Determination of Furosemide in Whole Blood using SPE and GC-EI-MS

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Abstract

A simple and rapid method was validated to determine furosemide in whole blood. The experimental work was performed so that all validation parameters are considered simultaneously in a one-day assay protocol. A solid-phase extraction procedure using BondElut®-LRC Certify columns was used to extract this compound from blood samples, while ketoprofen was used as an internal standard. The extracts were analyzed by gas chromatography–electron ionization–mass spectrometry after on-column derivatization with trimethylanilinium hydroxide (0.2M in methanol). Calibration curves were prepared daily, between 0.10 and 5.00 μg/mL, and the correlation coefficients were above 0.9910. The calculated limits of detection and quantitation were 0.010 and 0.045 μg/mL, respectively. Control samples at low, medium, and high concentrations (0.30, 0.75, and 3.00 μg/mL) of furosemide of an independent source were measured in the same day. Precision and trueness, calculated in terms of relative standard deviation (%), were less than 15% for all concentration levels. The relative recoveries calculated for the three levels of the control samples were 104%, 89%, and 91%, respectively. In general, a sensitive, specific, and reliable procedure has been developed for the determination of furosemide in whole blood samples and was found suitable for the application in postmortem forensic toxicology routine analysis.

Introduction

Furosemide (Figure 1, 4-chloro-N-furfuryl-5-sulfamoylanthranilic acid) is an anthranilic acid derivative with strong diuretic potential (1,2). It exerts its action on the luminal side of the thick ascending limb of the Henle’s loop by inhibiting active chloride transport (reabsorption) with sodium passively following (3–6). This action of furosemide has a vasodilator renal effect, resulting both in a decrease of the vascular resistance and in an increase in the renal blood flow. Therefore, the excretion of salts and water is enhanced (6,7). This potent loop diuretic used in adults and children is employed in the pharmacotherapy of various diseases, namely congestive heart failure, hypertension, renal diseases, cirrhosis, and some types of edema (8). After oral administration, furosemide is rapidly absorbed by the gastrointestinal tract with a bioavailability of about 65%. Up to 90% of an intravenous dose is excreted in urine, mainly as unchanged drug, and up to 14% is excreted as a glucuronide metabolite (8,9). Its plasma half-life is about 1 to 3 h, and increases in subjects with renal failure, congestive heart failure, liver disease, and in neonates (4,5). This substance extensively binds to plasma protein (about 90%) (10). Serum levels in the range of 2–10 μg/mL and 25–30 μg/mL are reported to be respectively therapeutic and toxic concentrations (11).

Although furosemide is included in the list of prohibited doping substances indicated by the World Anti-Doping Agency (WADA) (12), it is widely used in sports, mainly in competitions classified by weight or to avoid detection of other drugs (13,14).

Several methods for the detection of furosemide in urine, plasma, and serum using solid-phase extraction (SPE) and liquid chromatography or liquid–liquid extraction followed by gas chromatography (GC) have been described (6,8,10,15–20). To the best of our knowledge, the determination of this substance in whole blood has not been reported. This paper describes a reliable and reproducible methodology for furosemide quantitation in whole blood samples using a GC–electron ionization–mass spectrometry (EI-MS) procedure after SPE and on-column derivatization with trimethylanilinium hydroxide (TMAH) (21), which was rapid and sensitive enough for post-mortem forensic toxicology studies.

Figure 1. Non-derivatized furosemide and positions of the hydrogens, which can be potentially replaced by derivatization with TMAH (R1 = R2 = R3 = R4 = CH3).
Experimental

Samples

Human blood samples were obtained from the local blood bank (Portuguese Institute of Blood, Coimbra, Portugal) and were out of the stated period to be used in human transfusions. These samples were stored at -20°C until use.

An authentic blood sample was obtained at a medicolegal autopsy, from the Forensic Pathology of the Delegation of Coimbra of the National Institute of Legal Medicine in Portugal.

Reagents and materials

Ketoprofen (internal standard, IS) and two different lots of furosemide, one for calibrators and the other for controls, were obtained from Sigma Co. (St. Louis, MO). The derivatization agent (MethElute™ Reagent trimethylanilinium hydroxide 0.2M in methanol, TMAH) was obtained from Pierce (Rockford, IL).

All other chemicals and solvents, obtained from Merck Co. (Darmstadt, Germany) were of analytical grade.

BondElut® LRC 300 mg SPE columns and a Vac-Elut SPS 24 system coupled with a vacuum pump (AKNF) were purchased from Varian (Harbor City, CA).

Instrumentation and chromatographic conditions

Chromatography was conducted on a Hewlett-Packard (Waldbronn, Germany) 6890 series GC, equipped with a model HP 5973 mass selective detector (Waldbronn, Germany). A capillary column (12 m × 0.25-mm i.d., 0.25-μm film thickness) packed with 5% phenylmethylsiloxane (Ultra 2) supplied by J&W Scientific (Folsom, CA), was used. The carrier gas was helium at a constant flow rate of 1 mL/min. Chromatographic conditions were as follows: initial oven temperature was 160°C for 1 min, increased by 20°C/min to 270°C and held for 8.5 min. The temperatures of the injector and detector were set at 250°C and 280°C, respectively. The mass spectrometer was operated at 70 eV in the EI mode using selected ion monitoring (SIM) mode. The injection volume was 1 μL in the split mode at a ratio of 10:1. The ions were monitored at m/z 81, 372, and 96 for methylated furosemide (Figure 2) and at m/z 209, 105, and 268 for methylated IS.

Analytical Method

Standard solutions

Stock solutions of furosemide of both lots as well as that of IS were prepared in methanol at 1000 μg/mL. Subsequently, working solutions for calibration curves and control samples were obtained by serial dilutions with methanol at 100 and 10 μg/mL for furosemide, and at 10 μg/mL for IS. All solutions were protected from light and stored at 4°C until use.

Sample pre-treatment

A 50-μL aliquot of the IS solution (10 μg/mL) was added to 1 mL of human whole blood. The mixture was vortex mixed and sonicated in a sonic bath for 15 min at room temperature. After sonication, 6 mL of phosphate buffer (0.1M, pH 6.0) was added and the mixture was vortex mixed for 30 s before centrifugation at 3000 rpm for 15 min.

SPE

The extraction of pretreated samples was performed with a Vac-Elut system assembled with BondElut®-LRC Certify 300 mg SPE columns. The columns were conditioned with 2 mL methanol and 2 mL of 0.1M phosphate buffer (pH 6.0) before applying the samples, using reduced pressure at low flow speed. The columns were washed successively with 1 mL of a mixture of phosphate buffer (0.1M, pH 6.0) and methanol (4:1 v/v), 1 mL of acetic acid (1M), and 1 mL of n-hexane. After each washing...
step the columns were dried under full vacuum for 5, 10, and 2 min, respectively. The substances of interest were eluted with 4 mL of dichloromethane, and the solvent was subsequently evaporated to dryness in a vacuum rotary evaporator at 40°C.

**Derivatization**

The remaining residues were dissolved in 25 µL of TMAH, gently vortex mixed, and directly injected in the GC–EI–MS system in SIM mode. The TMAH reagent was specially formulated for use as a direct on-column methylating agent.

**Results**

**Validation of the analytical method**

The proposed strategy included the verification and determination of selectivity, linearity, limits of detection (LOD) and quantitation (LOQ), precision, trueness, and recovery in spiked blood samples. To evaluate the validation parameters, a one-day assay protocol was defined, based on the analysis of the calibration curve with three different concentrations of control samples. Five calibration curves were prepared on different days over several months.

**Selectivity**

Selectivity was examined by analyzing nine different blank blood samples in order to detect matrix interferences at the retention times and monitored ions. Figure 3 shows the chromatogram from a blank blood sample free of interferences at the retention time of methylated furosemide. Blank blood matrix was analyzed each day.

**Calibration curves and linearity**

Calibration curves (peak-area ratio between furosemide and IS versus furosemide concentration) were measured daily, using six levels of spiked blood samples prepared and analyzed in duplicate by the described procedure, between 0.10 and 5.00 µg/mL. Five replicates of control samples at low, medium, and high concentrations (0.30, 0.75, and 3.00 µg/mL) of furosemide were measured on the same day. The choice of concentration range was based on the therapeutic and toxic range values in serum published in the literature versus what was practically feasible under the applied GC conditions (11). For concentrations > 5 µg/mL it was decided that the analysis should be repeated after appropriate dilution with blank blood matrix.

Linearity was obtained in the range 0.10–5.00 µg/mL using a series of different levels in duplicate. The selected ions for quantitation were m/z 81 for methylated furosemide and m/z 209 for methylated IS. The calibration curve was submitted to linear regression analysis using the least-squares method to obtain the slope and intercept. The correlation coefficient was 0.9941. Because the calculated t-value was greater than the tabulated value the correlation is significant. This linear relationship demonstrated to be statistically significant by using an analysis of variance of the regression (F-test) to compare F values. The F-value obtained is much greater than the F-tabulated, so linearity exists in the studied concentration range. An example of the statistical evaluation of the standard curve is presented in Table I.

**LOD and LOQ**

LOD and LOQ were calculated based on the standard deviation of the response and the slope of the calibration curve. The following formulas were applied: LOD = (3.3 × SD)/b and LOQ = (10 × SD)/b, where SD was the standard deviation of the response and b the slope of the regression line (22,23). The thus calculated and extrapolated LOD and LOQ were 0.010 µg/mL and 0.045 µg/mL, respectively.

**Trueness and precision**

Precision and trueness were estimated at three different concentrations (0.30, 0.75, and 3.00 µg/mL) with five replicates for

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**Table I. Statistical Evaluation of a Calibration Curve**

<table>
<thead>
<tr>
<th>Linear model: ( y = a + bx )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( y ) variable: Furosemide/Ketoprofen</td>
</tr>
<tr>
<td>( x ) variable: Concentration (µg/mL)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regression Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple R ( = 0.997062249 )</td>
</tr>
<tr>
<td>R square ( = 0.994133128 )</td>
</tr>
<tr>
<td>Adjusted R square ( = 0.993714065 )</td>
</tr>
<tr>
<td>Standard error ( = 0.445319468 )</td>
</tr>
<tr>
<td>Observations ( = 16 )</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Coefficients</th>
<th>Standard error</th>
<th>t Stat</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept ( -0.407077164 )</td>
<td>( 0.163058982 )</td>
<td>( -2.4965 )</td>
<td>( 0.025639297 )</td>
</tr>
<tr>
<td>Slope ( 3.257683172 )</td>
<td>( 0.06688456 )</td>
<td>( 48.70606 )</td>
<td>( 5.02448E^{-17} )</td>
</tr>
</tbody>
</table>

**ANOVA**

<table>
<thead>
<tr>
<th>Degrees of Freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>( F )</th>
<th>Significance F</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>470.4454765</td>
<td>470.4455</td>
<td>2372.279926</td>
<td>( 5.02448E^{-17} )</td>
</tr>
<tr>
<td>Residual 14</td>
<td>2.776332</td>
<td>0.198309</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total 15</td>
<td>473.2218085</td>
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<td></td>
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</tbody>
</table>

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**Table II. Method Validation**

<table>
<thead>
<tr>
<th>Theoretical Concentration (µg/mL)</th>
<th>Intraday RSD (%)</th>
<th>Trueness Relative Error (%)</th>
<th>Interday RSD (%)</th>
<th>Trueness Relative Error (%)</th>
<th>Relative Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.30</td>
<td>6.52</td>
<td>3.82</td>
<td>11.58</td>
<td>3.52</td>
<td>104</td>
</tr>
<tr>
<td>0.75</td>
<td>4.82</td>
<td>-6.51</td>
<td>10.38</td>
<td>-10.55</td>
<td>89</td>
</tr>
<tr>
<td>3.00</td>
<td>7.94</td>
<td>-0.23</td>
<td>13.90</td>
<td>-8.63</td>
<td>91</td>
</tr>
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</table>

**Table III. Toxicological Findings in the Case Study**

<table>
<thead>
<tr>
<th>Furosemide (µg/mL)</th>
<th>Disopyramide (µg/mL)</th>
<th>Ethanol (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood concentrations</td>
<td>2.7</td>
<td>38.0</td>
</tr>
</tbody>
</table>
each level, analyzed in the same day (intraday precision) and on
five different days over five months (interday precision) using
one-way ANOVA. Precision was calculated in terms of relative
standard deviation (RSD, %), and trueness in terms of relative
error (%) between the measured and the spiked concentra-
tions. The obtained values for precision and trueness were less
than 15% for all concentration levels (Table II), falling within
the criteria accepted internationally (22,23).

Recovery
The relative recovery of the described method was calculated
by dividing the amount of the compound measured by the the-
oretical concentration. Results were 104%, 89%, and 91% for
0.30, 0.75, and 3.00 μg/mL controls, respectively (Table II).

Discussion
After several experiments it was verified that the use of soni-
cation and dilution of the blood sample before SPE was of cru-
cial importance because of strong plasma protein binding of
furosemide. This way homogenization was more effective, the
extracts were cleaner for chromatographic analysis, and the
extraction recovery was higher.
In recent years, several studies were published about the de-
termination of furosemide in plasma, serum, and urine in
doping control analysis. However, in the field of forensic toxi-
cology, blood samples are the most important matrices for
quantitative determinations, and most frequently the only avail-
able. The development of a methodology to determine
furosemide in authentic blood samples is an important and
useful tool, particularly in postmortem toxicology.

Application to an Authentic Sample
The developed experimental protocol was applied to an au-
thetic blood sample obtained from a medicolegal autopsy.
A 48-year-old female with a history of alcohol abuse and sui-
cide attempts was admitted to the hospital after consuming
multiple drugs and alcohol. She died 2 h later. At autopsy no evi-
dence of any disease that might have caused death was found,
no signs of violence were observed, and intoxication was sus-
ppected because of pulmonary edema and general congestion of
the inner organs. Histological findings revealed the presence
of liver disease in an initial phase. Samples taken for toxicological
analysis included tissues, gastric content (including pills), car-
diac blood, and femoral blood. The pathologist requested al-
cohol analysis and a drug screen with specific attention toward
furosemide. Ethanol was quantitated in femoral blood using GC–flame-ionization detection with a headspace autosampler.
After screening using high-pressure liquid chromatography–
photodiode array detector and GC–EI-MS, disopyramide (an
antiarrhythmic) was detected in the blood and in pills recovered
from the gastric contents. Furosemide was detected and quan-
titated using the described methodology. Table III shows the
 toxicological results.

Conclusions
The developed procedure showed to be appropriate to deter-
mine furosemide in whole blood samples. It offers time reduc-
tion in the derivatization step, simplicity, and sensitivity for
quantitative determinations of small concentrations of
furosemide.
The optimized and validated methodology is suitable for the
application in routine analysis in the field of forensic toxicology.

Acknowledgments
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ance with the bibliography.

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