Analysis of Flecainide and Two Metabolites in Biological Specimens by HPLC: Application to a Fatal Intoxication

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Abstract

A few days after her admittance to a hospital for a suicide attempt with benzodiazepines, a 15-year-old girl was found dead in bed. At autopsy, no specific anatomo-pathologic cause of death was identified. Systematic toxicological analysis (HPLC–DAD, GC–NPD, and GC–MS) of postmortem blood and urine revealed the presence of high concentrations of flecainide and its two major metabolites. Flecainide is a class IC anti-arrhythmic drug causing a decreased intracardiac conduction velocity in all parts of the heart. To identify and quantitate flecainide together with its metabolites in blood, urine, and other toxicologically relevant matrices, a new method was developed using high-performance liquid chromatography with diode-array detection. All compounds were separated on a Hypersil BDS phenyl column using water, methanol, and 1.5M ammonium acetate in a gradient system. Chromatographic analysis was preceded by an optimized solid-phase extraction procedure on RP-C18 extraction columns. The flecainide concentrations in blood and urine were 18.73 and 28.3 mg/L, respectively, and the metabolites were detected only in urine at the following concentrations: 9.4 mg/L for meta-O-dealkylated flecainide and 8.59 mg/L for meta-O-dealkylated lactam of flecainide. Based on these results, it was concluded that the suicide was consistent with an overdose of this anti-arrhythmic drug.

Introduction

Flecainide acetate, N-(2-piperidinylmethyl)-2,5-bis(2,2,2-trifluoroethoxy) benzamide monacetate, a class IC anti-arrhythmic used as racemate (Tambocor*), is a sodium blocking agent causing a decreased intracardiac conduction velocity, mainly by influencing the His-Purkinje system and is therefore used to treat ventricular arrhythmias (1).

Major noncardiac side effects include dizziness, visual disturbances, nausea, and vomiting, which seem to be dose-related. Long-term therapy can generate pulmonary complications. Cardiac adverse effects include negative inotropic action and depression of all major conduction pathways, leading to ventric-ular tachyarrhythmias and severe bradycardia (2).

Flecainide is rapidly and nearly completely absorbed orally (95% bioavailability) with minimal first-pass effect. Peak concentrations in plasma occur 3 to 4 h after oral intake, the therapeutic range in serum is 0.2–1.0 mg/L, and the plasma protein binding is about 40%.

Flecainide is biotransformed via O-dealkylation. The major metabolites are meta-O-dealkylated flecainide, which possesses minimal cardioactivity, and the meta-O-dealkylated lactam of flecainide, which displays no anti-arrhythmic activity (Figure 1). Both metabolites and the parent drug (about 10% of a dose) are recovered from urine (3).

The drug has a long half-life (12–27 h) in the therapeutic range. In toxic doses reduced hepatic and renal blood flow due to hypotension both lower the elimination and prolong the duration of toxicity. Hemodialysis and hemoperfusion are not effective for removing flecainide from the circulation because of its high apparent volume of distribution (8.7 L/kg). Moreover, a specific antidote is not known. Therefore, an acute overdose of flecainide acetate is frequently fatal (4).

Several analytical techniques have been used for research including pharmacokinetic studies of flecainide in plasma or serum, using capillary gas chromatography (GC) (5–7), and var-

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Figure 1. Metabolization of flecainide (3).
ious high-performance liquid chromatographic (HPLC) methods with either fluorescence or ultraviolet detection (7–19).

To the best of our knowledge all HPLC methods focused exclusively on the identification and quantitation of flecainide. This paper describes a new HPLC method and solid-phase extraction procedure for the simultaneous determination of flecainide and its two main metabolites in several toxicologic matrices.

Case History

On a Sunday, at about midday, a 15-year-old female was admitted to the hospital for observation after ingestion of about 10 to 15 “sleeping pills”. Flurazepam pills were ingested in the night of Friday to Saturday and possibly later in the course of Saturday. On arrival at hospital, she was obviously drowsy and, hence, an auto-intoxication was suspected. Urine was screened for the presence of medication and illegal drugs and was positive only for benzodiazepines. According to the statements of the nursing staff, the girl was severely depressed, and suicidal behavior was noticed by the neuropsychiatrist. On Monday, she complained a few times of non-specific “gastric” and “abdominal” pain, which was treated with an antacid. In addition, at about 4 p.m., she was crying with abdominal pain, which was interpreted as hysteria. In the night of Monday to Tuesday, at about midnight, the patient was found lying on the floor and was described as hysterical by the nurse who put her back to bed. At 2:30 a.m., a normally sleeping patient was observed. However, she was found dead in bed at 8:00 a.m.

Further inquiry revealed that the victim had phoned a friend on Monday evening, at about 10 p.m., and told her that she had stolen pills from the hospital without knowing what they were only for benzodiazepines. According to the statements of the nursing staff, the girl was severely depressed, and suicidal behavior was noticed by the neuropsychiatrist. On Monday, she complained a few times of non-specific “gastric” and “abdominal” pain, which was treated with an antacid. In addition, at about 4 p.m., she was crying with abdominal pain, which was interpreted as hysteria. In the night of Monday to Tuesday, at about midnight, the patient was found lying on the floor and was described as hysterical by the nurse who put her back to bed. At 2:30 a.m., a normally sleeping patient was observed. However, she was found dead in bed at 8:00 a.m.

On postmortem external examination the following day, the body weighed approximately 55 kg and was 168 cm tall. During the external examination, nothing conspicuous was noticed. No puncture marks were observed. On internal inspection, 150 mL bloody fluid was found in the pleural cavities, and both lungs weighed 880 g. Emphysema was remarked, and on incision, obvious congestion and edema were observed. White vomit particles were noticed in the trachea. A few Tardieu spots were present on the pericardium. The heart weighed 230 g and showed—except for a pale appearance of the myocardium—no anomalies. The stomach contained about 350 mL of hardly digested food and some small white granules compatible with medicines. The brain weighed 1330 g and, apart from slight congestion and edema of the white matter, no lesions were found. The remaining organs only showed vascular congestion.

On histologic examination, pronounced pulmonary congestion, hemorrhagic edema, and an obviously increased number of eosinophilic cells, scattered throughout the lung parenchyma, were remarked. The macroscopic and histologic findings of heart and lungs were consistent with acute to subacute cardiopulmonary failure. There were no arguments for vomit aspiration into the smaller bronchi and bronchioli. A few myocytes showed basophilic degeneration, and some leucocytic sludging in the veins was found. The portal spaces of the liver showed lymphocytic and eosinophilic infiltration but were not enlarged.

Bacteriologic investigations were negative. Biochemical screening of urine pointed to the absence of glucose and ketone bodies. Subclavial blood, urine, stomach contents, bile, vitreous humor, and samples of liver and kidney were taken for toxicologic investigation.

Experimental

Solvents and reagents
Flecainide and its metabolites, meta-O-dealkylated flecainide and the meta-O-dealkylated flecainide lactam, as well as the internal standard (IS), a positional isomer of flecainide (Figure 2) were a generous gift from 3M Pharmaceuticals (St. Paul, MN). Flurazepam was a gift from Madaus-Therabel (Brussels, Belgium). N-Desalkylflurazepam was obtained from Mikromol GmbH (Teltow, Germany). Tramadol and carbamazepine were obtained from Sigma-Aldrich Chemicals (Bornem, Belgium).

All standards were more than 99% pure and used without further purification.

β-Glucuronidase (EC 3.2.1.31), type HP-2 from H. pomatia (127,300 U/mL) was from Sigma-Aldrich. Sodium acetate was from UCB (Leuven, Belgium), and acetic acid, sodium hydroxide, di-sodium hydrogen phosphate 2-hydrate, and sodium dihydrogen phosphate 1-hydrate were purchased from Merck-Eurolab (Leuven, Belgium). Water and methanol were of HPLC gradient grade (Merck-Eurolab).

The solid-phase extraction columns (LiChrolut RP-C18 (200 mg)) were purchased from Merck-Eurolab.

Preparation of standards and buffer solutions
Separate stock solutions containing each of the previously mentioned standards and IS at a concentration of 1 g/L, were prepared in methanol. Working solutions containing 20 mg/L of

![Figure 2. Structure of flecainide and the internal standard.](image-url)
each drug were prepared by repeated dilutions of the stock solutions with a mixture of water/methanol/1.5M NH₄Ac (87:10:3, v/v).

Sodium acetate buffer (pH 4.5) for hydrolysis: 5.85 mL of 2M acetic acid was added to 4.15 mL of 2M sodium acetate (164.1 g C₂H₃NaO₂/L HPLC-grade water), and the solution was made up to 100 mL with HPLC-grade water.

Phosphate buffer (pH 6.8) for extraction: 25.5 mL of 0.2M NaH₂PO₄ (27.60 g NaH₂PO₄•H₂O/L) was added to 24.5 mL of 0.2M Na₂HPO₄ (35.61 g Na₂HPO₄•2H₂O/L), and the solution was made up to 100 mL with HPLC-grade water.

Quantitation

Extraction. One milliliter of postmortem fluid (blood, urine, bile, and vitreous humor) was spiked with 80 µL of a 20-mg/L internal standard solution. Blood samples were ultrasonicated for 10 min, and urine samples were hydrolyzed by enzymatic hydrolysis. To 1 mL of spiked urine in a 15-mL centrifuge tube, 2 mL of 0.2M sodium acetate buffer (pH 4.5) and 5500 U of H. pomatia β-glucuronidase were added. The tubes were mixed vigorously and incubated at 56°C for 1 h. Tissue samples (liver and kidney) and stomach contents were diluted (1:1, w/w) with HPLC-grade water and homogenized with an Ultra-Turrax mixer (Ika-Werke, Staufen, Germany) before being spiked with the IS. The homogenates were deproteinized by the addition of 0.5 mL ethanol followed by 10 min of ultrasonication. Thereafter, all samples were centrifuged at 2500 rpm for 5 min, and the upper layer was separated from the precipitate and brought to pH 6.8 by the addition of phosphate buffer. Samples were applied slowly on the SPE RP-C18 cartridges, which had been previously conditioned with 2 mL of methanol, followed by 2 mL of water. The columns were not allowed to dry before the addition of the samples. After a wash step with 2 mL of water followed by 1 mL of the water/methanol mixture (70:30, v/v), cartridges were dried under maximum vacuum for 20 min, and the analytes were eluted with 1 mL of methanol. The elution solvent was evaporated to dryness under a gentle stream of nitrogen. Extraction residues were reconstituted in 80 µL of the mobile phase (solvent A), and 50 µL was injected for HPLC analysis.

Figure 3. HPLC–DAD chromatogram (λ det: 230 nm) of the postmortem blood sample (A). Peak identification: 1, tramadol; 2, IS; 3, flecainide; 4, carbamazepine; and 5, N-desalkylflurazepam. Corresponding UV-spectrum (225–350 nm) (B) and mass spectrum (after GC–MS analysis) (C) of flecainide.
Chromatographic conditions. Analysis was performed on a ternary low-pressure gradient system (model 325, Kontron Instruments, Milan, Italy) equipped with a Beckman model 168 diode-array detector (Analys, Ghent, Belgium), operated in a 4-mm band-pass mode monitoring light from 225 to 350 nm. The display wavelength was 230 nm. Separation was performed on a Hypersil BDS phenyl column (53 × 7.0-mm i.d., 3 µm, Alltech, Deerfield, IL). The mobile phase consisted of a mixture of water/methanol/1.5M NH₄Ac (87:10:3, v/v) (solvent A) and a mixture of water/methanol/1.5M NH₄Ac (17:80:3, v/v) (solvent B). The following gradient conditions were used: a linear gradient starting from 0% B and going to 50% B in 8 min, followed by a hold at 50% B for 7 min and another linear gradient to 75% B in 1.5 min. After an isocratic period (75% B) of 1 min, the pump was programmed to regain the initial conditions (0% B) by a hold at 50% B for 7 min and another linear gradient to 75% B in 1.5 min. The total analysis time was 18.5 min at a flow rate of 1.5 mL/min.

Calibration. Calibration graphs for each compound were prepared in blank whole blood, urine, and liver. Blood was used as a representative matrix for bile and vitreous humor, and liver was a representative for kidney. Each sample was spiked with 80 mL of the 20-mg/L internal standard solution. The spiked compound levels for each calibration graph were 0.08, 0.16, 0.80, 1.6, 4.00, and 8.00 mg/L. Forensic case samples were analyzed twice. First, 1-mL or 2-g homogenous aliquots of all available postmortem matrices (blood, urine, vitreous humor, bile, stomach contents, liver, and kidney) were spiked with the internal standard solution and extracted. Following these orientating preliminary results, a second extraction was performed on appropriately diluted sample specimens.

Validation. The limits of detection (LOD) and quantitation (LOQ), calculated according to the method described by the European Pharmacopoeia (20), were estimated as 3 and 10 times the signal-to-noise ratio, respectively. Calibration graphs were prepared as described. Weighted linear regression was performed in an effort to account for data heteroscedasticity. The concentrations of all compounds were calculated from this regression line. The precision of the method was assessed by six replicate analyses containing all compounds in a concentration of 0.50 mg/L.

Absolute recoveries were determined by comparing the analyte peak height obtained after extraction of spiked samples to the peak height of known amounts of unextracted analyte.

### Results and Discussion

In accordance with the laboratory's operating procedures (21,22), a general screening of the blood, urine, and stomach contents was performed with an enzymatic test, enzyme multiplied immunoassay (EMIT®), and radioimmunoassay (RIA), yielding positive screening results for ethanol (0.14 g/L in blood), caffeine (0.66 mg/L in urine), carbamazepine (33.38 mg/L in stomach contents), and benzodiazepines (0.14 and 7.20 mg/L in blood and urine, respectively).

As a result of the initial HPLC screening on an alumina-based packing material (22), four different peaks were observed in the respective chromatograms of the alkaline extract of the blood, urine, and stomach contents. Based on retention and their UV-spectra these compounds were identified as flecainide, carbamazepine, N-desalkylflurazepam, and tramadol, confirming the EMIT screening results for carbamazepine and benzodiazepines. Furthermore, after extraction at neutral pH, the presence of flecainide was also confirmed in blood, urine, and stomach contents by routine gas chromatographic–mass spectrometric (GC–MS) analysis (Figure 3C). No other drugs

### Table I. Mean Recoveries and Coefficients of Variation

<table>
<thead>
<tr>
<th>Analyzed compounds</th>
<th>Blood (n = 6)</th>
<th>Urine (n = 6)</th>
<th>Liver (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flecainide</td>
<td>101.9 (8.2)</td>
<td>92.9 (3.9)</td>
<td>24.6 (10.4)</td>
</tr>
<tr>
<td>OH-Flecainide</td>
<td>99.2 (3.6)</td>
<td>97.0 (8.4)</td>
<td>94.3 (7.5)</td>
</tr>
<tr>
<td>OH-Flecainide lactam</td>
<td>94.0 (7.3)</td>
<td>91.2 (8.7)</td>
<td>67.7 (1.7)</td>
</tr>
</tbody>
</table>

Table II. Summary of the Quantitative Results

<table>
<thead>
<tr>
<th>Analyzed compounds</th>
<th>Blood (mg/L)</th>
<th>Urine (mg/L)</th>
<th>Liver (mg/kg)</th>
<th>Kidney (mg/kg)</th>
<th>Bile (mg/L)</th>
<th>Stomach contents (mg/L)</th>
<th>Vitreous humor (mg/L)</th>
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</thead>
<tbody>
<tr>
<td>Ethanol (g/L)</td>
<td>0.14</td>
<td>–*</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Caffeine</td>
<td>–</td>
<td>0.66</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>0.99</td>
<td>0.09</td>
<td>1.74</td>
<td>0.44</td>
<td>42.12</td>
<td>0.13</td>
<td>–</td>
</tr>
<tr>
<td>Tramadol</td>
<td>0.32</td>
<td>0.52</td>
<td>1.38</td>
<td>–</td>
<td>–</td>
<td>12.15</td>
<td>–</td>
</tr>
<tr>
<td>N-Desalkylflurazepam</td>
<td>0.07</td>
<td>0.38</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.22</td>
</tr>
<tr>
<td>Flecainide</td>
<td>18.73</td>
<td>26.3</td>
<td>18.21†</td>
<td>27.89†</td>
<td>23.78</td>
<td>133.62†</td>
<td>15.42</td>
</tr>
<tr>
<td>OH-Flecainide</td>
<td>–</td>
<td>9.4</td>
<td>–</td>
<td>–</td>
<td>6.68</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>OH-Flecainide lactam</td>
<td>8.59</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Not detected.
† Results obtained under low recovery conditions.

Table III. Flecainide Levels (mg/L) in Blood and Urine for the Presented Case and Previously Reported Fatal Cases

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Case</td>
<td>F-15y</td>
<td>F-22y</td>
<td>M-65y</td>
<td>M-36y</td>
<td>F-33y</td>
<td>M-15y</td>
<td>M-23y</td>
<td>M-18y</td>
</tr>
<tr>
<td>Blood</td>
<td>18.73</td>
<td>13</td>
<td>7.7</td>
<td>3.32</td>
<td>93.7</td>
<td>100</td>
<td>7.3</td>
<td>16.3</td>
</tr>
<tr>
<td>Urine</td>
<td>28.3</td>
<td>54</td>
<td>28.9</td>
<td>–*</td>
<td>82.4</td>
<td>–</td>
<td>117</td>
<td>–</td>
</tr>
</tbody>
</table>

* Not analyzed.
were detected with either gas chromatography–nitrogen-phosphorus detection (GC–NPD) or GC–MS.

For the accurate quantification of flecainide and its main metabolites in postmortem matrices, a suitable internal standard in combination with optimized chromatographic conditions and a specific extraction were required.

Previously published papers describing HPLC analysis of flecainide used internal standards such as amiodarone (23), N-methylflecainide (19), or a positional isomer of flecainide (7,24,25). The authors preferred the positional isomer of flecainide (15531-S) as an internal standard because of its predictable identical behavior during the whole analytical procedure.

Chromatographic separation using HPLC was examined on various columns with different mobile phases. The most important problems that appeared were the co-elution of the metabolites with the solvent peak and the poor separation of flecainide and the internal standard. Altering gradient steepness and solvent composition could not always ameliorate the resolution. Different columns were tested, and finally all compounds could be baseline separated on a Hypersil BDS phenyl column (53 × 7.0-mm i.d., particle size 3 µm) using mixtures of water, methanol, and 1.5 M ammonium acetate as mobile phase in a gradient system. The flow was set at 1.5 mL/min resulting in a total analysis time of 18.5 min (Figures 3A and B).

Chromatographic analysis was preceded by an optimized solid-phase extraction procedure. LiChrolut RP-C18 extraction columns yielded satisfactory results with recoveries in blood and urine, fluctuating around 100% for all compounds (Table I). Despite several efforts (addition of ethanol, 10% trichloroacetic acid, and ultrasonication), drug recoveries in liver, used as a representative matrix for all tissue analyses, were considerably lower but had an acceptable coefficient of variation (26).

The LODs for flecainide and its metabolites ranged from 2 to 8 µg/L (blood) and from 4 to 6 µg/L (urine). The LOQs ranged from 6.5 to 25 µg/L blood and from 13 to 20 µg/L urine. All calibration curves were linear in the selected range (0.08–8 mg/L) with correlation coefficients (r²) ranging from 0.998 to 0.999. The coefficient of variation for within-day precision (n = 6) in blood and urine was below 20% for the lowest calibration point and below 9% for the rest of the calibration points for all compounds.

All available postmortem samples were analyzed using the developed method, and the quantitative results are summarized in Table II. High concentrations of flecainide, consistent with overdose, were demonstrated in blood (18.73 mg/L). The high volume of distribution of the compound is reflected in the equally high concentrations found in all samples, even in the vitreous. The metabolites were only detected in urine and bile and the concentrations were 9.4 mg/L (urine) and 6.68 mg/L (bile) for meta-O-dealkylated flecainide and 8.59 mg/L for the corresponding lactam (urine).

A subtherapeutic concentration of carbamazepine and therapeutic concentrations of tramadol and N-desalkylflurazepam were also found in blood. To our knowledge, only a few suicides by flecainide overdose have been reported. Flecainide concentrations for our case as well as previously reported fatal cases are given in Table III. Comparison of our quantitative results to those found in literature shows that the measured values are in the same range.

Not only can flecainide intoxication cause new or worsened arrhythmias, but it can also cause confusion, stupor, convulsions, and death (27). Gastric complaints such as nausea and vomiting were frequently reported as early symptoms in non-lethal intoxications (28). In this case report, post-hoc, however, her complaint of “gastric” or “abdominal” pain could be related to a flecainide overdose.

Conclusions

In summary, a unique HPLC method and a simple solid-phase extraction procedure were developed for the simultaneous determination of flecainide and its two major metabolites in relevant matrices. In contrast to GC analysis, no time-consuming derivatization of the polar metabolites was required.

The obtained quantitative data clearly demonstrated that the described fatality was due to an oral overdose of flecainide. The other drugs were only present in therapeutic (tramadol and N-desalkylflurazepam) or subtherapeutic (carbamazepine) concentrations. The case history was consistent with suicide by drug-intake.

Acknowledgments

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