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Sensitive method for the quantitative determination of gemfibrozil in dog plasma by liquid–liquid cartridge extraction and liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive LC–MS/MS assay for the quantitative determination of gemfibrozil in dog plasma has been developed and validated and is described in this work. The assay involved the extraction of the analyte from 0.5-ml aliquots of dog plasma using Chem Elut cartridges and methyl *tert.*-butyl ether (MTBE). Chromatography was performed on a Metasil Basic column (50×2 mm I.D., 3 μm) using a mobile phase that consisted of 70:30 acetonitrile–ammonium acetate (1 m*M*, pH 5.0) with a flow-rate of 0.2 ml min^{-1}. The method showed excellent reproducibility with an inter- and intra-assay precision of <8.9% (%RSD), as well as excellent accuracy with an inter- and intra-assay accuracy between 99 and 101%. This method has a lower limit of quantitation (LLOQ) of 1.0 ng ml^{-1} with a linear calibration range from 1.0 to 250 ng ml^{-1}. This new assay offers higher sensitivity and a much shorter run time over earlier methods.
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1. Introduction

Gemfibrozil (Fig. 1) is a cholesterol-lowering agent that has been clinically proven effective at not only reducing serum cholesterol, triglyceride, and LDL levels, but has also proven effective at increasing serum HDL levels [1]. Mechanistically, gemfibrozil acts as an agonist for peroxisome proliferator-activated receptors (PPARs) [2]. Its high binding affinity for the PPAR receptors is thought to reduce cholesterol, triglyceride, and LDL levels by stimulating fatty acid oxidation and is thought to increase HDL levels by the induction of apolipoprotein-AI and -AII expression.

With current pharmacokinetic studies, it is very important to have an analytical method that is both sensitive and has short analytical run-times. A sensitive method will allow for the measurement of low levels of analytes which may be present at later post-dose time-points during terminal elimination. Short analytical run-times would allow for an increase in throughput which in turn allows for quick turn-around of large pharmacokinetic studies. Previous bioanalytical methods for determination of gemfibrozil in plasma focused on gas chromatographic techniques which included tedious derivatisation steps and high-performance liquid chromatographic techniques that utilized either UV or fluorescence

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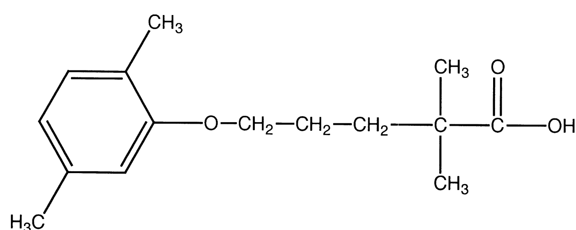


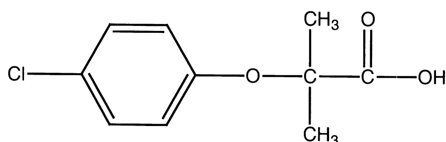


Fig. 1. The chemical structures of gemfibrozil and the internal standard clofibric acid. Gemfibrozil, $M_w = 250.3$; clofibric acid, $M_w = 214.6$.

detection. The limits of quantitation for these methods ranged from a low of 10 ng ml^{-1} to a high of 1000 ng ml^{-1}, and the chromatographic run-times for these methods have been in the range of 20 min [3–7]. This work describes a simple liquid–liquid cartridge extraction (LLCE) technique that utilizes liquid chromatography–tandem mass spectrometry (LC–MS/MS) detection for a more sensitive and higher-throughput determination of gemfibrozil in dog plasma. To our knowledge, this is the first reported use of this extraction and detection technique for the determination of gemfibrozil in biological matrices.

2. Experimental

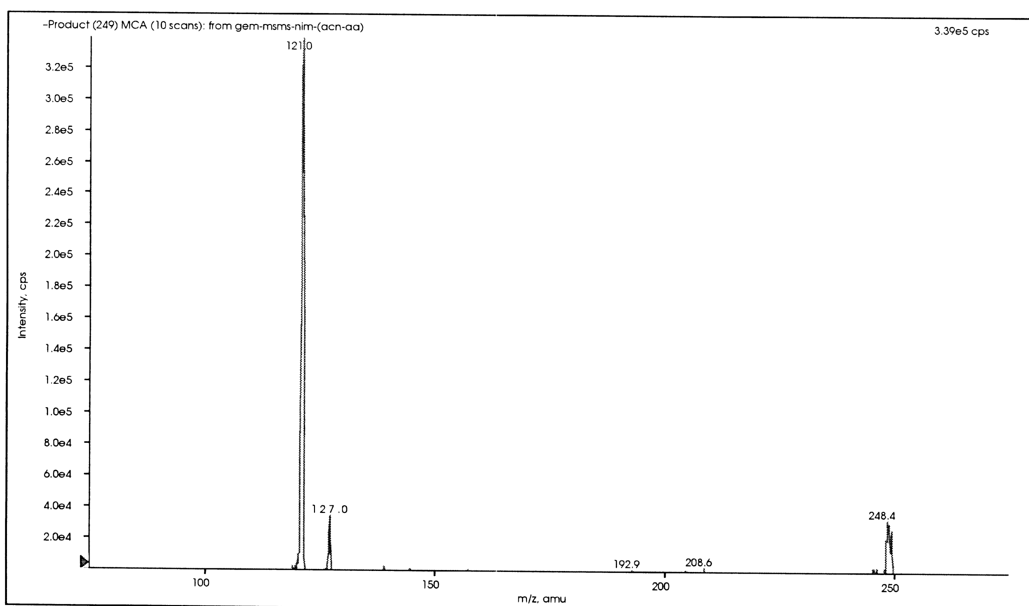
2.1. Chemicals and reagents

Gemfibrozil (99% purity) was purchased from Sigma (St. Louis, MO, USA) and clofibric acid (97% purity) and acetic acid (glacial) were purchased from Aldrich (Milwaukee, WI, USA). Ammonium acetate (HPLC grade) and acetonitrile (Optima grade) were obtained from Fisher Scientific (Fairlawn, NJ, USA), and methyl *tert*.-butyl ether (MTBE) (HPLC grade) from Burdick & Jackson (Muskegon, MI, USA). Regular control dog (Beagle) plasma (heparinized) was purchased from Biological Specialty (Lansdale, PA, USA). Nitrogen (refrigerated liquid) was obtained from Praxair (Danbury, CT, USA). De-ionized water was prepared using a Milli-Q Plus Ultra-Pure water system (Millipore, Bedford, MA, USA). All chemicals were used as received.

2.2. LC–MS/MS instrumentation and analytical conditions

A Perkin-Elmer Series 200 LC micropump and autosampler (Perkin-Elmer Instruments, Norwalk, CT, USA) was used as the liquid chromatographic system. Gemfibrozil and the internal standard (clofibric acid) were separated on a Metasil Basic column (50×2 mm I.D., 3 μm) (Metachem Technologies, Torrence, CA, USA) using a mobile phase that consisted of 70:30 (v/v) acetonitrile–ammonium acetate (1 m*M*, pH 5.0) at a flow-rate of 200 μl min^{-1} under ambient conditions. The mobile phase was prepared by mixing 700 ml of acetonitrile with 300 ml of 1 m*M* ammonium acetate with the pH adjusted to 5.0 using acetic acid. The temperature of the sample tray in the autosampler was maintained at 4 °C.

A PE Sciex API 365 tandem mass spectrometer (MSD SCIEX, Concord, ON, Canada) with a TurboIonSpray interface was used for mass analysis and detection. The TurboIonSpray temperature was optimized and maintained at 300 °C. Both gemfibrozil and the internal standard were detected in the negative ion mode. A Harvard Model 22 syringe pump (Harvard Apparatus, South Natick, MA, USA) was used for infusion of analyte and internal standard solutions for optimization of mass spectrometric conditions. Quantitation was performed using the selected reaction monitoring (SRM) of the precursor–product ion transitions at (m/z 249.0→121.0) for gemfibrozil (Fig. 2) and (m/z 213.0→126.8) for clofibric acid (Fig. 3). The dwell times for gemfibrozil and clofibric acid were both 500 ms. The



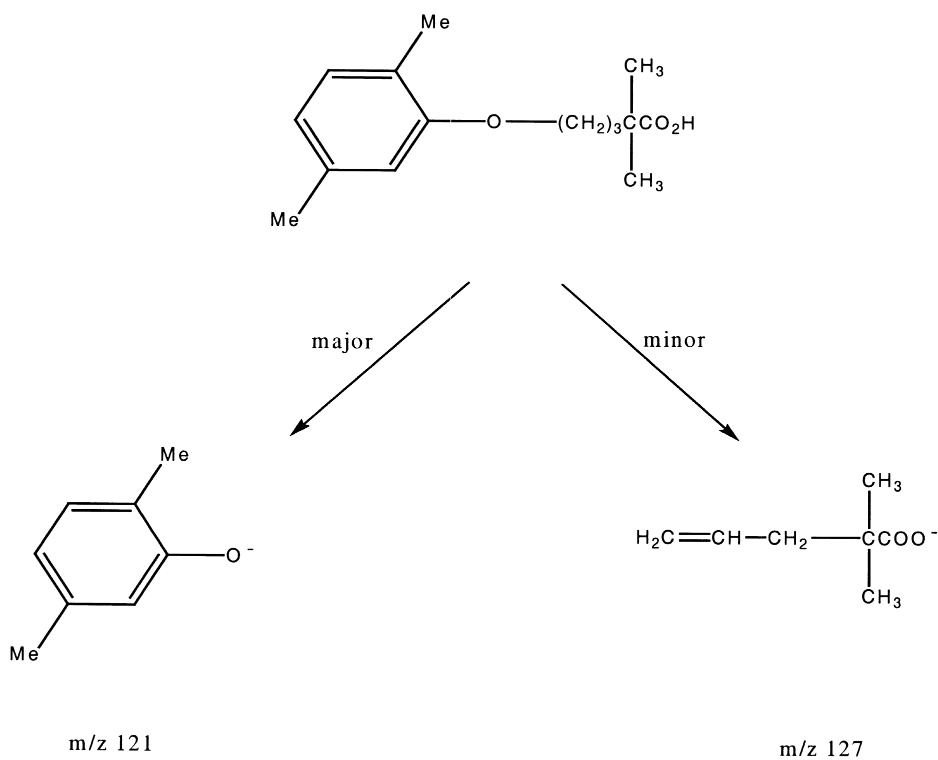
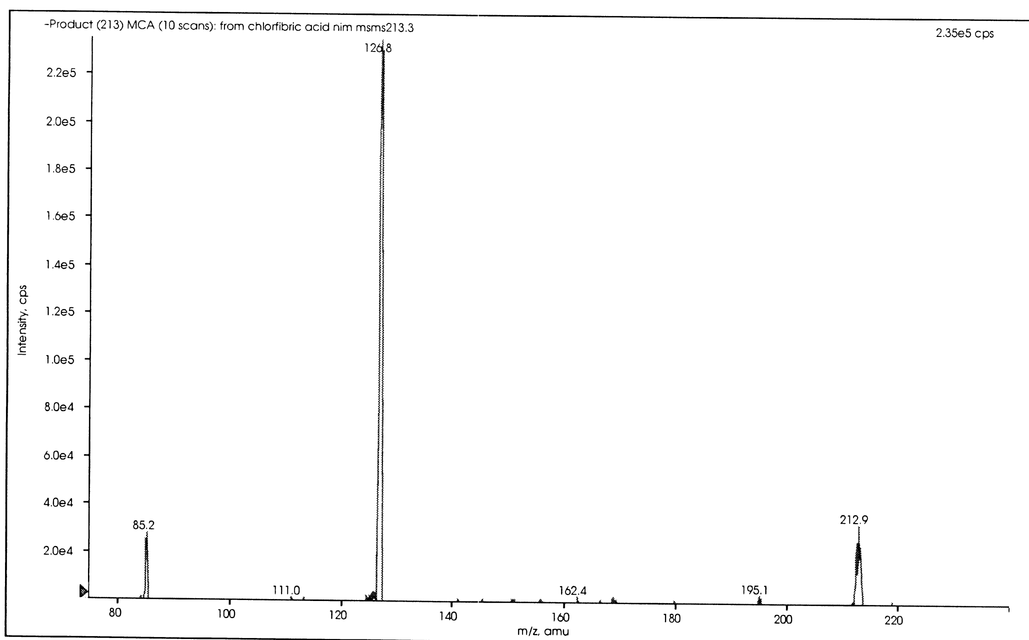


Fig. 2. The product ion spectrum for gemfibrozil and proposed fragmentation scheme.



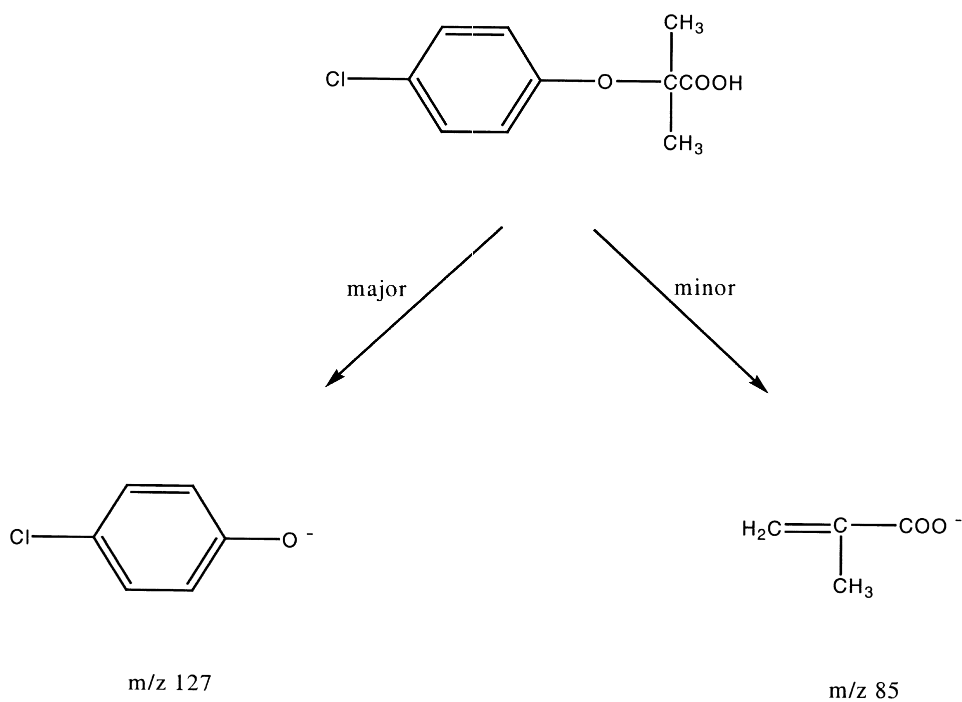


Fig. 3. The product ion spectrum for clofibric acid and proposed fragmentation scheme.

collision energy was optimized at 14.0 V (Q0–RO2). The mass calibration was performed by the infusion of a 10^{-4} *M* polypropylene glycol (PPG) solution into an ion spray source. The peak widths of precursor and product ions were maintained at ~0.7 amu at half-height in the SRM mode. The analytical run time was ~2 min per sample.

Data acquisition, peak integration, and calculations were performed using PE Sciex MassChrom software residing on a Macintosh 9500 computer. Peak area ratios of analytes to internal standards were utilized for the construction of calibration curves, using $1/x$ weighted linear least-squares regression of plasma concentrations and the measured peak area ratios. Concentrations of the analytes in the quality control samples (QC) or study samples were calculated by interpolation from the calibration curves.

2.3. Standards and quality control samples

Stock solutions of gemfibrozil (1.0 mg ml^{-1}) and clofibric acid (1.0 mg ml^{-1}) were prepared by dissolving accurately weighed standard compounds in acetonitrile–water (60:40, v/v) to yield a concentration of 1.0 mg ml^{-1}. Diluted solutions of each at a concentration of 10 μg ml^{-1} were prepared by mixing 200 μl of the appropriate stock solutions with 19.8 ml of acetonitrile–water (60:40, v/v).

Standard working solutions of gemfibrozil at concentrations of 2500, 1000, 500, 250, 100, 50, and 10 ng ml^{-1} were prepared by serial dilutions of the 10 μg ml^{-1} gemfibrozil standard stock solution with acetonitrile–water (60:40, v/v). An internal standard working solution of 500 ng ml^{-1} was prepared by mixing 1.0 ml of the 10 μg ml^{-1} clofibric acid stock solution with 19 ml of an acetonitrile–water (60:40, v/v) solution. QC working solutions at concentrations of 2000, 800, and 200 were prepared by successively diluting the 10 μg ml^{-1} QC stock solution of gemfibrozil (prepared separately) with acetonitrile–water (60:40, v/v).

Plasma standards of gemfibrozil were prepared fresh daily by spiking 50 μl of the appropriate working solutions into 500 μl of dog control plasma to yield calibration concentrations of 250, 100, 50, 25, 10, 5, and 1 ng ml^{-1}. Acetonitrile–water (60:40, v/v) was added to control plasma, at 100 and 50 μl, to make up plasma double blank and blank samples, respectively.

Plasma QC samples were prepared by adding 200 μl of the appropriate QC working solutions to 19.8 ml of control dog plasma to yield concentrations of 200, 80, and 20 ng ml^{-1}. After preparation, the bulk QC samples were aliquoted in 0.5-ml volumes and stored at −70 °C until needed.

2.4. Plasma extraction

A 0.5-ml aliquot of dog plasma was acidified with 100 m*M* ammonium acetate, pH 4.0, mixed, and loaded on a 1.0-ml Chem Elut extraction cartridge packed with diatomaceous earth (Varian Sample Preparation Products, Harbor City, CA, USA). After about 5 min, the analytes were eluted off the cartridge with 3×4 ml volumes of MTBE and were concentrated to dryness under a stream of nitrogen at 40 °C. The residues were then reconstituted with 100 μl of 70:30 acetonitrile–ammonium acetate (1 m*M*, pH 5.0). A 20-μl aliquot was then used for the LC–MS/MS analysis.

3. Results and discussion

3.1. LC–MS/MS

Due to the presence of carboxylic acid groups in both molecules, gemfibrozil and clofibric acid exhibited favorable sensitivity in the negative ion mode under TurboIonSpray ionization conditions. The product ion spectrum of $[M-H]^-$ for gemfibrozil showed only one major fragment ion peak, located at m/z 121.0. Also observed in the product ion spectrum for gemfibrozil was a minor fragment ion peak located at m/z 127.0. Fig. 2 shows the product ion spectrum and the proposed major and minor collision-induced dissociation (CID) fragment ion pathways for gemfibrozil. The major fragmentation ion peak at m/z 121.0, formed by the loss of the dimethyl-pentanoic acid group, was chosen as the product ion. The product ion spectrum of $[M-H]^-$ for the internal standard, clofibric acid, also showed a major and a minor fragment ion peak located at

m/*z* 126.8 and 85.2, respectively. Fig. 3 shows the product ion spectrum and the fragmentation pathways for the product ions of clofibric acid. The major fragment ion peak at *m*/*z* 126.8, which was formed by the loss of the dimethyl-propionic acid group, was chosen as the product ion for this method.

The analytical column, the mobile phase buffer pH, and the mobile phase composition were evaluated and optimized to give adequate resolution between the analyte and internal standard as well as the best sensitivity for the analyte. Gemfibrozil and clofibric acid had retention times of 1.29 and 0.59 min, respectively, under these chromatographic conditions. Fig. 4 shows an SRM mass chromatogram of a typical dog plasma blank and Fig. 5 shows an SRM mass chromatogram of a typical dog plasma sample at 12 h post-dose with 60 mg kg^{-1} gemfibrozil.

3.2. Extraction recovery

LLCE on Chem Elut cartridges packed with diatomaceous earth was selected as the extraction technique for this method. This technique was previously shown to provide very simple, fast, and efficient sample extraction [8]. The procedure and extraction conditions adapted well for gemfibrozil with very little optimization needed.

The extraction recovery for gemfibrozil at three QC concentration levels was determined by comparing the peak area ratios of the analyte to internal standard obtained from plasma samples with the analytes spiked before extraction to those that were spiked after the extraction. The internal standard was spiked after extraction in each case. The extraction recovery for the internal standard was assessed in a similar way using the middle QC as a reference. The

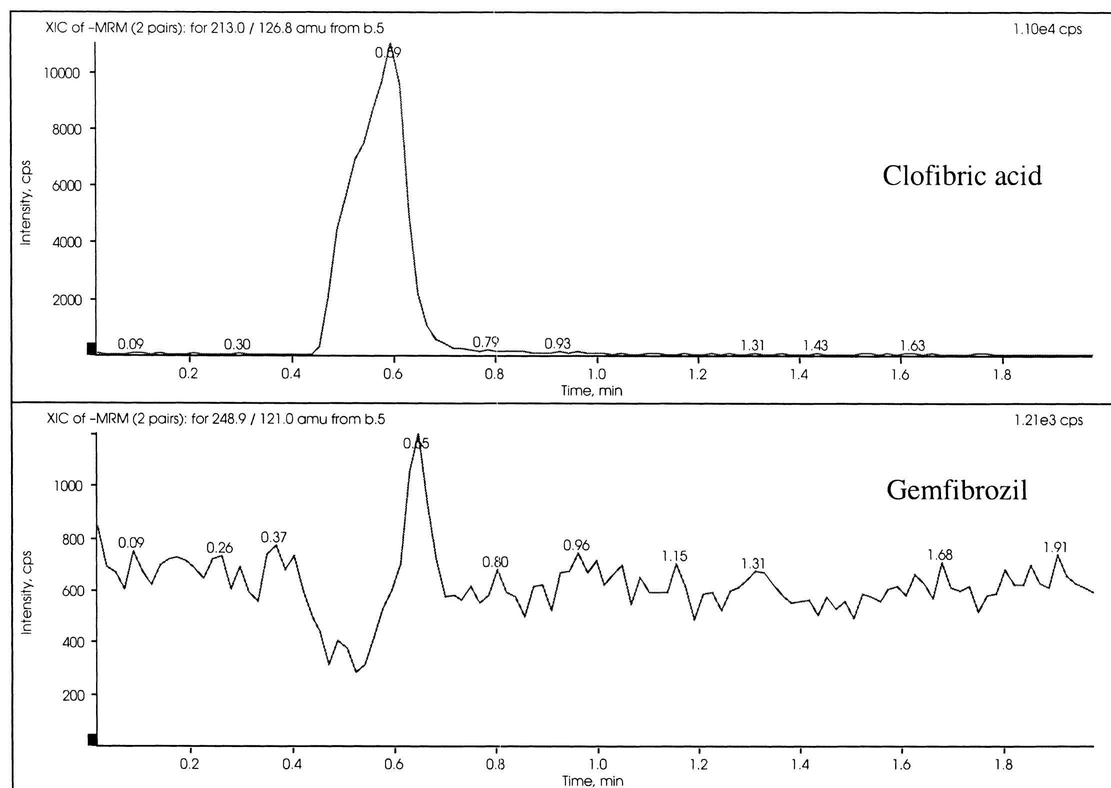


Fig. 4. The SRM mass chromatograms of a typical dog plasma blank sample.

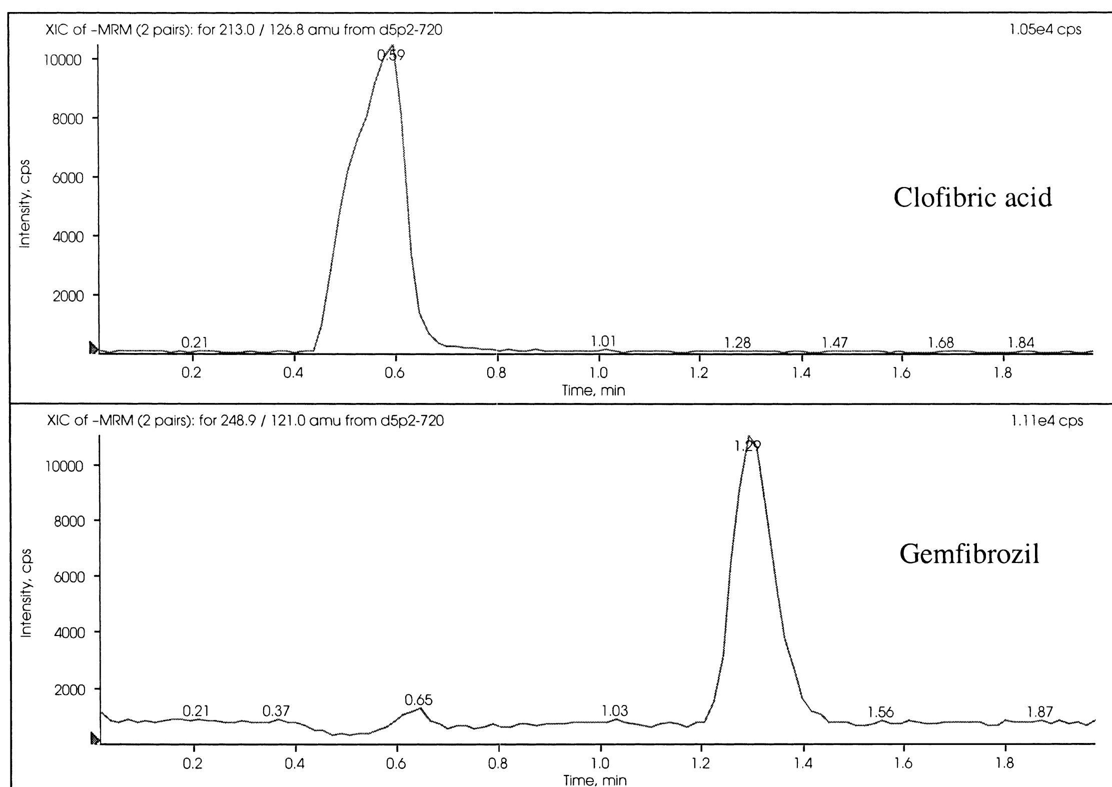


Fig. 5. The SRM mass chromatograms of a typical dog plasma sample at 12 h post-dose with 60 mg kg^{-1} gemfibrozil.

mean extraction recovery for gemfibrozil, calculated as the average recoveries from the three QC levels, was 83.1%. The extraction recovery for the internal standard was 78.2% (Table 1).

3.3. Specificity

Assay specificity was assessed by analyzing drug-free pooled plasma and plasma from five individual dogs and checking for peaks that interfered with the gemfibrozil and clofibric acid. There were no chromatographically interfering peaks observed at the retention times of either the analyte or internal standard in any of those samples. The possibility of interference occurring between the minor gemfibrozil product ion m/z 127 and the major clofibric acid product ion m/z 126.8 was minimized due to the chromatographic resolution between both components.

3.4. Sensitivity

The LLOQ for this assay was determined by measuring the peak-area ratio of decreasing and known concentrations of gemfibrozil. An LLOQ of 1.0 ng ml^{-1} was determined to be the lowest concentration of gemfibrozil that could be measured with a precision better than ±15% and an accuracy of at least ±15% of nominal at a signal-to-noise ratio of 10.

3.5. Linearity

A weighted ($1/x$) least-squares linear regression of response vs. concentration was used for the calibration. Good linearity was obtained in the range of 1.0–250 ng ml^{-1} with typical correlation coefficients between 0.9993 and 1.0000, and an average slope and intercept of $0.0354 \pm 8.32 \times 10^{-4}$ (mean±SD,

Table 1
Extraction recovery of gemfibrozil and its internal standard clofibric acid in dog plasma by LLCE

	Mean peak area ratio[a]		Mean recovery (%)[b]
	Pre-spiked	Post-spiked	
Gemfibrozil			
Concentration of QC sample (ng ml^{-1})			
20	0.57046	0.69782	81.7
40	1.13666	1.30638	87.0
80	2.27100	2.82303	80.4
Overall mean recovery			83.1
Clofibric acid			
Concentration of I.S. in sample (ng ml^{-1})			
50	0.60201	0.76984	78.2

[a] $n=5$. Calculated as (mean peak area of gemfibrozil)/(mean peak area of internal standard) for gemfibrozil and vice versa for clofibric acid.
[b] Calculated as [(pre-spiked mean peak area ratio)/(post-spiked mean peak area ratio)]×100%.

$n=5$) and $0.00205 \pm 3.70 \times 10^{-4}$ (mean±SD, $n=5$), respectively. A wider linear calibration range could have been obtained if a better choice for internal standard such as stable-isotope labeled gemfibrozil was available, but a 1.0–250 ng ml^{-1} range turned out to be good enough for our assay.

3.6. Precision and accuracy

The intra-assay precision and accuracy were assessed by extracting and analyzing five replicates of the plasma standards at all of the concentration levels used in the calibration curve. The intra-assay precision (expressed as percent relative standard deviation, %RSD) ranged from 3.5 to 8.9% and the intra-assay accuracy (expressed as % of nominal values) ranged from 99.2 to 100.7% (Table 2). The initial inter-assay precision and accuracy were determined by analyzing five replicates of the quality control samples at concentrations of 20, 80, and 200 ng ml^{-1} through three assay runs. The method showed excellent reproducibility with an inter-assay precision ranging from 4.9 to 5.4% RSD (Table 3). The inter-assay accuracy ranged from 99.1 to 100.5%.

3.7. Analyte stability

The stability of gemfibrozil was evaluated under a variety of conditions (Table 4). Gemfibrozil was

Table 2
Intra-assay precision and accuracy of the measurement of gemfibrozil in dog plasma

Nominal concentration (ng ml^{-1})	Mean calculated concentration (ng ml^{-1})[a]	Accuracy (%)[b]	Precision (%)[c]
1.0	1.01	100.6	8.9
5.0	5.03	100.7	4.3
10	9.92	99.2	5.6
25	24.9	99.6	3.5
50	49.9	99.7	4.0
100	100	100.2	4.9
250	250	100.1	3.8

[a] From the linear least-squares regression of the standard line using all points ($n=5$) at all concentrations.
[b] Calculated as [(mean calculated concentration)/(nominal concentration)]×100%.
[c] Expressed as %RSD.

Table 3
Inter-assay precision and accuracy of the measurement of gemfibrozil in dog plasma

Nominal concentration (ng ml^{-1})	Mean calculated concentration (ng ml^{-1})[a]	Accuracy (%)[b]	Precision (%)[c]
20	19.8	99.1	5.4
80	80.4	100.5	5.3
200	199	99.7	4.9

[a] $n=3$ days with five replicates per day.
[b] Calculated as [(mean calculated concentration)/(nominal concentration)]×100%.
[c] Expressed as %RSD.

found to be stable in dog plasma while stored at −70 °C for at least 8 days. It was also found to be stable in plasma for at least 3 h under bench top (4 °C) storage conditions and was found to be stable after three freeze (−70 °C)–thaw (4 °C) cycles. Additionally, gemfibrozil was also found to be stable in the reconstitution solution under autosampler storage conditions (4 °C) for at least 24 h (Table 4).

3.8. Matrix effect

The reliability of quantitative LC–MS/MS data obtained from the determination of drugs in biological matrices can be adversely affected by endogenous components in the biological fluids. These matrix effects generally take the form of either ion suppression or ion enhancement and may vary between different sources of plasma. Our approach to determining the effect of different lots of plasma on the performance of this method was twofold: (1) determine to what extent, if any, ion suppression or enhancement played on this method. This was determined based on the differences between the peak area ratios of neat standards and that of post-extraction spiked control plasma samples analyzed at each of the three QC levels. (2) Then, investigate the effect, if any, that different sources of plasma had on the quantitation of the method. This was determined by measuring the peak area ratios of five replicates of the plasma QC samples that were extracted from different plasma lots originating from different animals.

Table 4
The stability of gemfibrozil under a variety of conditions

	−70 °C Storage stability (8 days)	Benchtop storage stability		Freeze–thaw stability (3 cycles)	Autosampler storage stability (24 h)
		($T=1.5$ h)	($T=3$ h)		
	20 ng ml^{-1}				
Mean[a]	20.6	18.9	19.8	19.8	20.4
SD	1.4	0.5	0.8	0.8	0.7
%RSD	7.0	2.7	4.3	4.3	3.4
% Accuracy[b]	103.3	94.5	99.0	99.0	101.8
	40 ng ml^{-1}				
Mean[a]	39.0	40.5	40.4	39.8	44.1
SD	3.1	2.9	2.6	3.0	1.3
%RSD	8.1	7.2	6.4	7.6	2.9
% Accuracy[b]	97.5	101.2	101.0	99.6	110.3
	80 ng ml^{-1}				
Mean[a]	79.5	82.8	78.8	78.7	87.0
SD	6.3	4.1	3.5	4.3	3.5
%RSD	7.9	5.0	4.4	5.4	4.1
% Accuracy[b]	99.3	103.5	98.5	98.4	108.8

[a] $n=5$.
[b] Calculated as [(mean calculated concentration)/(nominal concentration)]×100%.

Table 5
Relative matrix effect and inter-lot variation of the measurement of gemfibrozil in dog plasma

Nominal concentration ($ng\ ml^{-1}$)	Relative matrix effect (%)[a]	Inter-lot variation (%)[b]
20	131	8.1
80	145	3.5
200	133	8.4

[a] Calculated as [(mean peak area ratio of post-spiked plasma sample)/(mean peak area ratio of neat sample)]×100% ($n=5$).
[b] Expressed as %RSD ($n=5$).

Based on the results listed in Table 5, the amount of ion enhancement (expressed as (mean peak area ratio of post-extraction spiked plasma samples/mean peak area ratio of neat samples)×100) is in the range of 131 to 145% across the QC levels. The inter-lot variation on peak area ratio (expressed as percent relative standard deviation, %RSD), however, was found to be in the range of 3.5 to 8.4% which not only falls within the acceptance criteria of ≤10%, but is also comparable to the inter-assay precision listed in Table 3 that was obtained from the same source of plasma. Based on these results any variation between different sources of plasma should have little effect on the quantitated results.

4. Conclusion

A sensitive and reliable method for the determination of gemfibrozil in dog plasma utilizing LLCE and LC–MS/MS was developed and validated. This method has an LLOQ of 1.0 $ng\ ml^{-1}$ using a 0.5-ml aliquot of plasma and has a linear calibration range from 1.0 to 250 $ng\ ml^{-1}$. Compared to previous methods for gemfibrozil, this method provided a very simple sample preparation procedure, a much shorter analytical run time (2 min vs. ~20 min), and much better (at least 10-fold) sensitivity for the determination of gemfibrozil in dog plasma.

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