# Simultaneous determination of unbound thyroid hormones in human plasma using high performance frontal analysis with electrochemical detection

Tomoko Kimura[a], Keiko Nakanishi[a], Terumichi Nakagawa[b],
Akimasa Shibukawa[c], Katsumi Matsuzaki[a],*

[a] Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan
[b] Faculty of Pharmaceutical Sciences, Setsunan University, 451 Nagao tiuge-cho, Hirakata 573-0101, Japan
[c] Faculty of Pharmaceutical Sciences, Chiba Institute of Science, Chosi 288-0025, Japan

## Abstract

A direct injection HPLC method in combination with high-performance frontal analysis (HPFA) and electrochemical detection (ECD) was developed for the simultaneous and sensitive determination of unbound thyroid hormones (thyroxine, triiodothyronine, and reverse triiodothyronine) in human plasma. The present on-line HPLC/HPFA system consists of an HPFA column, an extraction column and an analytical HPLC column connected through a column-switching device, and the eluent from the analytical column was monitored by ECD. The calibration lines showed good linearity (rsq. > 0.999) within 7.4–148.2 pM for T4 and 1.5–74.1 pM for T3 and rT3. Unbound T4 and T3 concentrations determined by the present system were $16.4 \pm 2.4$ pM ($n = 15$) and $7.14 \pm 1.04$ pM ($n = 15$), which were in agreement with those determined by the EIA method. The unbound rT3 concentration was $2.30 \pm 0.27$ pM ($n = 15$). The CV% values of intra-day and inter-day assays ($n = 15$) were less than 14.9% for T4, 14.5% for T3 and 13.2% for rT3. The present system was also applied to a competitive binding study of these thyroid hormones in human plasma.
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## 1. Introduction

Drugs and endogenous compounds such as hormones are bound to proteins in plasma, and bound and unbound forms are in binding equilibria. Plasma protein binding significantly influences their distributions in the body and the exhibition of pharmacological and biological activities, and the therapeutic monitoring of the unbound concentration is often an important clinical issue, especially in the case of highly protein bound compounds [1,2]. Methodologies for determination of unbound concentrations include equilibrium dialysis [3] and ultrafiltration [4]. However, these conventional methods involve problems, such as the adsorption of drug on the membrane and the leakage of the bound form through the membrane. In addition, the protein binding equilibrium may deviate from the true one due to changes in the sample concentration during filtration. To overcome these problems, we developed a novel analytical method named high-performance frontal analysis (HPFA).

HPFA has several advantages over conventional methods. HPFA allows analysis following direct sample injection analysis without any pretreatment process such as deproteinization and extraction. HPFA is free from overestimation of unbound concentration due to protein leakage which is a troublesome problem in the conventional ultrafiltration
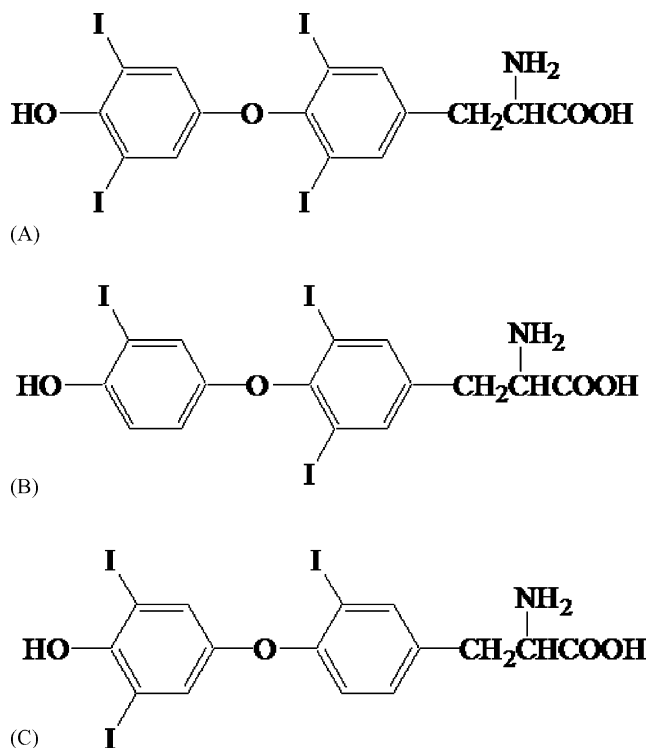
Fig. 1. Chemical structures of (A) thyroxine, (B) triiodothyronine, and (C) reverse triiodothyronine.

method. In addition, HPFA can be easily incorporated into an on-line HPLC system and is suitable for the analysis of strong protein binding [5–8].

Thyroxine (3,3′,5,5′-tetraiodothyronine, T4 Fig. 1(A)) and triiodothyronine (3′,5,3-triiodothyronine, T3 Fig. 1(B)) are major thyroid hormones. T4 and T3 regulate a number of biological processes, including growth and development, carbohydrate metabolism, oxygen consumption, protein synthesis and fetal neurodevelopment [9–11]. T4 with a direct hormonal activity is locally converted to the active form, T3 or the inactive form, reverse triiodothyronine (3′,5′,3-triiodothyronine, rT3 Fig. 1(C)) in some tissues. Alternative routes of enzymatic deiodination can also produce either the biologically potent product, T3 or the very weak agonist rT3 [12].

The unbound concentrations of these compounds are versatile indexes to assess thyroid functions. For example, since T3 is the active form of the thyronine, low serum T3 level would favor the protein-sparing phenomenon, probably being required for survival in many systemic illnesses [13]. The magnitudes of the relative changes in T3, T4 and rT3 levels are indicative of severity of these non-thyroidal diseases and, therefore, are useful to prognose the outcome of the primary illness [14].

The unbound concentrations of these thyroid hormones are usually measured by radioimmunoassay or by enzyme immunoassay. Although these methods have several advantages such as high sensitivity and high throughput,

they are not suitable for competitive binding study because competitive compounds cannot be simultaneously analyzed by these methods. In addition, radioimmunoassay requires a special facility in a controlled area. Electrochemical detection is a sensitive and selective method and is applied to HPLC analyses of several redox compounds such as catecholamines, *p*-nitrophenol [15] and levodopa [16].

Electrochemical detection has been also used in the determination of total T4 concentration in plasma [17]. However, it has not been applied for sensitive determination of the unbound thyroid hormones in plasma. In this paper, a novel on-line HPLC system incorporating the HPFA system and electrochemical detection is developed for the simultaneous determination of unbound T4, T3 and rT3 in human plasma.

## 2. Experimental

### 2.1. Samples and apparatus

T4, T3 and rT3 were purchased from Sigma (St. Louis, MO, USA). Human plasma samples were collected from healthy man and were shaken at 37 °C for 30 min before analysis. Develosil 100 Diol 5 and Develosil ODS-10 were purchased from Nomura chemicals (Seto, Japan), and YMC-Pack ODS-AM from YMC (Kyoto, Japan). TOSOH II FT4 and TOSOH II FT3 assay kits were obtained by TOSOH Corporation (Tokyo, Japan).

### 2.2. On-line HPFA/HPLC system

Fig. 2 shows a schematic of the on-line HPFA/HPLC system in this study, where a HPFA, extraction and analytical columns were connected via four-port and six-port switching valves. The instruments used were as follows: HPLC pumps, LC 9A (Shimadzu, Kyoto, Japan); UV detectors, SPD-10A (Shimadzu); injector, Rheodyne Type 8125; integrated data analyzer, Chromatopac C-R8A (Shimadzu); column oven, CS-300 C (Chromato-Science, Osaka, Japan). An ESA Coulochem II Model 5200 electrochemical detector (ESA, MA, USA) was equipped with a Model 5020 guard cell working at 550 mV and a Model 5010 analytical cell operating in the oxidation screening mode with the potential of the first electrode set at 300 mV and the second electrode set at 500 mV based on good selectivity and optimized signal-to-noise ratios at LOQ. The sensitivity of the detector was set at 2 μA.

Table 1 lists the HPLC conditions of the present system. Sodium phosphate buffer of physiological pH (7.4) was used as the mobile phase for HPFA without addition of any organic modifier so as not to disturb the protein binding equilibrium. Since hydrophobic diol-silica materials were employed as the HPFA column, T4, T3 and rT3 were eluted out under mild mobile phase conditions.
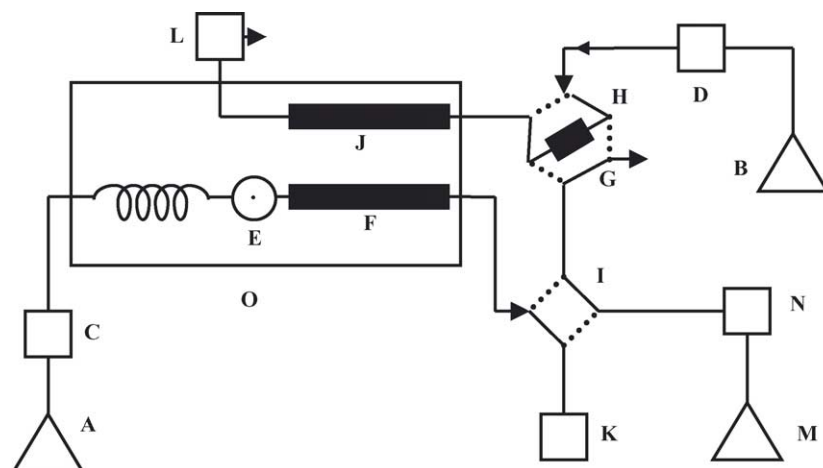
Fig. 2. Schematic diagram of on-line HPFA/HPLC system.

## 2.3. Determination of unbound T4, T3 and rT3 concentrations by HPLC/HPFA–ECD system

Plasma samples (3.2 mL) were directly injected into the HPFA column. After plasma proteins are size-excluded, T4, T3 and rT3 were eluted out as a zonal peak with a plateau region. Due to the principle of frontal analysis, the T4, T3 and rT3 concentrations in this plateau region were equal to the unbound concentrations in the sample solution. An amount of 2.8 mL volume of this plateau region was transferred into the extraction column by switching the four-port valve via the "heart-cut" procedure after which the unbound thyroid hormones in this fraction were trapped on the extraction column. Next, the mobile phase for the analytical column was introduced into the extraction column by switching the six-port valve, and the trapped unbound hormones were completely transferred to the analytical column. The extraction column

was then washed with distilled water for 30 s before and after the heart-cut procedure. The HPFA column, the analytical column and the injector loop were kept at 37 °C in a column oven at all times during this procedure. The flow rate before plasma elution was 0.2 mL/min to avoid plugging of plasma proteins in the column.

For HPFA, the sample solution should be injected without diffusion. Otherwise, the binding equilibrium is disturbed and the plateau region disappears. However, a part of the sample solution often diffuses in the injector loop during the sample injection process. To avoid the introduction of this diffused part into the HPFA column, the "injector-reswitching technique" [18,19], which was adopted in this study, is useful. The injector loop was loaded with a 3.2 mL portion of the sample solution, connected to the mobile phase flow (0.2 mL/min) for 14 min, and then disconnected. The actual injection volume was therefore 2.8 mL.

## 2.4. Method validation

The intra- and inter-day precisions of the method were evaluated by replicate analyses of the same plasma. The acceptance criteria were calculated by taking $S/N = 10$ for the limit of quantitation and $S/N = 3$ for the limit of detection.

The percent recovery of T4 from the extraction column was determined using standard samples at 0.05, 0.5 and 1 μM. The recovery of T3 and rT3 was determined using standard solutions at 0.01, 0.05 and 0.5 μM. The peak-area ratios of three extracted samples were then compared with the unextracted samples to determine the percent recovery. The same three concentrations used for the recovery experiments were used to examine the inter-day and intra-day precision. The accuracy was evaluated by back-calculation and expressed as the percent deviation between the amount found and the amount added for each T4, T3 and rT3 for the three concentrations examined. Five samples for each concentration were extracted for each of three consecutive days.

Table 1
HPFA conditions for the determination of unbound T4, T3 and rT3 in human plasma

| Subsystem | |
| --- | --- |
| **HPFA** | |
| Column | Develosil 100-Diol-5 (15 cm × 6 mm i.d.) |
| Mobile phase | Sodium phosphate buffer (pH 7.4, $I = 0.17$) |
| Flow rate | 0.2 mL/min and after elution of proteins, 1.5 mL/min |
| Temperature | 37 °C |
| **Extraction** | |
| Column | Develosil ODS 10 (1 cm × 4.6 mm i.d.) |
| **Analytical HPLC** | |
| Column | YMC-Pack ODS-AM (15 cm × 4.6 mm i.d.) |
| Mobile phase | Sodium phosphate buffer (pH 6.0): $CH_3CN = 72.5:27.5$ (V:V) |
| Flow rate | 1.0 mL/min |
| Temperature | 37 °C |

Detection, UV 230 nm. Electrochemical detector (ECD) E1 = +300 mV, E2 = +500 mV.

The stability of stock solutions was assessed for 6 months at $-20\,^{\circ}C$.

The calibration lines were prepared as follows. The HPFA column was removed from the on-line system, and a 5 mL injector loop was replaced by a 20 μL loop. After washing the extraction column with distilled water for 30 s, each 10 μL portion of a series of standard methanol solutions (0.05, 0.1, 0.15, 0.25, 0.5 and 1 μM for T4 and 0.01, 0.02, 0.03, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 μM for T3 and rT3) was injected directly into the extraction column. Because the heart-cut volume was 45 mL, the concentrations in the standard solutions corresponded to 7.4–148.2 pM in plasma for T4, and 1.5–74.1 pM in plasma for T3 and rT3. After perfusing the extraction column with water for 30 s, the adsorbed standard solutions were back-flushed into the analytical column by the column-switching procedure. The calibration line was prepared by plotting peak area versus injected amount. The stock solution A was stable for 6 months.

## 2.5. Determination of unbound T4 and T3 concentrations by EIA assay

The normal plasma samples were determined by EIA to evaluate the unbound T3 and T4, respectively. All assays were performed according to the manufacture's instructions.

## 2.6. Statistics

Statistical significance ($P < 0.05$) was determined using the Student's *t*-test.

## 3. Results and discussion

### 3.1. HPFA profile

The unbound concentrations of endogenous substances such as thyroxine are very small. Therefore, a long HPFA column was selected for this assay to extract large volumes of unbound thyroid hormones. For HPFA, it is essential to obtain a plateau region, for which the HPLC conditions including the sample injection volume should be properly optimized. If
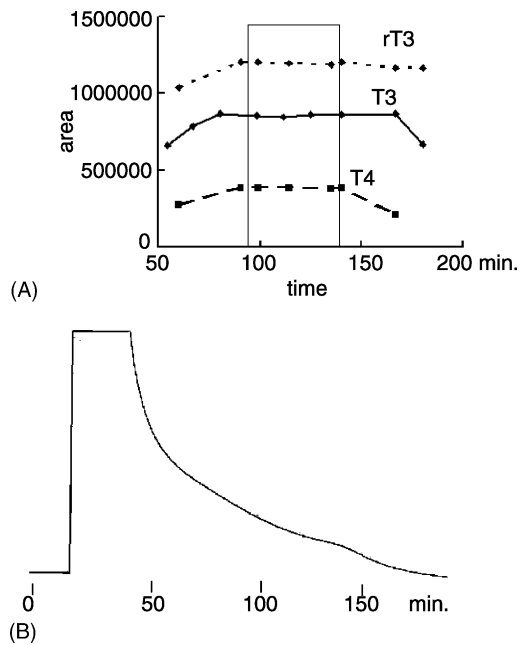
(A)

(B)

Fig. 3. (A) Plateau region of plasma sample spiked with T4, T3 and rT3; (B) HPFA profile.

the injection volume is insufficient or if the elution time of the analyte is too short or long, a clear plateau region cannot be obtained and the unbound concentration cannot be correctly determined. In order to confirm the appearance of plateau regions, the concentrations of three thyroid hormones in the eluent from the HPFA column were monitored (Fig. 3(A)). Although the unbound plasma concentrations of these thyroid hormones can be determined after preconcentration in a large heart-cut volume (67.5 mL), accurate determination was difficult with a much smaller heart-cut volume. Therefore, each plasma sample was spiked with 20 μM T4, 10 μM T3 and 10 μM rT3 to facilitate the detection with a smaller heart-cut volume of 3 mL. Fig. 3(B) shows the HPFA profile using the same sample solution. As shown in Fig. 3, the plateau regions were clearly observed in 90–140 min for T4, 80–170 min for T3 and 90–140 min for rT3. Based on these results, the heart-cut time was set at 98–143 min. Fig. 4 shows the time schedule for the valve switching.
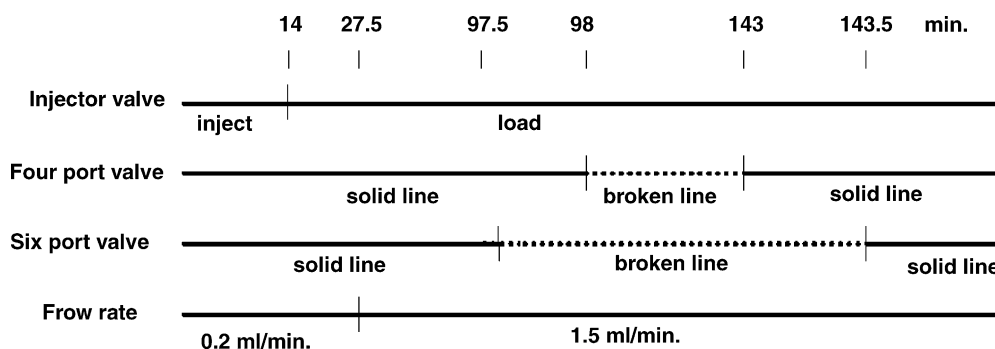
Fig. 4. Time schedule for the valve switching.

Table 2
Accuracy and intra- and inter-day precisions for the assay in standard solutions

| Concentration (μM) | R.S.D. (%) | | Accuracy (%) |
| --- | --- | --- | --- |
| | Inter-day ($n = 3$) | Intra-days ($n = 15$) | |
| T4 | | | |
| 0.05 | 6.8 | 5.8 | 90.2 |
| 0.5 | 7.4 | 4.6 | 89.5 |
| 1 | 1.5 | 4.2 | 98.2 |
| T3 | | | |
| 0.01 | 7.2 | 2.8 | 90.8 |
| 0.05 | 3.5 | 3.7 | 95.4 |
| 0.5 | 4.2 | 5.3 | 94.2 |
| rT3 | | | |
| 0.01 | 7.5 | 7.3 | 93.2 |
| 0.05 | 6.4 | 4.9 | 94.5 |
| 0.5 | 6.3 | 5.2 | 93.8 |

## 3.2. Recovery, accuracy and precision

The recoveries for T4 averaged 98% over the three concentrations, for T3 they were 98.8% and for rT3 they were 97.6%. The accuracy and the intra-day and inter-day precision in standard solutions are presented in Table 2. The calibration lines showed good linearity (rsq. > 0.999). The stock solutions were stable for 6 months.

## 3.3. Limits of quantitation and detection

The limits of quantitation were 4.44, 0.998 and 1.23 pM for T4, T3 and rT3, while the limits of detection were 0.10, 0.020 and 0.025 pM for T4, T3 and rT3. These low levels of unbound hormones could be determined without using RI markers because of the high sensitivity of the electrochemical detection, and the on-line pre-concentration of a large volume of the plateau zone containing unbound thyroid hormones.
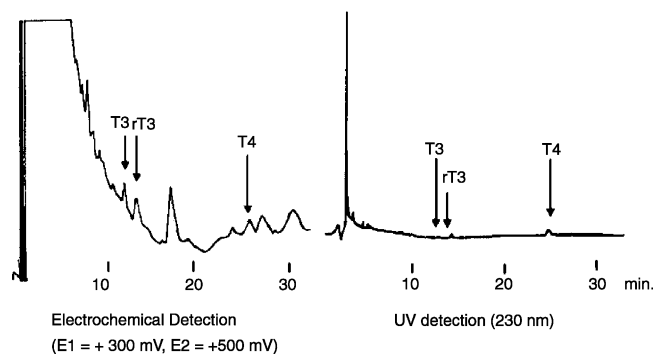


Fig. 5. Chromatograms of unbound thyroid hormones in normal human plasma.

## 3.4. Chromatographic results

Fig. 5 shows the chromatograms of thyroid hormones in normal human plasma analyzed by the present system. To identify each thyroid hormone, the chromatogram of normal human plasma was compared with that spiked with standard T4, T3 or rT3. Each peak in normal plasma with the standard became higher than that without the standard. The electrochemical detection (left) was about five times more sensitive than the UV detection (right). The intra-day and inter-day precisions were determined by analyzing five replicates on three non-consecutive days (Table 3). Precision was expressed in coefficients of variation (CV %). The results of the HPFA assay were comparable with those determined by the EIA method, although the CV % values of the latter were smaller. The unbound concentration of T3 obtained by EIA was smaller than the value obtained by the HPLC/HPFA–ECD method probably because the unbound concentrations by this method can be affected by several analytical artifacts, including the effects of albumin concentration [20], abnormal concentrations or functions of binding proteins [21] and the presence of anti-thyroxine antibodies

Table 3
Unbound concentrations of thyroid hormones in normal human plasma determined by HPFA/LC and EIA method

| HPFA/LC | T4 | | T3 | | rT3 | |
| --- | --- | --- | --- | --- | --- | --- |
| | Mean (pM) | CV (%) | Mean (pM) | CV (%) | Mean (pM) | CV (%) |
| First day ($n = 5$) | 17.9 | 12.2 | 6.97 | 13.9 | 2.40 | 13.2 |
| Second day ($n = 5$) | 16.3 | 11.3 | 7.08 | 13.6 | 2.52 | 5.83 |
| Third day ($n = 5$) | 15.1 | 14.0 | 7.38 | 13.9 | 2.20 | 10.0 |
| Mean | 16.4 | 14.9 | 7.14 | 14.5 | 2.31 | 11.9 |
| EIA ($n = 3$) | 16.3 | 4.63 | 4.44 | 4.85 | | |

Table 4
Changes in the unbound concentrations of thyroid hormones in human plasma spiked with 0.1 μM T4

| | T4 | | T3 | | rT3 | |
| --- | --- | --- | --- | --- | --- | --- |
| | Mean (pM) | CV (%) | Mean (pM) | CV (%) | Mean (pM) | CV (%) |
| Plasma spiked T4 | 33.6 | 14.1 | 40.9 | 13.9 | 22.2 | 13.2 |
| Untreated plasma | 16.3 | 11.3 | 7.08 | 13.6 | 2.52 | 5.83 |
| Ratio | 2.0 | | 5.8 | | 8.8 | |

Table 5
Changes in the unbound concentrations of thyroid hormones in human plasma spiked with 0.01 μM T3

|  | T4 | | T3 | | rT3 | |
|---|---|---|---|---|---|---|
|  | Mean (pM) | CV (%) | Mean (pM) | CV (%) | Mean (pM) | CV (%) |
| Plasma spiked T3 | 19.1 | 3.14 | 28.6 | 8.25 | 17.3 | 11.4 |
| Untreated plasma | 16.3 | 11.3 | 7.08 | 13.6 | 2.52 | 5.83 |
| Ratio | 1.2 | | 4.0 | | 7.3 | |

[22]. The advantage of this method is that the unbound concentrations of these thyroid hormones can be determined by a single analysis.

### 3.5. Competitive binding study

Changes in the unbound concentrations of thyroid hormones in plasma after the addition of T4 or T3 were also investigated using the present system. The unbound concentrations were increased by 2.0, 5.8 and 8.8 times for T4, T3 and rT3, respectively, after the addition of 0.1 μM T4 (Table 4). The unbound concentrations were increased by 1.2, 4.0 and 7.3 times for T4, T3 and rT3, respectively by the addition of 0.01 μM T3 (Table 5).

T4 and T3 are very hydrophobic and bound strongly to plasma proteins such as albumin, thyroxine-binding globulin and transthyretin. The spike of T4 or T3 increased the concentrations of not only T4 but also T3 and rT3, indicating that the protein binding of these hormones is competitive. In addition, the enhancement ratios for T4 were smaller than those for T3 and rT3, suggesting that T4 was more strongly bound to plasma proteins (Tables 4 and 5).

Human serum albumin (HSA) is the most abundant protein found in human plasma. A wide variety of drugs and endogenous compounds including T4 and T3 are bound to HSA. The present results were in accordance with the reported binding constants of HSA about $1.4$–$5.7 \times 10^5 \, M^{-1}$ for T4, $1.7 \times 10^4$ to $2.5 \times 10^5 \, M^{-1}$ for T3 at $37\,^\circ C$ [23].

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