td>
<td>9.4</td>
</tr>
<tr>
<td>MRT (hrs)</td>
<td>15.7</td>
</tr>
<tr>
<td>Cl/F (ml/min per kg)</td>
<td>6470.5</td>
</tr>
<tr>
<td>Vd/F (l/kg)</td>
<td>86629.7</td>
</tr>
</tbody>
</table>

$C_{\text{max}}$, peak plasma concentration; $t_{\text{max}}$, time to reach peak plasma concentration; AUC$_{0\rightarrow \infty}$, area under the concentration-time curve from zero to infinity; AUC$_{0\rightarrow t}$, area under the concentration-time curve from zero to the last measurable plasma concentration; AUC$_{t\rightarrow \infty}$, area under the concentration-time curve from the last measurable plasma concentration to infinity; $t_1/2$, elimination half-life; MRT, mean residence time; Cl/F, clearance; Vd/F, volume of distribution.
Fig. 1. Chemical structure of etamsylate
Fig. 1. Calibration curve of etamsylate in spiked plasma (mean ± S.D.; n=8).
Fig.  Representative chromatograms for blank plasma sample and blank plasma spiked with etamsylate to produce a drug concentration of 10 μg/mL and the internal standard (IS).
Fig. 4. Mean plasma concentrations of etamsylate in 24 healthy volunteers after a single oral dose administration of one Dicynone® tablet (500 mg etamsylate) (mean ± standard error).