DEVELOPMENT AND VALIDATION OF A RP-HPLC METHOD FOR DETERMINATION OF SOLUBILITY OF FUROSEMIDE

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Abstract
The objective of the Biopharmaceutics Classification System is to allow prediction of in vivo pharmacokinetic performance of drug products from in vitro measurements; therefore, it is important to determine the solubility and permeability of drug substances. Furosemide (FSM) is a loop diuretic commonly used in the treatment of edematous states associated chronic renal failure, hypertension, congestive heart failure and cirrhosis of the liver. The aim of this study was to develop and validate an HPLC method for quantification of FSM in the samples obtained from the in vitro solubility studies performed at five different pH values (pH 1.0, 2.9, 3.9, 4.9 and 7.5). Chromatographic separation of FSM was achieved on a reverse phase column (Waters Spherisorb ODS2 C18 250x4.6 mm 5 μm) with a mobile phase consisted of 0.01M KH₂PO₄ (pH 5.5) and methanol (70:30 v/v). Analyses were run at a flow rate 1 mL/min and UV detection was performed at 235 nm. Under these conditions, the retention time of FSM was about 7.0 min. The method was linear in the concentration range of 0.5 to 50 μg/mL, and limit of quantification was 320 ng/mL. Developed and validated HPLC method was proved to be simple, reliable and also suitable as a single method for studying the solubility of FSM as a function of pH. Finally, based on our results, solubility of FSM was dependent on pH. Its solubility was low between pH 1.0 and 4.9, and was high at pH 7.5.

Key words: Furosemide, HPLC method, BCS, Solubility class, Dose number

Furosemidin Cozunurlugunun Tayini icin Bir RP-HPLC Metodun Gelistirilmesi ve Validasyonu

Biyofarmasotik Sınıflandırma Sisteminin amacı ilaç ürünlerinin invitro ölçülmlerinden invivo farmakokinetik performansının öngörümesine olanak sağlamaktır; bu nedenle ilaçların çözünürlük ve permeabilitelerinin tayini önemlidir. Furosemid (FSM) karaciğer sirozu, konjestif kalp yetmezliği, hipertansiyon ve kronik böbrek yetmezliği ile ilişkili odemlerin tedavisinde kullanılır. Bu çalışmanın amacı bu farklı pH (pH 1.0, 2.9, 3.9, 4.9 and 7.5) değerlerinde gerçekleştirilmiş in vitro çözünürlük deneylerinden elde edilen örneklerden FSM’in tayini için bir HPLC yöntemi metodu geliştirmek ve valide etmektir. FSM’in kromatógraflık ayırımı bir ters faz kolonuya (Waters Spherisorb ODS2 C18 250x4.6 mm 5 μm), 0.01M KH₂PO₄ (pH 5.5) ve metanol (70:30 v/v) içeren mobil faz ile gerçekleştirilmiştir. Bu koşullar altında FSM’in altkınya zamanı yaklaşık 7.0 dk’dır. Yöntem 0.5 to50 μg/mL konsantrasyon aralığında doğruşaldır ve ölçülebilir alıt sınır 320 ng/mL’dir. Geliştirilen ve valide edilen HPLC yönteminin FSM’in, pH’ın fonksiyonu olarak, yapılan çözünürlük çalısmaları için basit, güvenilir ve de kullanışlı bir yöntem olduğunu kanıtlanmıştır. Son olarak, sonuçlarınza göre; FSM’in çözünürlüğü pH’ya bağlıdır. FSM’in çözünürlüğü pH 1.0-4.9 arasında düşük ve pH 7.5’de yüksektir.

Anahtar kelimeler: Furosemid, HPLC yöntemi, BCS, Çözünürlük sınıfı, Doz sayısı

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INTRODUCTION

The oral absorption of a drug is fundamentally dependent on that drug’s aqueous solubility and gastrointestinal permeability. Extensive research into these fundamental parameters by Amidon et al. (1) led to the Biopharmaceutics Classification System (BCS) that categorizes drugs into four groups, Class 1 (high solubility and high permeability), Class 2 (low solubility and high permeability), Class 3 (high solubility and low permeability), and 4 (low solubility and low permeability). In 2000, the FDA promulgated the BCS as a science based approach to allow waiver of in vivo bioavailability and bioequivalence testing for immediate release solid dosage forms for Class 1 compounds, highly soluble and highly permeable drugs, when such drug products also exhibit rapid dissolution (2). The objective of the BCS is to allow prediction of in vivo pharmacokinetic performance of drug products from in vitro measurements; therefore, it is important to determine the solubility and permeability of drug substances.

Furosemide (FSM; Figure 1) is a loop diuretic used in adults, infants and children for the treatment of edema associated with congestive heart failure, cirrhosis of the liver and renal disease. Oral furosemide may be used in adults for the treatment of hypertension of alone or in combination with other antihypertensive agents (3-5). The therapy should be individualized according to patient response to gain maximal therapeutic response and to determine the minimal dose needed to maintain that response. The usual dose of FSM in edema is 20 to 80 mg given as a single dose. If needed, the same dose can be administered 6 to 8 hrs later or the dose may be increased (4-6). In the case of hypertension, the usual initial dose is 80 mg, usually divided into 40 mg twice as day.

![Figure 1. Chemical structure of FSM (3).](image_url)

Due to its weak acidic properties (pKa=3.9; (7)) FSM is mostly absorbed from stomach and upper small intestine (8). Bioavailability of FSM from tablets varies from 37% to 70% (6-11). Plasma peak concentration (C_{max}) is occurred between 48-90 min (6, 8, 12). The plasma half-life (t_{1/2}) of FSM in healthy subjects is about 30-90 mins (13).

Several HPLC methods have been reported for determination of FSM from tablets (14-18), human serum (14, 19-21), dog plasma (22), rabbit plasma (16), human urine (21, 23, 24), intestinal perfusion (25, 26) and milk (27, 28) samples (Table 1). All these methods differ with respect to the mobile phases, columns and detector systems used for the analysis of the compound. Some of the methods have not been formally validated. On the other hand, although the retention time of FSM is about 2 min in the HPLC method reported by Semaan et al. (15), the column used in that method is not commercially available.

The initial aim of this study was to develop a simple, rapid and validated HPLC method for quantification of FSM in the samples obtained from the in-vitro solubility studies, as the HPLC methods reported in the literature are not specific for our purpose. The second aim was to apply
the developed method for determination of FSM solubility as a function of pH which recommended by FDA Guidance (2).

EXPERIMENTAL

Chemicals and Reagents
FSM was kindly supplied by Sanofi-Aventis (Türkiye). All HPLC grade solvents (methanol, acetonitrile) were purchased from Sigma-Aldrich (Germany). The water used in the HPLC analyses was deionized water obtained from Millipore Simplicity 185 Water Purification Systems (France). All other chemicals were of analytical reagent grade or better and purchased commercially.

Chromatographic System and Conditions
The HPLC system comprised of Water 2690 Separations Module equipped with a Waters 2996 Photodiode Array Detector (Waters, USA). FSM was separated using a Waters Spherisorb ODS2 C18 (250x4.6 mm 5 μm; USA) column. The HPLC system was operated at room temperature using a mobile phase was consisted of phosphate buffer (0.01M; pH 5.5) and methanol (70:30, v/v). After mixing, the mobile phase was filtered through a 0.45 μm membrane filter and degassed before use. Analyses were run at a flow rate 1 mL/min and UV detection was performed at 235 nm. Drug concentrations in samples were calculated from peak areas obtained from analyses using Empower™ software.

Standard Stock Solutions
Standard stock solution of FSM was prepared in methanol at concentration of 1 mg/mL. This stock solution was then diluted with the mobile phase to obtain secondary standard stock solution (0.1 mg/mL).

Calibration and Validation
The secondary standard stock of FSM was diluted with the mobile phase to obtain the calibration standards at concentrations of 0.5, 1.0, 2.0, 5.0, 7.5, 10, 20, 30, 40, 50 μg/mL FSM. The peak area was plotted against the corresponding concentration to obtain the calibration graph. Calibration curve and corresponding determination coefficient ($r^2$) were then calculated by least squares linear regression analysis. The method was validated according to the International Conference on Harmonization (ICH) Guideline and Text on Validation of Analytical Procedures: Text and Methodology Q2R1 (29).

Standard specificity was examined in relation to interference from matrix components in drug free buffers used for the solubility of FSM. Linearity of the method was assessed by the calibration equation which was characterized by determination coefficient, slope and intercept. Repeatability was assessed at two levels; method and injection repeatabilities. Method repeatability was determined by single injections from six independent standard solutions containing 10 μg/mL FSM, whereas, injection repeatability was determined from six consecutive injections from the same standard solution containing 10 μg/mL FSM. Repeatability was assessed by calculating the relative standard deviation (RSD %) of the measurements. Inter- and intra-day precision and accuracy of the analytical method were determined at three test concentrations (1, 10, 40 μg/mL) and calculated as percentage recovery. The sensitivity of the analytical method was evaluated by determining the limits of detection (LOD) and quantitation (LOQ). Based on FDA guidance, the signal-to-noise ratios of 3:1 and 10:1 were taken as LOD and LOQ, respectively.
Table 1. Chromatographic conditions of the reported methods for the separation and determination of FSM.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Column</th>
<th>Detection</th>
<th>Mobile Phase</th>
<th>Flow Rate (mL/min)</th>
<th>Extraction (+/-)</th>
<th>LOD/LOQ (μg/mL)</th>
<th>Retention Time (min)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tablet Human Plasma</td>
<td>Purosphere C18 (250x4.6mm, 5μm)</td>
<td>Ultraviolet 232 nm</td>
<td>MeOH:Water:ACN (pH 3.0) (75:25:5)</td>
<td>0.8</td>
<td>-</td>
<td>0.84/2.55</td>
<td>4.2</td>
<td>(14)</td>
</tr>
<tr>
<td>Human Urine</td>
<td>Purosphere C18 (250x4mm, 5μm)</td>
<td>Ultraviolet 234 nm</td>
<td>0.05% TFA:ACN:MeOH Gradient</td>
<td>1.0-1.4</td>
<td>+</td>
<td>0.02/0.05</td>
<td>38.5</td>
<td>(23)</td>
</tr>
<tr>
<td>Dog Plasma</td>
<td>Spherisorb ODS 25μm</td>
<td>Ultraviolet 235 nm</td>
<td>Water:ACN (70:30) (pH 6.5)</td>
<td>1.0</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(22)</td>
</tr>
<tr>
<td>Intestinal Perfusion</td>
<td>Symmetry Shield C18 (150x4.6mm, 5μm)</td>
<td>Ultraviolet 228 nm</td>
<td>KH2PO4:MeOH (0.01M, pH 3.5) Gradient</td>
<td>1.5</td>
<td>-</td>
<td>0.125/-</td>
<td>&lt;7.0</td>
<td>(25)</td>
</tr>
<tr>
<td>Intestinal Perfusion</td>
<td>Shimpack ODS 250x4mm, 5μm</td>
<td>Ultraviolet 280 nm</td>
<td>ACN:Water:GAA:TEA (41.5:57.4:0.9:0.1)</td>
<td>1.0</td>
<td>-</td>
<td>2.4/7.2</td>
<td>8.2</td>
<td>(26)</td>
</tr>
<tr>
<td>Human Urine</td>
<td>Spherisorb ODS C18 (125x4mm, 5μm)</td>
<td>Ultraviolet 270 nm</td>
<td>Ammonium Acetate:ACN (pH 3.0) Gradient</td>
<td>1.5</td>
<td>+</td>
<td>0.001/0.003</td>
<td>7.9</td>
<td>(19)</td>
</tr>
<tr>
<td>Human Urine</td>
<td>Kromasil 100-5 C18 (150x4.6mm, 5μm)</td>
<td>Fluorescence Exc:268 nm Em:410 nm</td>
<td>KH2PO4:ACN (66:34) (0.02M, pH 3.0)</td>
<td>1.0</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(18)</td>
</tr>
<tr>
<td>Milk</td>
<td>Kromasil C18 (150x4.6mm, 5μm)</td>
<td>Amperometric</td>
<td>NaH2PO4:ACN (75:25) (0.005M)</td>
<td>1.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(27)</td>
</tr>
<tr>
<td>Tablet Rabbit Plasma</td>
<td>Nucleosil C18 (250x4.6mm, 10μm)</td>
<td>Ultraviolet 235 nm</td>
<td>KH2PO4:ACN (80:20) (0.02M, pH 4.5)</td>
<td>3.0</td>
<td>+</td>
<td>0.1/0.04</td>
<td>8.1</td>
<td>(16)</td>
</tr>
<tr>
<td>Human Plasma</td>
<td>Bondapak C18 150x3.9mm, 5μm</td>
<td>Fluorescence Exc:230 nm Em:410 nm</td>
<td>KH2PO4:ACN (65:33:3) (0.02M, pH 3.0)</td>
<td>1.0</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(20)</td>
</tr>
<tr>
<td>Tablet</td>
<td>Bondapak C18</td>
<td>Amperometric</td>
<td>KH2PO4/K2HPO4:ACN (30:70) (0.05M, pH 3.5)</td>
<td>1.0</td>
<td>+</td>
<td>0.0015/-</td>
<td>-</td>
<td>(17)</td>
</tr>
<tr>
<td>Human Urine Tablets</td>
<td>Bondapak C18 (35°C)</td>
<td>Amperometric</td>
<td>KH2PO4/K2HPO4:ACN (40:60) (0.05M, pH 3.5)</td>
<td>1.0</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(18)</td>
</tr>
<tr>
<td>Milk</td>
<td>Spherisorb 5 ODS2 (30°C)</td>
<td>Fluorescence Exc:272 nm Em:410 nm</td>
<td>KH2PO4:ACN (70:30) (pH 3.0)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>(28)</td>
</tr>
<tr>
<td>Human Plasma</td>
<td>C18 Column</td>
<td>Fluorescence Exc:235 nm Em:389 nm</td>
<td>NaH2PO4:MeOH (55:35) (0.01M, pH 3.5)</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(21)</td>
</tr>
<tr>
<td>Human Urine</td>
<td>C18 Column</td>
<td>Fluorescence Exc:235 nm Em:389 nm</td>
<td>Acetic Acid:CH3OH (60:40) (pH 3.5)</td>
<td>3.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(21)</td>
</tr>
</tbody>
</table>

Solubility Studies
The method was applied to determine the in-vitro solubility of FSM. The saturation solubility of the compound was determined at five different pH values (1.0, 2.9, 3.9, 4.9, 7.5) according to FDA guidance (2). Solubility studies were repeated six times at each pH conditions. Five different pH mediums were prepared according to USP 29 (30). Excess amount of FSM was added in a suitable buffer solution and agitated overnight in a horizontal shaker (100 rpm; 37±0.5°C). Sample (1 mL) was withdrawn at the end of the experiment (24 h) and filtered through a La-Pha-Pack HPLC syringe filter (0.45 µm) which has no absorption for FSM. An aliquot (200 µL) of filtered sample was diluted with mobile phase to a final volume of 1 mL, and then 10 µL was injected into HPLC system.

Data Analysis
Saturation solubility of FSM was determined as a function of pH values using the calibration curves. The volume of the buffer required to solve the highest dose strength of FSM was then calculated using these solubility values. For each condition, dose number (Do) was calculated using the dose (D) of FSM, volume (V=250 mL) and solubility (Eq. 1) (31).

\[ D_o = \frac{D}{V} \]  

Eq.1

All tabulated results were expressed as mean ± standard error (S.E.)

RESULTS AND DISCUSSIONS
Method Development
In order to achieve acceptable peak shape and perform the separation on a suitable run time, various buffer systems were tried systematically. The mixture of a mobile phase was consisted of phosphate buffer (0.01M; pH 5.5) and methanol (70:30 v/v) was capable of a good separation and defined as the optimum conditions. Under optimum conditions, FSM was successfully separated by C_{18} (Waters Spherisorb ODS2 C_{18} 5 µm 250x4.6 mm) column from the matrix components with the retention time of 6.9±0.3 min. Total run time for an assay was approximately 10 min, and the interval between two consecutive injections was 1 min. The representative chromatograms obtained in the standard stock solution of FSM at different pH values (1.0, 2.9, 3.9, 4.9, 7.5) are depicted in Figure 2. FSM was well separated from the matrix components with no interfering peaks in the relevant portion of the trace, and the retention time was not influenced by the pH value of the sample.

Validation of the Method
Linearity
The calibration curve for FSM constructed under optimum conditions and linearity of the method was determined by performing injections at ten different concentration levels in the linear range over 6 different days. The peak area of FSM was plotted against the corresponding nominal concentration to obtain calibration graph. Thus, the method was evaluated linear in the range of 0.5 to 50 µg/mL for FSM. The regression equation data are given in Table 2.

Sensitivity
The sensitivity of the analytical method was evaluated by determination of the limits of detection (LOD) and quantitation (LOQ). The signal-to-noise ratios of 3:1 and 10:1 were taken as LOD and LOQ, respectively. The values of LOD and LOQ for FSM were 80 ng/mL and 320 ng/mL, respectively and given in Table 2.
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Figure 2. FSM containing sample chromatograms at five different pH values.

**Repeatability**

In order to measure the method repeatability of the developed method, single injections were made into the HPLC system from six independent standard solutions containing 10 μg/mL FSM. Injection repeatability was determined from six consecutive injections from the same standard solution (10 mg/mL). The relative standard deviations (RSD %) determined for the method and injection repeatabilities were less than 1% and 0.2%, respectively. All these results can be taken as an indication of high repeatability.

**Table 2. Linearity and sensitivity data of the developed method (n=6)**

<table>
<thead>
<tr>
<th>Data</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression equation*</td>
<td>$y = 47526x - 5652$</td>
</tr>
<tr>
<td>Standard error of intercept</td>
<td>575</td>
</tr>
<tr>
<td>Standard error of slope</td>
<td>4133</td>
</tr>
<tr>
<td>Determination coefficient ($r^2$)</td>
<td>0.9989</td>
</tr>
<tr>
<td>Linearity range (μg/mL)</td>
<td>0.5 – 50</td>
</tr>
<tr>
<td>Number of data points</td>
<td>10</td>
</tr>
<tr>
<td>LOD (ng/mL)</td>
<td>80</td>
</tr>
<tr>
<td>LOQ (ng/mL)</td>
<td>320</td>
</tr>
</tbody>
</table>

*where y is peak area and x is concentration in μg/mL of FSM
Precision and Accuracy

Three different concentrations of standard FSM solutions (within the linear range) were analyzed six consecutive days (inter-day precision) and six times within the same day (intra-day precision). The RSD values for intra- and inter-day precision were less than 2%, and corresponding Bias values were 15% (Table 3) indicating that the precision and accuracy of the method were satisfactory.

Table 3. Precision and accuracy data of the developed method

<table>
<thead>
<tr>
<th>Added (µg/mL)</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found* (µg/mL)</td>
<td>Precision RSD%</td>
</tr>
<tr>
<td>1</td>
<td>1.09 ± 0.01</td>
<td>1.60</td>
</tr>
<tr>
<td>10</td>
<td>9.93 ± 0.08</td>
<td>1.94</td>
</tr>
<tr>
<td>40</td>
<td>40.3 ± 0.28</td>
<td>1.72</td>
</tr>
</tbody>
</table>

* mean ± standard error (n=6), Bias % = [(Found-Added)/Added] x 100

Application to Solubility Studies

It is recommended that the pH-solubility profile of the test drug substance should be determined at 37 ±1°C in aqueous media with a pH in the range of 1.0-7.5. According to the FDA Guidance (2), the number of pH conditions for a solubility determination can be based on the ionization characteristics of the test drug substance. When the pKa value of drug is in the range of 3-5, the solubility should be determined at pH=pKa, pH=pKa+1, pH=pKa-1 and at pH = 1.0 and 7.5. FSM has a pKa value of 3.9 (7). Therefore, we determined the solubility of the drug in aqueous media at pH values of 1.0, 2.9, 3.9, 4.9 and 7.5. Our experimental results showed that FSM has pH dependent solubility. Solubility of FSM increased from 0.028 mg/mL to 6.411 mg/mL (Table 4) when the pH medium increased from 1 to 7.5 (Figure 3).

Figure 3. pH solubility profile of FSM (mean ± SE, n=6).
Table 4. Solubility, volume required to solve the highest dose strength of FSM and dose numbers

<table>
<thead>
<tr>
<th>Medium (pH)</th>
<th>Solubility$^1$ (µg/mL)</th>
<th>Volume required to dissolve FSM$^2$ (mL)</th>
<th>Dose Number$^3$ (Do)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>28.4 ± 4.7</td>
<td>2816</td>
<td>11.79</td>
</tr>
<tr>
<td>2.9</td>
<td>27.6 ± 1.0</td>
<td>2898</td>
<td>12.76</td>
</tr>
<tr>
<td>3.9</td>
<td>28.5 ± 3.0</td>
<td>2807</td>
<td>11.77</td>
</tr>
<tr>
<td>4.9</td>
<td>63.3 ± 4.7</td>
<td>1263</td>
<td>5.24</td>
</tr>
<tr>
<td>7.5</td>
<td>6410.8 ± 262.1</td>
<td>12</td>
<td>0.05</td>
</tr>
</tbody>
</table>

$^1$Determined from solubility experiments; $^2$Volume required to dissolve 80 mg of FSM; $^3$Dose number was calculated using Equation 1.

CONCLUSIONS

Determination of solubility class of a drug substance according to FDA guidance is very important for the pharmaceutical companies which are taking bio waiver for the immediate release oral solid drug products. Although there are various HPLC methods available for determination of FSM, in this study, a validated HPLC method was for quantification of FSM for particularly solubility studies. Developed and validated method was proved to be simple, reliable and also suitable as a single method for studying the solubility of FSM as a function of pH. Finally, based on our results, solubility of FSM was dependent on pH. Its solubility was low between pH 1.0 and 4.9, and was high at pH 7.5.

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