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Extraction and Determination of Fexofenadine and Levocetirizine in Human Plasma by Hollow Fiber Liquid Phase Microextraction and High Performance Liquid Chromatography

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ABSTRACT

A simple, inexpensive and efficient method was used for extraction and determination of Fexofenadine and Levocetirizine with three–phase hollow fiber liquid-phase microextraction and high-performance liquid chromatography. Several parameters influencing the extraction recoveries such as the nature of organic extraction solvent, pH of donor and acceptor phases, stirring rate, extraction time and salt addition to the sample solution (donor phase) were investigated and optimized. Under the optimized conditions (toluene as organic solvent, pH_{donor} = 2.5, $pH_{acceptor}$ = 12.0, stirring rate of 1000 rpm, extraction time of 30 min, with no salt addition), extraction recoveries were 64% for Fexofenadine and 79% for Levocetirizine. A good clean-up sample with excellent linearities in the range of 1–500 ng/mL for Fexofenadine (R^2=0.997) and 0.6-600 ng/ml for Levocetirizine (R^2=0.998) were obtained. The limits of detection were 0.3 ng/mL for Fexofenadine and 0.2 ng/mL for Levocetirizine.

KEYWORDS: Levocetirizine; Fexofenadine; Hollow Fiber Liquid Phase Microextraction; HPLC.

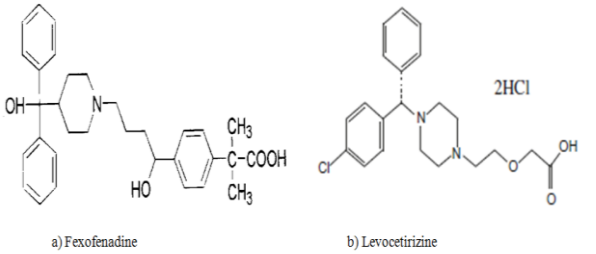
1. INTRODUCTION

Allergic rhinitis (AR) is a global health problem with increasing prevalence, affecting between 10% and 40% of the world's population [1-2]. In some countries it is now approaching epidemic proportions and is becoming a significant public health concern [3]. The cumulative symptoms of allergic rhinitis can be troublesome [4] and may impair daily activities and sleep patterns, resulting in a significant effect on patients health-related qualities of life (HRQoL) [5-7], learning and psychomotor performance [8] and thereby in an economic effect on society in terms of both direct and indirect costs [9]. Rhinitis is also associated with other conditions such as asthma, sinusitis and otitis media [10-14].

First and second-generation oral antihistamines are generally viewed as being more effective in controlling the nasal itching, sneezing and rhinorrhea associated with allergic rhinitis than in relieving nasal congestion [15-18]. Some recent evidence suggests that the newer second-generation agents such as fexofenadine and levocetirizine have anti-inflammatory effects that may relieve nasal congestion [19-20].

Fexofenadine, -α, α-dimethyl- 4- [1- hydroxy-4 -[4-(hydroxyldiphenylmethyl) –1-piperidinyl] butyl] benzene acetic acid (Fig. 1a), a selective H_1-receptor antagonist, is the principal active metabolite of terfenadine and it bears antihistaminic properties of the parent compound. Since Fexofenadine exists in zwitter-ionic form, it cannot pass through brain-biood barries and therefore does not cause sedation [21-22]. Fexofenadine displays some anti-inflammatory properties and it also has another advantage as it lacks the cardiotoxic side effects (fatal arrythmy) associated with terfenadine [21-22].

Levocetirizine dihydrochloride, (2-[4-[(R)-(4-chlorophenyl) phenylmethyl]-1-piperazinyl] ethoxy]-acetic acid dihydrochloride) (Fig. 1b) is the active enantiomer of cetirizine dihydrochloride.



a) Fexofenadine b) Levocetirizine

Fig. 1. Chemical structure of (a) fexofenadine and (b) levocetirizine.

In many cases, the two racemic enantiomers differ in their pharmacokinetic and pharmacodynamic properties. Replacing existing racemates with single isomers has resulted in improving the safety and/or efficacy profile of various racemates [23]. Levocetirizine has the priority of higher efficacy, less

side effects and longer duration upon other antihistamines and has begun to replace cetirizine in clinical therapy stepwise. It has been chemically proved that the half dosage form of Levocetirizine (2.5 mg) has a comparable antihistaminic activity with normal amount (5.0 mg) of cetirizine in the treatment of allergic rhinitis and chronic idiopathic urticaria [24].

Biological sample analysis without sample preparation, because of some reasons such as low analyte concentrations, complex sample matrixes and limited sample volumes available for the determinations, is very difficult [25]. Therefore, a sample isolation as well as pre-concentration technique is necessary. Sample preparation is traditionally carried out by liquid-liquid extraction (LLE) or by solid-phase extraction (SPE). LLE offers a high reproducibility and a high sample capacity, but it is a tedious, time-consuming procedure, which can produce emulsions and requires large amounts of high purity organic solvents for analyte extraction [26]. Although the consumption of organic solvents is relatively low in SPE, it may require a lengthy processing (i.e., conditioning, washing, eluting and drying) [27].

To overcome or reduce these problems, alternative sample preparation methods for drug analysis such as hollow fiber liquid phase microextraction (HF-LPME) have been developed. This technique was introduced by Pedersen-Bjergaard and Rasmussen in 1999 [28]. There are two modes of HF-LPME: two-and three-phase HF-LPME. In two-phase the analytes are extracted from an aqueous sample matrix within an organic receiving phase and in three-phase mode, three liquid phases participate in analyte extraction: (1) the sample solution (donor phase), with a pH that is adjusted to keep analytes neutrally charged, (2) the water immiscible organic extractor phase, which is immobilized in the wall pores of the hollow fiber and (3) the aqueous acceptor phase, with a pH that is adjusted to ionize the analytes. Compounds in their non-ionized form are extracted from the sample within the organic solvent and are subsequently drained into the acceptor phase. This extraction mode is used for basic or acidic analytes with ionizable functionalities. The major advantages of HF-LPME are simplicity, negligible volume of used solvents, high enrichment, low cost and excellent clean-up sample ability. The carry-over sample can be avoided because the hollow fibers are inexpensive enough to be single-used and disposable. In addition, HF-LPME tolerates a wide pH range, a feature which could not be done using solid phase extraction (SPE) and solid phase microextraction (SPME).

In the present study, three-phase HF-LPME followed by HPLC-UV was applied for extraction and determination of fexofenadine and levocetrizine in the human plasma sample. Various parameters which affect the extraction efficiency of Fexofenadine and Levocetirizine including pH of the donor and acceptor phases, the nature of organic phase, salt concentration, stirring rate and extraction time were studied and optimized in aqueous solutions of Fexofenadine and Levocetirizine. Finally, the optimized condition was applied to determine Fexofenadine and Levocetirizine in human plasma sample from a patient under the treatment of these drugs.

2. EXPERIMENTAL

2.1. Materials

Fexofenadine (≥98%) and Levocetirizine dihydro-chloride (≥98%) were bought from cipla (Cipla Ltd, India). Methanol and acetonitril (HPLC-grade), sodium hydroxide, hydrochloric acid, phosphoric acid, formic acid, sodium chloride, 1-octanol and toluene were obtained from Merck (Darmstadt, Germany). Benzyl alcohol, ethyl acetate and undecanol were purchased from Fluka (Buchs, Switzerland). Water used was double distilled deionized. The Accurel Q3/2 polypropylene hollow fiber (200 µm wall thickness, 600 µm internal diameter and 0.2 µm average pore size), purchased from Membrana GmbH (Wuppertal, Germany), was used for all HF-LPME experiments.

2.2. Instrumentation

Chromatographic measurements were carried out using a HPLC system equipped with a series 10-LC pump, UV detector model LC 95 set at 226 nm and model 7725i manual injector with a 20 µl sample loop (Perkin-Elmer, Norwalk, CT, USA). Column used was C_{18} (250 × 4.6 mm, 10 µm particle size) from Dr. Maisch GmbH (Ammerbuch-Entringen, Germany). A mixture of methanol, acetonitrile and 0.5% aqueous solution of triethylamine (20:30:50, v/v) at a flow rate of 1.0 ml/min were used as mobile phase at room temperature. Measurement of solutions pH were done by a 3030 Jenway pH meter (Leeds, UK).

2.3. Extraction procedure

The hollow fiber was cut manually into 8.8 cm length pieces. Before using them, each piece was sonicated for 5 min in acetone to remove any possible contaminants in the fiber and then, dried in air. For each experiment, 11 ml of the sample solution was poured into a 12 ml sample vial. Then 30 µl of the acceptor phase (pH = 12.0) was withdrawn into the Hamiltone microsyringe and its needle was inserted into the lumen of the hollow fiber. The hollow fiber was submerged in the organic solvent (toluene) for 10 second to impregnate its pores with organic solvent. The acceptor phase in the syringe was completely injected into the lumen of the hollow fiber and the end of the hollow fiber was sealed by a piece of aluminum foil. The fiber was bent to a U-shape and submerged in 11 ml of the sample solution. During extraction, sample was stirred using a 14 ×4 mm magnetic bar at 1000 rpm. After 30 min, the fiber was removed from the sample solution, its closed end was opened, the extract was withdrawn into the microsyringe and directly injected into the HPLC system. A fresh hollow fiber was used for each extraction to decrease the memory effect.

2.4. Preparation of standard solutions, real samples and mobile phase

Stock standard solutions of fexofenadine and levocetrizine (100.0 µg/ml) were prepared separately by dissolving proper amounts of each drug in methanol and were stored at 4 ˚C. The stock solutions are stable for two month. The mixed working solutions were made from these stock solutions with a concentration of 0.1µg/mL whenever needed using deionized water.

Blank plasma was provided by healthy donors. Real plasma sample was obtained from a female patient under fexofenadine and levocetrizine treatment. In order to eliminate the protein binding of the drug in plasma (greater than 99%), the pretreatment was performed as outlined in the work of Tahmasebi et al. [29-30]. For this purpose, 3 ml methanol was added to 2 ml of the plasma and the resulting mixture was strongly vortexed for 10 min. The mixture was placed for 10 min in an ice bath, followed by 10 min at ambient temperature and then centrifuged at 3500 rpm for 10 min. The supernatant was transferred into a 12 ml volumetric flask and diluted to the mark with double distilled deionized water. pH of the final solution was adjusted to 2.5 using 3 M HCl solution and the extraction procedure was done under the optimized conditions.

To select the proper mobile phase (based on retention time, peak shape and resolution) for sepearation of Fexofenadine and Levocetirizine different mobile phase compositions were made and tested by mixing of MeOH:H_2O, CH_3CN:H_2O, MeOH:H_2O:acetate buffer, MeOH:H_2O:phosphate buffer and MeOH:CH_3CN: 0.5% aqueous solution of triethylamine (20:30:50, v/v).

3. RESULTS AND DISSCUTIONS

3.1. Optimization of HF-LPME

The effect of experimental variables such as the nature of organic solvent, pH of the donor and acceptor phases, stirring rate, extraction time and salt addition to the sample solution on recovery were investigated and optimized. For this purpose, HF-LPME was carried out using 11 ml of aqueous solution containing 0.1 µg/ml of Fexofenadine and Levocetirizine and each experiment was repeated at least three times.

3.1.1. Selection of the organic extraction solvent

Selection of suitable organic solvent in HF-LPME is of great importance for efficient analyte preconcentration. There are several requirements for organic solvent selection. Firstly, it should be easily immobilized in the hollow fiber pores. Secondly, it should be nonvolatile to prevent solvent loss during the extraction and immiscible with water because it serves as a barrier between the two aqueous phases (the donor and the acceptor phases).

Organic solvent is also used to promote analyte diffusion from the donor phase into the acceptor phase through the pores of the hollow fiber which was filled with organic solvent. Based on these considerations, in the present research, 1-octanol, undecanol, benzyl alcohol, ethyl acetate and toluene were evaluated as the organic solvents for HF-LPME. The hollow fiber, impregnated with the organic solvent and filled with 0.1 mM NaOH, was inserted as U-shape into the vial and the extraction occurred for 30 min. Based on the obtained results, toluen showed the best extraction recovery. Therefore, toluene was selected as membrane solvent for further experiments. No extraction was occurred in the presence of benzyl alcohol, ethyl acetate as the membrane solvents, indicating that, organic solvents with lower polarity are suitable for the extraction of the mentioned drugs.

3.1.2. Optimization of sample solution pH

To obtain high recovery for acidic compounds, the sample solution should be acidic to effectively deionise the analytes and consequently reduce their solubility in the sample solution. For this purpose, three different acids including hydrochloric, phosphoric and formic acid were tested to adjust the solution at pH = 3.0. The highest recovery was obtained using HCl. Therefore, hydrochloric acid was used to adjust acidity (pH = 2 - 4.5). Results in Fig. 2 show that the highest extraction recovery were obtained at pH = 2.5 for Fexofenadine and pH = 3.0 for Levocetirizine. In a more acidic solution, a decrease in the extraction recovery was observed. This can be attributed to protonation of the carbonyl groups [31] of Fexofenadine and Levocetrizine. Finally pH=2.5 were chosen for sample solution.

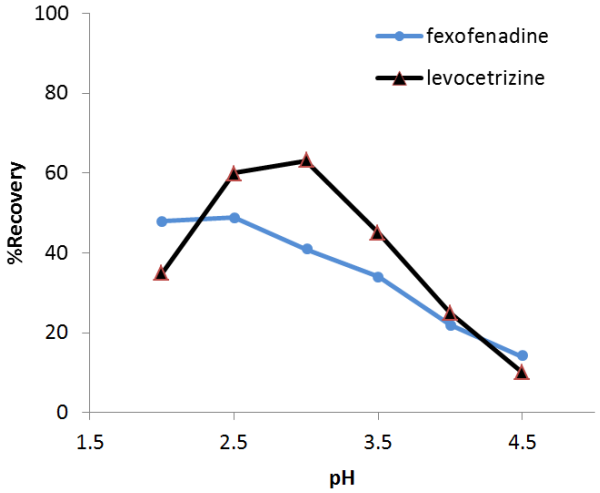


Fig. 2. Effect of donor phase pH on the extraction recovery of Fexofenadine and Levocetirizine. Extraction conditions: toluen as organic extraction solvent, donor phase, 11 mL of aqueous solution containing 0.1 µg/mL of fexofenadine and levocetrizine; extraction time = 30 min; stirring rate = 250 rpm; acceptor phase, 30 µl of 0.1 mM NaOH.

3.1.3. Optimization of acceptor phase

The acceptor solution should be basic to provide high solubility for the acidic analytes and ionize the analytes to prevent them re-entering the organic phase. In general, the pH of the acceptor phase was required at least 3.0 pH units higher the pK_a of acidic analytes. To select the composition of acceptor phase, sodium

hydroxide and ammonium hydroxide were tested (at pH = 10.0). Higher recovery was obtained with sodium hydroxide. Therefore, sodium hydroxide was used to adjust the pH of acceptor phase in the range of 7.0–12.0. Results (Fig. 3) showed that, the highest recovery for these compounds were obtained at pH = 12.0. Therefore, this pH was selected as the optimum pH value for further study.

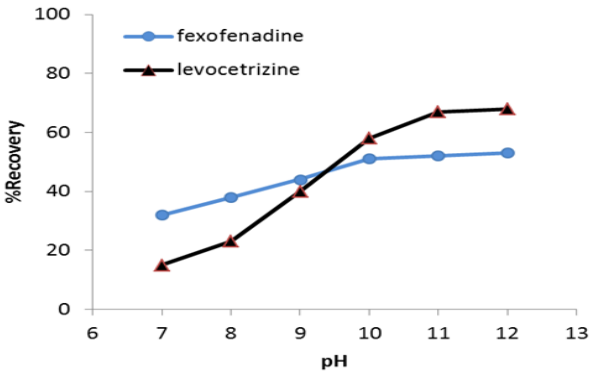


Fig. 3. Effect of the acceptor phase pH on the extraction recovery of Fexofenadine and Levocetirizine. Extraction conditions: pH donor = 2.5. Other conditions as Fig. 2.

3.1.4. Effect of stirring rate

Increasing stirring rate can speed up the thermodynamic equilibrium, thus enhance the extraction recovery [32]. The effect of stirring rate in the range of 100-1200 rpm was evaluated (Fig. 4). The extraction recovery increased with higher stirring speed [33-34]. However, overhigh speed would produce excessive air bubbles and lose solvent that could affect the precision [35]. Therefore, 1000 rpm was chosen as a suitable stirring rate in the experiments based on experimental resuts.

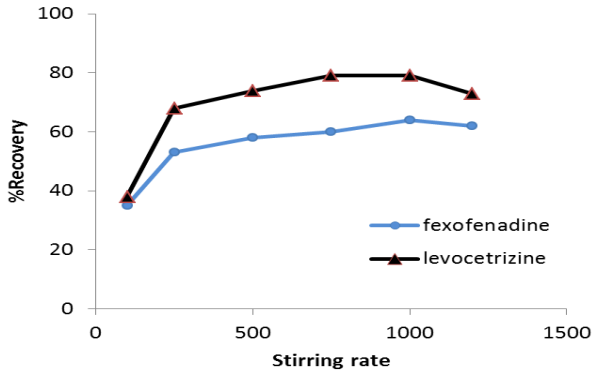


Fig. 4. Effect of stirring rate on the extraction recovery of Fexofenadine and Levocetirizine.
Extraction conditions: $pH_{acceptor}$ = 12.0. Other conditions as Fig. 3.

3.1.5. Effect of extraction time

The extraction time is an important parameter in HF-LPME procedure because it influences the partition of the analyte between the donor phase and organic phase and, subsequently, between the organic phase and acceptor phase. In this study, recovery of Fexofenadine and Levocetrizine were investigated as a function of extraction time in the range of 15–120 min (Fig. 5) under the 15 min extraction recovery of Fexofenadine was very low. For Fexofenadine the recovery increased by the increasing of the extraction time until 30 min and then decreased. For Levocetirizine the recovery decreased by the increasing the time. A marked decrease in the extraction recovery is due to extraction solvent dissolution [36]. Based on this observation the extraction time of 30 min was selected for all experiments.

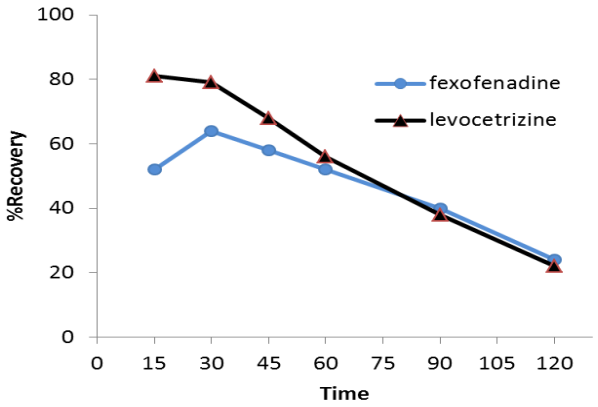


Fig. 5. Effect of extraction time on the extraction recovery of Fexofenadine and Levocetirizine. Extraction conditions: stirring rate = 1000 rpm. Other conditions as Fig. 4.

3.1.6. Effect of salt addition

The addition of salt improves the ionic strength of the donor phase and reduces the affinity of the organic compounds in the aqueous phase, which would improve the extraction efficiency of organic analytes in many conventional extraction techniques [37]. NaCl is commonly added to analytical samples.

In this study, the ionic strength of the sample solution was optimized by adding different amounts of NaCl into sample solution in the range 0.0 to 2.5 M. Results showed that NaCl had almost no salting-out effect on the extraction recovery. Therefore, this HF-LPME system was conducted without the addition of salt.

3.2. Calculation of recovery and enrichment factors

Extraction recovery % (ER %) and enrichment factor (EF) were calculated according to the following equations for the analytes [28, 38].

$$ER\% = \frac{n_{a,final}}{n_{s,initial}} \times 100 = \frac{C_{a,final}V_a}{C_{s,initial}V_s} \times 100 \qquad (1)$$

$$EF = \frac{C_{a,final}}{C_{s,initial}} = \frac{V_s}{V_a} \times \frac{ER}{100} \qquad (2)$$

where $n_{s,initial}$ and $n_{a,final}$ are the number of moles of analyte present in the initial sample and analyte finally collected in the acceptor phase, respectively. V_s ,V_a , $C_{s,initial}$ and $C_{a,final}$ are the volume of sample, volume of the acceptor phase, initial analyte concentration within the sample and final concentration of analyte in the acceptor phase, respectively. Under the optimized conditions, enrichment factor of 290 with Extraction recovery equel 79% for Levocetirizine and enrichment

factor of 235 with Extraction recovery equel 64% for Fexofenadine were achieved.

3.3. Method validation

To evaluate the analytical performance of the HF-LPME technique, figures of merit of this method including limit of detection (LOD), linear range, repeatability, extraction recovery and enrichment factor were investigated for extraction of fexofenadine and levocetirizine and summarized in Table 1. Limit of detection (based on S/N = 3) for Fexofenadine and Levocetirizine were 0.3 and 0.2 ng/ml, respectively. The calibration curves were linear in the range of 1–500 ng/mL for Fexofenadine (R^2=0.997) and 0.6-600 ng/ml for Levocetirizine (R^2=0.998).

Table 1. Linear range, limits of detection (LOD), repeatability, reproducibility o and other detailes for extraction and determination of fexofenadine and levocetirizine using HF-LPME and HPLC.

	FEX	LCT
LOD (ng/ml)	0.3	0.2
Linear range (ng/ml)	1.0-500	0.6-600
R^2	0.997	0.998
EF	235	290
ER%	64	79
RSD% (intra-day, n = 5)	3.6	3.5
RSD% (inter-day, n = 5)	8.1	7.7

3.4. Application of HF-LPME for analysis of fexofenadine and levocetirizine in plasma sample

Due to the importance of analysis of fexofenadine and levocetirizine in plasma samples, the optimized method (toluene as organic solvent, pH_{donor} = 2.5, $pH_{acceptor}$ = 12.0, stirring rate of 1000 rpm, extraction time of 30 min, with no salt addition) was applied to determine the concentration of Fexofenadine (60 mg) and Levocetirizine (5 mg) in the plasma sample from a patient under these drugs treatment. The concentration of Fexofenadine and Levocetirizine in plasma were 385.6 and 107.2 ng/ml, respectively. The chromatograms of HF-LPME extracts from blank plasma sample, patient plasma sample and spiked patient plasma sample are shown in Fig. 6 (a–c) using standard addition method.

4. CONCLUSIONS

Hollow-fibre LPME method was successfully applied for extraction of fexofenadine and levocetirizine from human plasma sample. This method is simple, rapid, inexpensive, precise and sensitive analytical procedure. The disposable nature of the hollow fiber completely eliminates the possibility of sample carryover and incorporates the use of a porous hollow fiber membrane to support the solvent during extraction. In addition, the small pore size of hollow fiber prevents large molecules in the matrix and unsolved particles in the donor solution to enter to the acceptor phase, thus yielding a very clean extract. So the HF-LPME is an effective method to extract Fexofenadine and Levocetirizine from the biological sample prior to HPLC analysis.

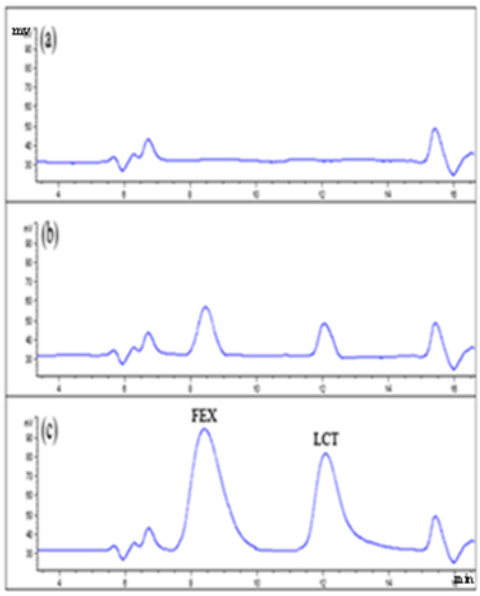


Fig. 6. HPLC chromatograms for Fexofenadine and Levocetirizine after extraction at optimum conditions (toluen as organic solvent, pH_{donor} = 2.5, $_{pHacceptor}$ = 12.0, stirring rate of 1000 rpm, extraction time of 30 min, with no salt addition. (a) blank plasma sample (b) patient plasma sample and (c) 300 ng/mL spiked patient plasma sample. Mobile phase: mixture of methanol, acetonitril and 0.5% aqueous solution of triethylamine (20:30:50, v/v); flow rate: 1.0 mL/min; column: C18 (250 × 4.6 mm, 10µm); λ =226 nm at room tempreture.

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