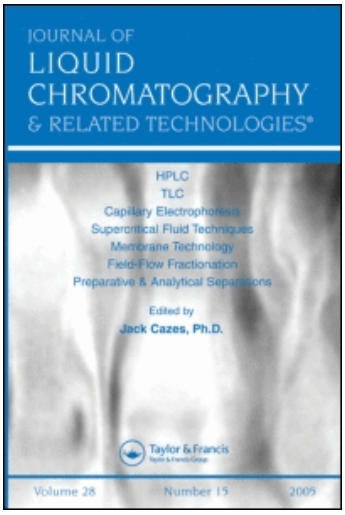

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Abstract: A sensitive and simple high performance liquid chromatography (HPLC) with fluorescence detection was developed for the determination of gemfibrozil in a small plasma sample. The plasma sample was analyzed by a deproteination method that had very simple pretreatment steps. Ibuprofen was used as an internal standard. The analysis of gemfibrozil in the plasma sample was carried out using a reverse phase C_{18} column with fluorescence detection (a maximum excitation at 242 nm and a minimum emission at 300 nm). A mixture of acetonitrile–0.4% phosphoric acid solution (53 : 47, v/v) was used as a mobile phase. The detection limit of this method was 10 ng/mL using only 100 μL of plasma sample. The standard curve was an excellent linear fit over a range of 0.05–15 μg/mL of gemfibrozil in human plasma ($r^2 = 1$). The inter- and intra-day precision (coefficient of variation, CV%) did not exceed 15%. This simple, sensitive, and rapid method was successfully applied for the pharmacokinetic study following a single oral administration of 300 mg gemfibrozil.

Keywords: Gemfibrozil, HPLC, Fluorescence detection, Pharmacokinetics

INTRODUCTION

Gemfibrozil, 5-(2,5-dimethylphenoxy)-2,2-dimethyl-pentanoic acid (Fig. 1a), is a cholesterol-lowering agent that has been clinically proven to be effective not only in reducing serum cholesterol, triglyceride, and LDL level, but also

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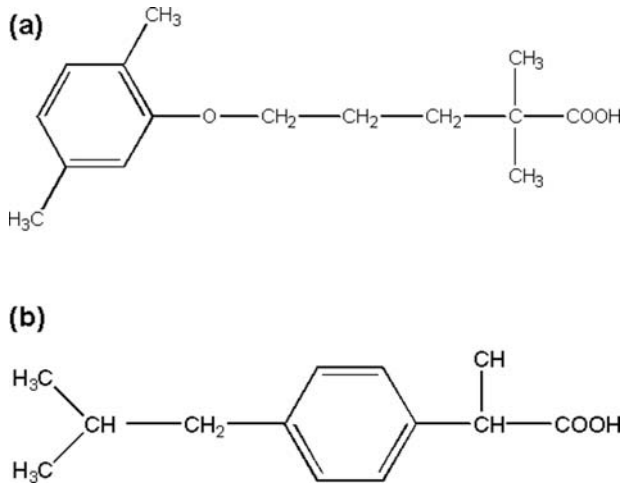

Figure 1. Chemical structures of (a) gemfibrozil and (b) ibuprofen.

increasing serum HDL levels.[1,2] Apart from a new HMG CoA reductase inhibitor, gemfibrozil is still a drug of choice in the treatment of hyperlipidaemias involving raised triglyceride levels, and has been effective in reducing the incidence of coronary heart disease.[3]

With current pharmacokinetic studies, it is very important to have an analytical method that is both sensitive and simple. A sensitive method would allow for the measurement of low levels of gemfibrozil. A simple method would allow for an increase in throughput, which in turn allows for quick turn-around in a large number of pharmacokinetic studies.

Several analytical methods have been developed and published for the determination of gemfibrozil in biological fluids, including high performance liquid chromatography (HPLC) with UV and fluorescence detection[4–6] and HPLC tandem MS methods.[2] However, the shortcomings of the published HPLC-UV or fluorescence methods seemed to be the complicated sample pretreatment steps, such as extraction and concentration with relatively large sample volumes in order to improve sensitivity and specificity. Thus, these methods might be time consuming and laborious. Although the HPLC tandem MS (MS/MS) method has the advantage of rapid and sensitive quantification of gemfibrozil, the method is sophisticated and complicated compared with HPLC-UV or fluorescence methods and the apparatuses are too expensive.

Therefore, there was a need to develop and validate a more sensitive method for pharmacokinetic studies of gemfibrozil in plasma than the previous papers. The objectives of this study were to establish a sensitive, specific, and reliable HPLC assay with fluorescence detection to quantify plasma concentrations of gemfibrozil, using only small plasma volumes

with simple pretreatment steps, which could be used for the pharmacokinetic studies of gemfibrozil in human volunteers.

EXPERIMENTAL

Chemicals and Materials

Gemfibrozil and ibuprofen as internal standards (I.S.) were purchased from Sigma (St. Louis, Mo, USA). Chemical structures were presented in Fig. 1. HPLC grade acetonitrile and methanol were purchased from J. T. Baker (Phillipsburg, NJ, USA) and water was purified by a Milli-Q system (Millipore Corp., Bedford, MA, USA). All other reagents were analytical reagent grade and used without further purification.

Apparatus and Chromatographic Conditions

The analyses were performed on a Hitachi chromatographic system (Tokyo, Japan) equipped with an L-7100 pump, F-1050 fluorescence detector, and L-7200 autosampler. The signals were processed by dsChrome software (Donam Int., Seoul, South Korea). The fluorescence detection was performed at excitation and emission wavelengths of 242 nm and 300 nm. A Capcell Pak C_{18} MG column (250 × 4.6 mm I.D., 5.0 μm particles, Shiseido Inc., Tokyo, Japan) was used for the chromatographic separation. The plasma samples were separated by isocratic elution of the mobile phase, which consisted of 0.4% phosphoric acid solution–acetonitrile (47 : 53, v/v), at the flow rate of 1.2 mL/min at room temperature.

Stock Solution and Standards

Stock solutions of gemfibrozil (1 mg/mL) and ibuprofen (10 mg/mL), as an internal standard, were prepared by dissolving the gemfibrozil in acetonitrile and then storing frozen. Standard solutions of gemfibrozil in human plasma for the calibration curve were prepared by spiking the appropriate volumes of diluted stock solutions, giving trial concentrations of 0.05, 0.1, 0.5, 1, 5, and 15 μg/mL.

Sample Preparation

Human plasma samples of 100 μL were placed in eppendorf tubes. The internal standard solution of 100 μL (ibuprofen, 100 μg/mL in acetonitrile) was added and vortexed for 10 min. Then the mixtures were centrifuged at

9503 g for 15 min, and clear supernatants were filtered through a 0.2 μm cellulose syringe filter (National Science, Seoul, Korea). The filtered aliquot of 40 μL was directly injected into the HPLC system for analysis.

Validation of the Method

Specificity

Specificity was assessed by the examination of peak interference from an endogenous substance. Drug-free human plasma was tested for interference using the HPLC method with fluorescence detection, and the result was compared with those obtained from gemfibrozil and the internal standard.

Linearity

The linearity of the calibration curve was assessed at six gemfibrozil concentrations: 0.05, 0.1, 0.5, 1, 5, and 15 μg/mL in plasma samples. Peak area ratios of gemfibrozil to internal standard were plotted versus gemfibrozil concentrations in μg/mL. The calibration curves were obtained by linear regression (no weighing factor).

Precision and Accuracy

The intra- and inter-day precisions expressed as coefficients of variation (CV%) and inter-day accuracies expressed as a percentage of the measured concentration to the theoretical concentration, were determined by the analysis of plasma samples spiked at 0.05, 0.1, 0.5, 1, 5, and 15 μg/mL. The intra-day precisions were determined by analyzing five replicates on the same day. The inter-day precisions and accuracies were determined by analyzing five calibration curves on five different days.

Sensitivity

The limit of detection (LOD) was defined as the lowest amount of analyte in the plasma sample with the peak of at least five times signal-to-noise ratio but not necessarily quantified. The limit of quantification (LOQ) was defined as the lowest concentration at which the precision was lower than 20%, the accuracy was within 80–120% and the ratio of signal to noise was better than 10.

Robustness and Ruggedness

Robustness is the capacity to remain unaffected by small but deliberate variations in method parameters.[7] It was determined by comparing the results under different conditions with precision under normal conditions.[8] It was

carried out to evaluate the influence of the mobile phase, pH of mobile solution, and column length.

Ruggedness of the method is the degree of reproducibility of test results under a variety of conditions such as different laboratories, analysis, reagents, and instruments.[8] It was tested by different operators in the laboratory and changes of the reagents and solvents sources and columns of the same type and manufacturer.

Recovery

The absolute recoveries of gemfibrozil from human plasma were performed at the three gemfibrozil concentrations of 0.05, 1, and 15 μg/mL. This was established by comparing absolute responses of gemfibrozil and internal standard in human plasma with those in mobile solutions, which represent 100% recoveries.

Stability

The stabilities of gemfibrozil and internal standard in human plasma were assessed by placing the standard solutions containing gemfibrozil at the concentrations of 0.05, 0.5, and 15 μg/mL under ambient conditions for 24 hr. The freeze-thaw stabilities were obtained over three freeze-thaw cycles, by thawing at room temperature and refreezing at −70°C for 24 hr. The short term stabilities were examined by keeping plasma samples at room temperature for 24 hr. The long term stabilities were assessed after storage at −70°C for 2 months. The stabilities of stock solutions were tested at room temperature for 6 hr in daylight. The autosampler stabilities were tested by the analysis of processed samples, which were stored in the autosampler tray for 24 hr.

Pharmacokinetic Studies of Gemfibrozil in Humans

The validated method was applied to assess the pharmacokinetics of gemfibrozil after an oral dose of 300 mg gemfibrozil. Six healthy male volunteers participated in the study. After an overnight fast, a catheter was introduced in a forearm vein and a pre-dosing blood sample was collected. Each volunteer was then orally administered one capsule of gemfibrozil, 300 mg, namely Lopidcapsule® (Je-Il Pharm. Co., Seoul, Korea) with 240 mL of water. Blood samples were collected in heparinized tubes at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, and 24 hr following the administration. The collected blood was separated by centrifugation at 2000 g for 20 min, and then the plasma was stored at −70°C until the analysis. The pharmacokinetic parameters were calculated by a bioavailability analytical program, WinNonlin™ (version 3.1, Pharsight Co., Mountainview, CA, USA).

RESULTS AND DISCUSSION

Chromatography

Symmetrical and well-resolved peaks were obtained for gemfibrozil and ibuprofen. The Capcell Pak C_{18} MG column has been used as the analytical column, since it provided excellent peak symmetry owing to the dense silicone polymer layer formed on high purity silica and minimized peak tailing from the effects of residual silanols and metallic impurities in the silica. The column showed no decrease in efficiency after more than 300 injections of plasma samples (equivalent to about 12 mL plasma). Representative chromatograms of processed human blank plasma and blank plasma spiked with gemfibrozil (5 μg/mL) and ibuprofen as an internal standard (100 μg/mL) are shown in Fig. 2. The endogenous impurities did not interfere with the regions of the analyte and ibuprofen peaks. Ibuprofen and gemfibrozil had retention times approximately of 5.5 and 7.3 min, respectively. The chromatographic run time was 12 min for the plasma sample analysis.

Validation

Specificity

As mentioned above, drug-free human plasma was screened and no endogenous interference was detected at the retention time of gemfibrozil and internal standard.

Linearity

The calibration curve was linear in the validated range. The mean equation of the calibration curve, including six points, was $y = 0.3321(\pm 0.0048)x - 0.0027\ (\pm 0.0068)$ with the correlation coefficient as $r^2 = 1(\pm 0.00004)$, where y represents the peak area ratio of gemfibrozil and internal standard and x represents the gemfibrozil concentrations in μg/mL.

Precision and Accuracy

The results of intra- and inter-day precisions and accuracies for gemfibrozil in human plasma are presented in Table 1. In all cases, the intra- and inter-day precisions were acceptable at a CV of 6.1% or less. In addition, the accuracies ranged from 91.69 to 100.99%.

Sensitivity

The estimated LOD was 10 ng/mL using only 100 μL of plasma samples. It was near two times a lower value than that obtained by Gonzalez-Penas

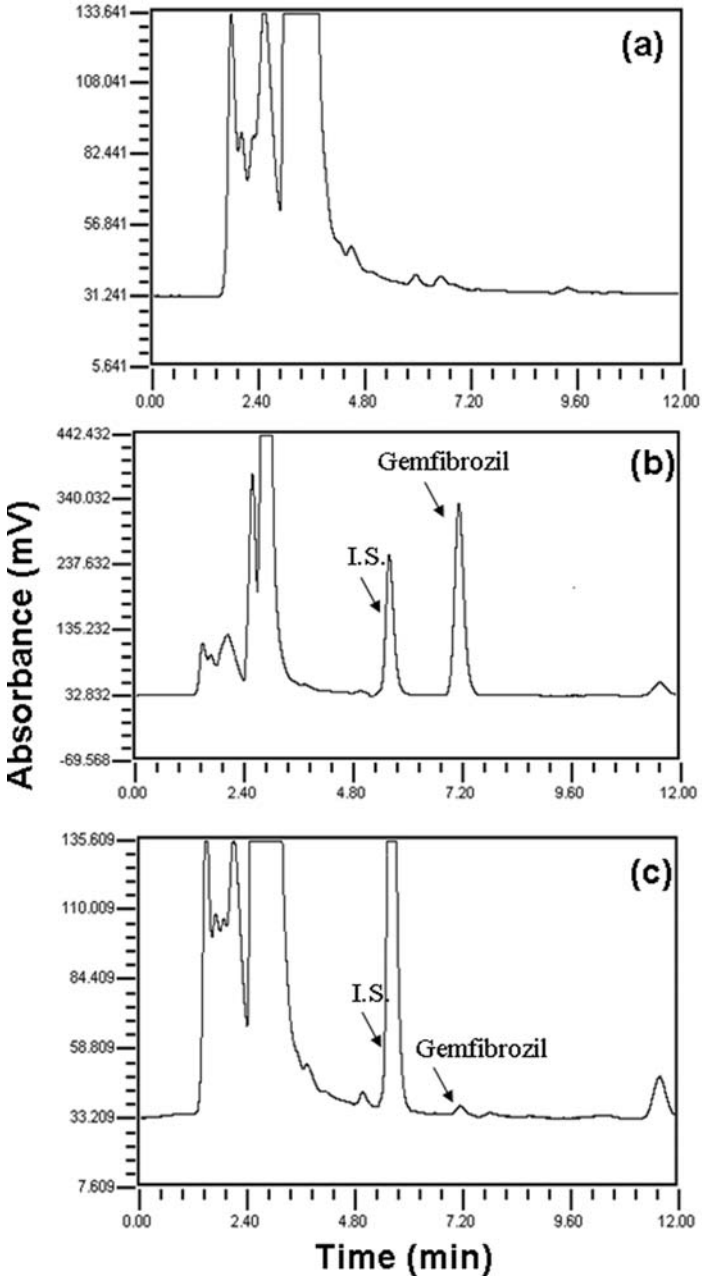

Figure 2. Chromatograms of (a) blank plasma, (b) blank plasma spiked with gemfibrozil (5 μg/mL) and ibuprofen as an internal standard (I.S.) (100 μg/mL), and (c) blank plasma spiked with gemfibrozil (0.05 μg/mL) and ibuprofen as an internal standard (I.S.) (100 μg/mL).

Table 1. Precisions and accuracies of gemfibrozil in human plasma (n = 5)

Gemfibrozil concentrations (μg/mL)	Precision (CV%)		Accuracy (%)
	Intra-day	Inter-day	
0.05 (LOQ)	5.794	6.139	92.34
0.1	0.779	4.758	91.69
0.5	0.354	4.654	95.79
1	0.458	1.400	99.92
5	3.266	1.038	100.99
15	1.117	0.106	99.90

et al., who used 500 μL of plasma samples to obtain the estimated LOD value of 25 ng/mL.[6] Moreover, we presented high sensitivity for the determination of gemfibrozil in plasma with the rapid sample pretreatment steps without extraction. The minimum sample requirement of 100 μL and low LOD value for gemfibrozil analysis are especially beneficial when analyzing plasma from small animals where only a small volume of blood can usually be collected. The LOQ of gemfibrozil was estimated to be 50 ng/mL as shown in Table 1 and Fig. 2(c). This method was sufficiently sensitive for the analysis of gemfibrozil in plasma.

Robustness and Ruggedness

We tested the influence of acetonitrile content (40–60%) in the mobile phase on the retention times, peak shapes, and peak heights of gemfibrozil and internal standard. The acetonitrile content in the mobile phase was found to be critical in the separation from endogenous compounds, and formation of symmetric peak shape. The clear peak shape was achieved at the acetonitrile content of 53% (v/v). When the acetonitrile content was below 53% (v/v), the retention time of the gemfibrozil peak was correspondingly increased and the peak shape was asymmetric. On the other hand, when the acetonitrile content was increased to above 53% (v/v), the retention time decreased and peak height of gemfibrozil increased. The analytical column packed with the same stationary phase but with a different length of 150–250 mm (Capcell Pak C_{18} MG column, 4.6 mm I.D., 5.0 μm particles, Shiseido) was investigated at a flow rate of 1.2 mL/min. The retention times for gemfibrozil and internal standard were shortened by reducing precolumn length. The pH had little influence in our system. Adjusting the percentage of acetonitrile to 53% and column length to 250 mm was associated by enhancement of the peaks symmetry, improvement of the resolution of the analytes, and shortening of the retention time.

Table 2. Analytical recoveries of gemfibrozil and internal standard, ibuprofen (n = 3)

Compound	Concentration (μg/mL)	Recovery (%) ± S.D.
Gemfibrozil	0.05	95.92 ± 13.13
	1	103.82 ± 1.67
	15	109.32 ± 29.44
Internal standard	100	104.37 ± 16.59

The ruggedness of the method proved to be adequate for routine laboratory use.

Recovery

The absolute recoveries of gemfibrozil and internal standard were presented in Table 2. The average absolute recovery values of gemfibrozil were approximately higher than 95% at low, medium, and high gemfibrozil concentrations. The mean recovery for internal standard was 104.37%. Sufficient recoveries were achieved to perform bioavailability studies.

Stability

The stabilities of gemfibrozil were shown in Table 3. All samples carried out for stability analysis showed no significant degradation under the conditions previously described above.

Application to the Pharmacokinetic Properties of Gemfibrozil

The method was applied to the analysis of gemfibrozil in human plasma after the single oral administration of a gemfibrozil capsule with the dose of 300 mg

Table 3. Stability of the samples (stability% ± S.D., n = 3)

	Gemfibrozil concentrations (μg/mL)		
Stability	0.05	1	15
Freeze-thaw	91.28 ± 9.91	87.57 ± 0.28	103.16 ± 2.39
Short-term	98.05 ± 4.78	92.73 ± 0.38	100.10 ± 0.50
Long-term	89.26 ± 11.95	99.97 ± 0.62	99.06 ± 0.19
Stock solution	92.09 ± 5.10	101.42 ± 0.47	99.60 ± 0.20
Autosampler	97.48 ± 8.68	100.20 ± 1.50	93.69 ± 0.20

in human volunteers. Plasma chromatograms of a volunteer administered gemfibrozil are shown in Fig. 3, which represents the typical chromatograms of gemfibrozil in human plasma at 0 hr (a), and 1.5 hr (b), after the administration in six healthy volunteers.

Figure 4 showed the changes of the plasma gemfibrozil concentrations after the oral administration of 300 mg gemfibrozil in human subjects. The C_{max} of gemfibrozil was reached at 1.33 ± 0.26 hr after the administration with the gemfibrozil concentration of $10.65 \pm 1.32\,\mu g/mL$. The area under the curve from 0 to 24 hr ($AUC_{0-24\,hr}$) was $30.94 \pm 3.31\,\mu g\,hr/mL$. The pharmacokinetic parameters of gemfibrozil were shown in Table 4.

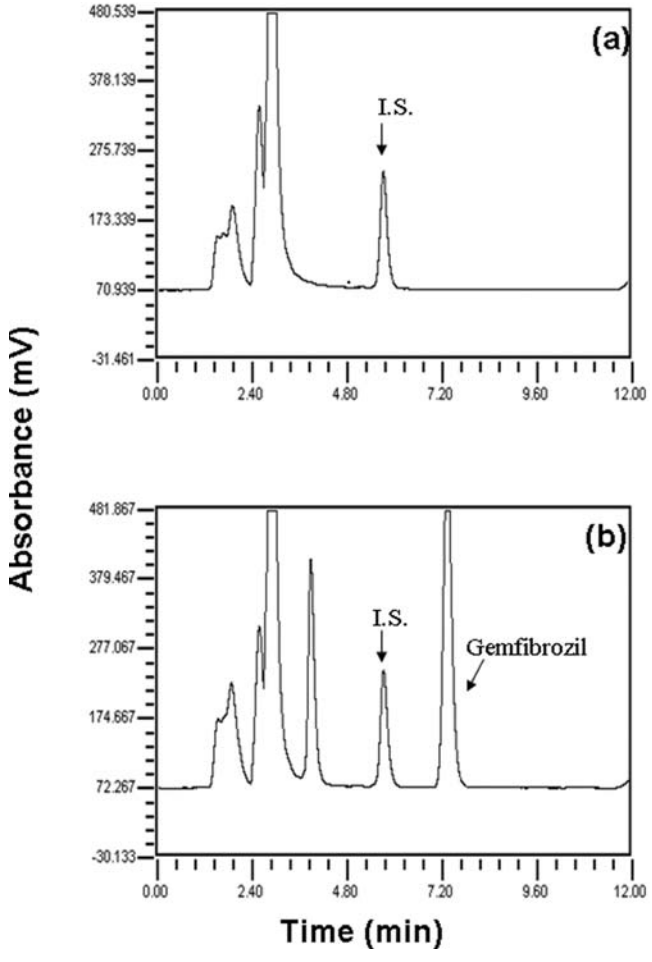

Figure 3. Chromatograms of (a) human blank plasma and (b) plasma sample from a human subject at 1.5 hr after an oral administration of 300 mg gemfibrozil.

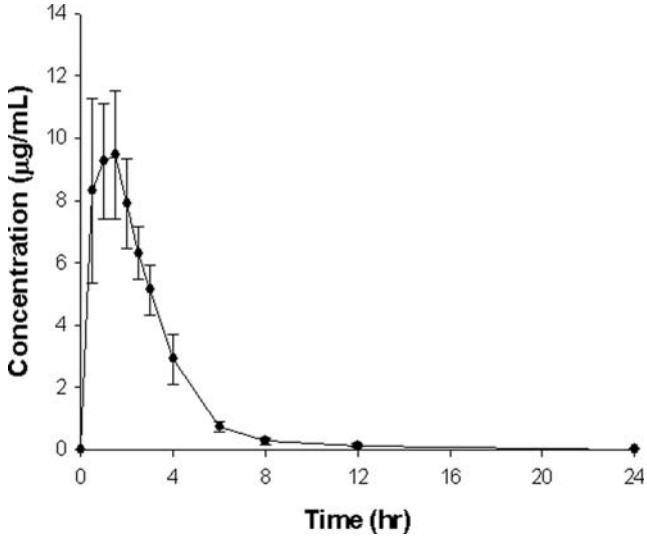

Figure 4. Mean plasma concentration of gemfibrozil in human subjects after oral administration of 300 mg gemfibrozil. The results represent the mean ± S.D. (n = 6).

These results are similar to those reported in previous studies.[6,9] From the results, it might be suggested that the present HPLC with fluorescence detection could be applied to the routine determination of gemfibrozil in biological fluid.

CONCLUSION

A sensitive, simple, specific, and rugged HPLC fluorescence method has been successfully developed and validated for the determination of gemfibrozil in human plasma. The LOD was 10 ng/mL, and LOQ was 50 ng/mL using

Table 4. Pharmacokinetic parameters of gemfibrozil in plasma of six healthy subjects after an oral administration of 300 mg gemfibrozil

Parameter	Mean ± S.D.
C_{max} (μg/mL)	10.65 ± 1.32
T_{max} (hr)	1.33 ± 0.26
$AUC_{0-24\,hr}$ (μg hr/mL)	30.94 ± 3.31
K_e (hr^{-1})	8.14 ± 1.16
$t_{1/2}$ (hr)	3.72 ± 1.72

only 100 μL of plasma sample. This analytical method has been successfully used to evaluate the pharmacokinetics of gemfibrozil in healthy volunteers. It can be very useful, and an alternative to performing pharmacokinetic studies in determination of gemfibrozil in the future.

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