

A Fluorescent Probe for Diacetyl Detection

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Abstract A water-soluble fluorescent probe, rhodamine B hydrazide (RBH), was prepared and its properties for recognition of diacetyl were studied. The method employs the reaction of diacetyl with RBH, a colorless and non-fluorescent rhodamine B spiro form derivative to give a pink-colored fluorescent substance. In weakly acidic media, RBH reacts more selectively with diacetyl than with other carbonyls, causing a large increase in fluorescence intensity and thereby providing an easy assay for the determination of diacetyl.

Keywords Fluorescent probe · Rhodamine B hydrazide · Diacetyl · Cell medium

Introduction

Diacetyl (2, 3-butanedione), is produced naturally in many foods, especially in fermented dairy products, and also in many bacteria [1–4]. Therefore, it plays an important role in microbiology and in fermentation technology [1–3]. Literature indicates that the diacetyl contained in food additives may be hazardous when inhaled over a long period, and diacetyl is further suspected to induce various toxic responses, such as lung disease, Alzheimer's disease, mutagenesis, and carcinogenesis [5, 6]. Hence, diacetyl determination is attractive, also because its concentration is one of the parameters on which lactic acid bacteria are characterized and evaluated [7].

Therefore, it is necessary to develop efficient methods for determination of diacetyl.

So far, a number of assays have been developed for diacetyl. Among them, there are two spectrophotometric methods. One is based on the conversion of diacetyl into dimethylglyoxime and subsequent conversion into a colored metal complex. Alternatively, a colored condensation product with urea is formed. The formation of dimethylglyoxime is slow and requires prolonged heating, which is time consuming and cannot be performed *in vivo* [8]. The other method, the Westerfeld method [9], was developed to measure diacetyl levels in blood. This original colorimetric assay for diacetyl and acetoin is very simple. Both diacetyl and acetoin react with creatine in the presence of α -naphthol to form a chromogenic compound that can be quantified spectrophotometrically. Diacetyl reacts comparatively faster than acetoin, and based on this property, diacetyl was determined successfully in presence of acetoin [8, 10, 11]. Later, when GC was introduced, it became the most frequently used method for diacetyl determination [12–14], and sometimes HPLC is also used [15]. By virtue of high sensitivity and the strong spatial resolution of fluorescence measurements, much interest has been shown in pre-column fluorescent derivatization methods in chromatography [16]. Currently, most of the commonly used derivatization reagents can form quinoline derivatives with α -dicarbonyl compounds, such as 2,3-diaminobenzene [17, 18]. Another way of derivatization is based on the reaction of hydrazide-containing fluorophores with carbonyls. In this way ketones and aldehydes are detected fluorescently [19, 20].

However, up to now, the fluorescent reagents developed for diacetyl are mostly excited at very short wavelengths, such as 2,3-diaminobenzene, ABD-H or DBD-H [17–19],

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which may suffer from interference by scatter and tissue absorbance. Therefore, long-wavelength fluorescent probes with excitation wavelengths in the visible region would represent a large progress. Among the long-wavelength fluorescent probes, rhodamine derivatives are widely used in biological systems due to their good photostability, water solubility and high quantum yield in aqueous solution [21]. Many unique signaling systems have been developed based on rhodamine B hydrazide, which is a classical example of Cu^{2+} -selective fluorescent chemodosimeter [22, 23].

Our aim is to develop a fluorescent method to detect diacetyl in cancerous cells. The non-fluorescent spiro dye rhodamine B hydrazide (RBH) was prepared and its selectivity for diacetyl was studied. It was found that RBH reacts with diacetyl at slightly acidic pH and undergoes a large increase in fluorescence intensity. pH, buffer and reaction time were optimized and a novel fluorescent method was developed for diacetyl determination. We show its potential for determination of diacetyl in cancerous cells with strong matrix effects by standard addition experiments in cell nutrition medium.

Experimental

Apparatus

Absorption spectra were recorded on a Cary 50 Bio UV-Vis spectrophotometer (Varian, Australia, www.varian.com). Luminescence spectra were recorded on an Aminco-Bowman AB 2 luminescence spectrometer (www.thermo.com) equipped with a 150-W continuous wave xenon lamp as excitation light source. All spectra are uncorrected. Measurements in cells were performed on a BMG Fluostar Optima (www.bmg-labtech.com) at $\lambda_{\text{exc}}=560$ nm and $\lambda_{\text{em}}=590$ nm in transparent flat bottom 96-well plates from nunc (www.nunc.de). pH was measured with a pH meter CG 842 from Schott (www.schott.com) at room temperature. The ESI mass spectra were taken on a ThermoQuest TSQ 7000 (www.thermo.com) mass spectrometer. The ^1H NMR spectra

were acquired on an Avance 300 MHz NMR Spectrometer (Bruker-BioSpin GmbH, www.bruker-biospin.com).

Reagents

Rhodamine B and diacetyl were purchased from Aldrich (www.sigmaaldrich.com). Diacetyl was distilled before use. Stock solutions of RBH (1.0 mmol/L) and diacetyl were prepared by dissolving an appropriate quantity of the reagents in ethanol.

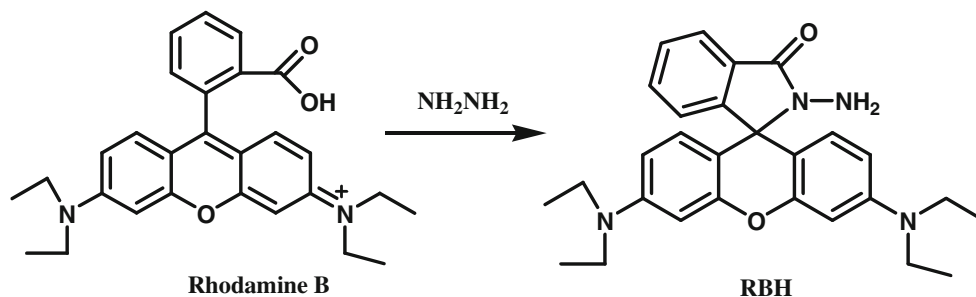
Synthesis of RBH

RBH was synthesized according to a method reported in literature [23]. Specifically, 0.5 g of rhodamine B were dissolved in 20 ml of methanol, an excess of hydrazine hydrate (0.6 mL) was added, and then the reaction solution was refluxed until the pink color disappeared (Scheme 1). The cooled reaction mixture was then poured into distilled water and extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate, filtered and the solvent was evaporated. The product was recrystallized from methanol-acetone as a colorless solid and the purity is above 99% by TLC. Yield: 0.36 g (70%) of RBH. MS (ES) m/z : 457.2, ($[\text{M}+\text{H}]^+$); calculated: 456.2 for M^+ . ^1H NMR (CDCl_3) δ 7.90 (m, 1H, ArH), 7.42 (m, 2H, ArH), 7.10 (m, 1H, ArH), 6.44 (d, 2H, xanthene-H), 6.40 (d, 2H, xanthene-H), 6.28 (dd, 2H, xanthene-H), 3.58 (s, 2H, NH_2), 3.32 (q, 8H, NCH_2CH_3), 1.15 (t, 12H, NCH_2CH_3).

Derivatization procedure for diacetyl

Twenty microliters of a solution of RBH (1 mmol/L in ethanol) was added to citric acid- Na_2HPO_4 buffer of pH 3 (50 mmol/L), followed by addition of diacetyl solution (in ethanol) containing no more than 20 $\mu\text{mol/L}$, and the volume of each diacetyl solution was made up to 1 mL. The same volume of ethanol as the sample solution was added to the control containing RBH only. The tube was then kept at 37 ± 1 °C for 3 h before the fluorescence spectra and/or intensities were measured at 563 nm (exc.) and 586 nm (em).

Scheme 1 Synthesis of RBH



Results and discussion

Fluorescence spectra of RBH

The stock solution of RBH in ethanol is colorless, and shows no detectable fluorescence. After addition of a small portion of RBH stock solution into pH 3 citrate–phosphate buffer and shaking, a faint pink color shows up almost immediately and weak fluorescence can also be detected. In aqueous buffers of pH 7 no such phenomenon appears. The fluorescence spectra of RBH with or without diacetyl are shown in Fig. 1. RBH and diacetyl in different concentrations were reacted for 3 h at 37 °C in pH 3 citrate–phosphate buffer solutions. As can be seen from Fig. 1, RBH shows weak fluorescence with $\lambda_{\text{exc/em}}$ at 560/590 nm in pH 3 media. This is due to the formation of a small portion of the ring-opened amide form of RBH in acidic condition (Scheme 2). After addition of diacetyl to the solution in $\mu\text{mol/L}$ quantities, the characteristic fluorescence emission of rhodamine B was observed and its pink color recovered to a large extent. Accordingly, the fluorescence intensity increases fourfold. This is accompanied by a 5 nm red-shift of the emission maximum from 581 nm to 586 nm in presence of 200 $\mu\text{mol/L}$. The wavelength of the excitation maximum remains almost unchanged. The fluorescence increase seen upon reaction of RBH with increasing amounts of diacetyl reaches saturation at a molar ratio of diacetyl to RBH of 1:1. If the concentration of diacetyl is 220 $\mu\text{mol/L}$ or higher, almost no more fluorescence increase appears. We therefore propose that only a 1:1 product is formed, but no product with two molecules of RBH reacted with one diacetyl. The reasons may be increased steric hindrance for an additional attack at the 1:1 product and a

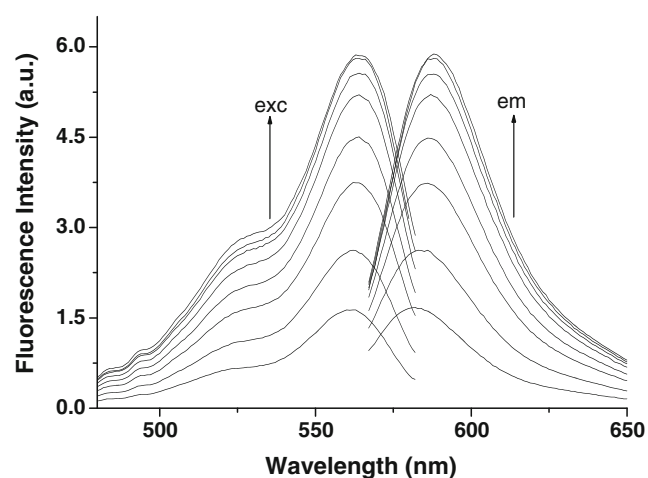


Fig. 1 Fluorescence spectra of RBH before and after reaction with diacetyl. The spectra from *bottom to top*: 200 $\mu\text{mol/L}$ of RBH with 0, 40, 90, 120, 150, 180, 200, 220 $\mu\text{mol/L}$ diacetyl in citric acid- Na_2HPO_4 buffer of pH 3, respectively

reduced nucleophilicity of the remaining carbonyl carbon of diacetyl in the 1:1 product. Thus, the probe shows a fluorescence-on response to diacetyl and may be used as a probe for diacetyl.

Effect of pH and reaction time

The effect of pH on the reaction of RBH with diacetyl was investigated. Several buffer systems, such as citrate buffer, citrate–phosphate buffer, acetate buffer and phosphate buffer from pH 2 to pH 5, were tested with 200 $\mu\text{mol/L}$ of RBH and diacetyl at 37 °C after a 2.5 h reaction. It was found that the best buffer system for this derivatization reaction is citric acid- Na_2HPO_4 of pH 3, in that it gives a more pronounced fluorescence signal than the other systems (Fig. 2).

The reaction rate of 200 $\mu\text{mol/L}$ of RBH with 100 $\mu\text{mol/L}$ of diacetyl was also examined at 37 °C and pH 3. Figure 3 shows that after 2.5 h incubation the derivatization is completed. Due to the pronounced increase of fluorescence intensity, one could also use a kinetic determination after 1.5 h, where more than half of the total fluorescence increase has already appeared.

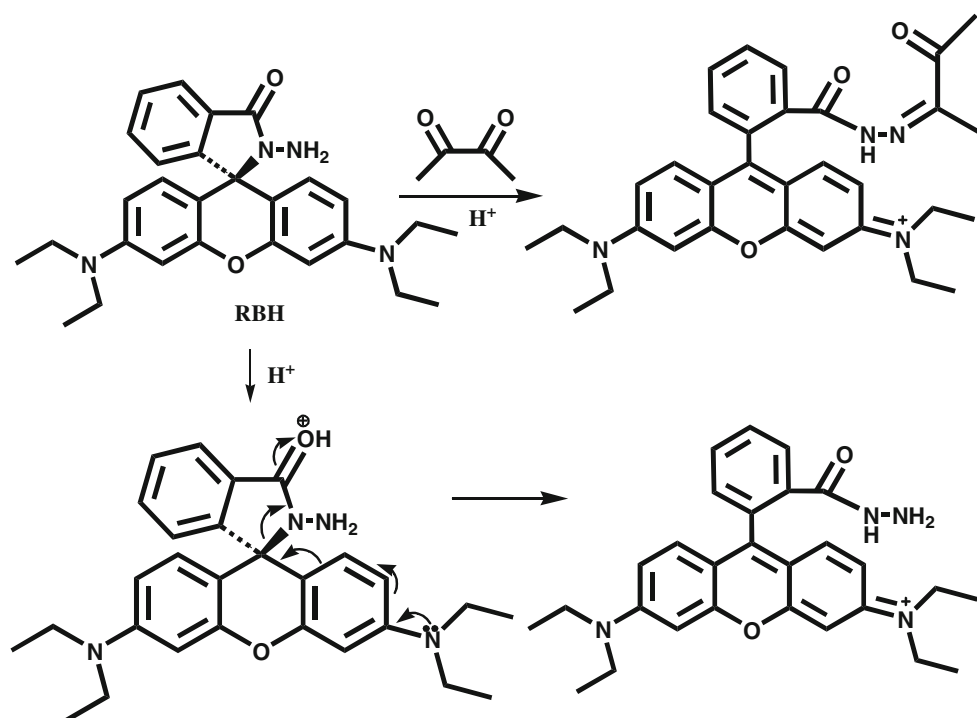
Absorption spectra of RBH before and after reaction

The absorption spectra of RBH (200 $\mu\text{mol/L}$) before and after the reaction with 100 $\mu\text{mol/L}$ of diacetyl at 37 °C in pH 3 citrate–phosphate buffer solution (50 mmol/L) are shown in Fig. 4. In accordance with the change of fluorescence, the absorbance also increases to almost the same degree after reaction with diacetyl. The absorption maximum only slightly changes from 559 nm to 562 nm. Obviously, the reaction of diacetyl with RBH causes a structural change in the molecule, as shown in Scheme 2.

Interference by other carbonyls

The reaction of RBH with various carbonyls was examined under the above conditions to determine the selectivity of the probe for different carbonyls. Ten micromolar per liter of diacetyl and a twice to 13.6-fold molar excess of interferent was reacted with RBH in pH 3 citrate–phosphate buffer solution (50 mmol/L) at 37 °C for 3 h. Then, the enhancement of the emission intensities was compared. Figure 5 indicates that RBH has a much higher selectivity for diacetyl than for other carbonyls, except that benzaldehyde shows noticeable interference. However, there is no report which shows that benzaldehyde is found as a result of metabolism in any physiological process. As we aim to find a reagent to determine diacetyl at physiological concentrations, the interference of benzaldehyde is not relevant for our purposes. Apart from benzaldehyde,

Scheme 2 Reaction mechanism of RBH with diacetyl and RBH hydrolysis in acidic buffer



pyruvic acid shows interference if it is present at 20 $\mu\text{mol/L}$. If the emission intensity of pyruvic acid is calculated for the same molar concentration (10 $\mu\text{mol/L}$) as for diacetyl, however, the signal of pyruvic acid is tenfold lower than that of diacetyl. This 10 $\mu\text{mol/L}$ is the concentration of pyruvic acid one can expect to occur in cell medium [24] or from cell influx and efflux [25]. The selectivity towards other carbonyls on a molar basis varies between 35:1 for glyoxal and 200:1 for acetaldehyde. Although glyoxal and pyruvic acid are more electrophilic compounds than diacetyl and thus should show faster reaction kinetics with

RBH, lower emission intensities of the respective reaction products after a reaction time of 3 h are found, reproducibly. We currently do not have an explanation for this phenomenon. Therefore, interferences are unlikely to be a problem for this probe to be applied for diacetyl detection in the cases where diacetyl is the dominant carbonyl species to be determined.

Preliminary test in cell medium

The derivatization method was tested in the supernatant of some cell lines. Three different cell lines (SW620, LS174 and SW837) were allowed to undergo their usual metabolism activity in RPMI 1640 Biochrom medium for 2 days. During this period, the medium is enriched with metabolism products and low-molecular weight carbonyl compounds released from the cells. This increases the matrix effect of the supernatant, additionally. RPMI as such is a strong matrix itself because it contains buffer salts and salts for adjustment of ionic strength in g/L concentrations, all 20 amino acids in up to hundreds of mg/L, the vitamins B₁, B₂, B₆, B₁₂; biotin, folic acid and other compounds in lower quantities [26, 27]. After 2 days, the cells were removed by centrifugation and the medium was adjusted to pH 3 with HCl. Unspiked cell medium was tested on its effect on RBH by mixing of 100 μL of cell supernatant, 40 μL of RBH and 60 μL of citric acid-Na₂HPO₄ buffer of pH 3. From the solid lines in Fig. 6 (curves I, III and V), it is obvious that luminescence increased by a factor between 2 and 5. This points out, that certain amounts of carbonyls were present in the

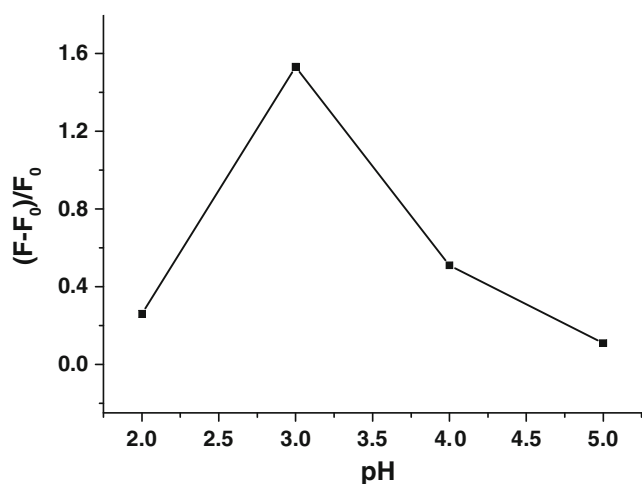


Fig. 2 Effect of pH on the reaction of RBH with diacetyl. HCl, 0.01 mol/L, was used as pH 2 buffer. Concentration of both RBH and diacetyl is 200 $\mu\text{mol/L}$

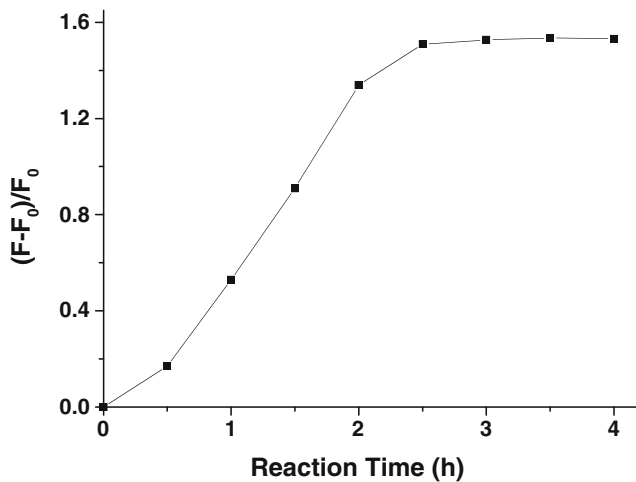


Fig. 3 Effect of reaction time on the reaction of RBH with diacetyl. Conditions: 200 μmol/L of RBH, 100 μmol/L of diacetyl, reacted at 37 °C in pH 3 citrate-phosphate buffer solution (50 mmol/L)

supernatants. The increase in luminescence is completed after about 1 h. This is a shorter time compared to the detection of pure diacetyl and hints to the existence of more reactive carbonyl than diacetyl. We then spiked the cell supernatant with diacetyl (60 μL of supernatant, 40 μL of RBH, 40 μL of 200 μmol/L of diacetyl and 60 μL of buffer) to judge on the capability of RBH to detect diacetyl in the presence of a potentially strongly interfering matrix. On comparing the luminescence of the supernatants spiked with diacetyl with the unspiked samples (I and II, III and IV, V and VI), it is visible that for each cell line, there is a

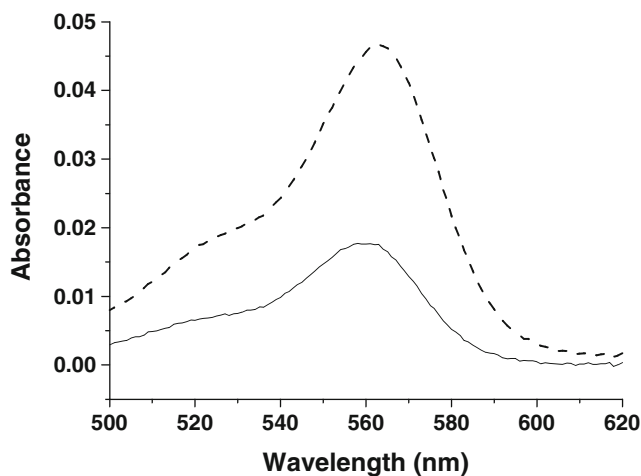


Fig. 4 Absorption spectra of RBH before (solid line) and after (dashed line) reaction with diacetyl. Conditions: 200 μmol/L of RBH, 100 μmol/L of diacetyl; reacted in pH 3 citrate-phosphate buffer (50 mmol/L) at 37 °C for 3 h

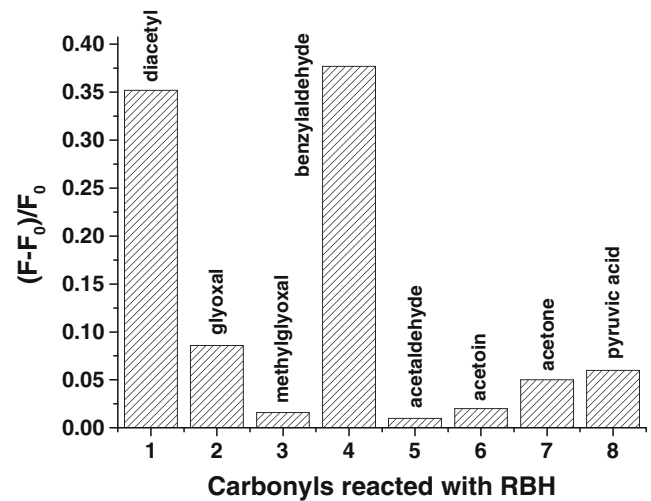


Fig. 5 Fluorescence increase of RBH caused by diacetyl or other carbonyls as potential interferents after reaction in pH 3 citrate-phosphate buffer (50 mmol/L) at 37 °C for 3 h. The concentration of diacetyl is 10 μmol/L. For other carbonyls the concentration is 87.5, 65, 60, 100, 50, 136 and 20 μmol/L for glyoxal, methylglyoxal, benzylaldehyde, acetaldehyde, acetoin, acetone and pyruvic acid, respectively

luminescence increase compared to the unspiked supernatant. As the reaction of the spiked samples still takes 3 h to be completed, we deduce that the additional fluorescence increase compared to unspiked samples is due to the presence of diacetyl. This shows that RBH can be used as a fluorescent probe for diacetyl in cell medium as well as in other matrix-containing media in the μmol/L-concentration range, and thus might become a suitable reagent for a rapid screening test for cancerous cells.

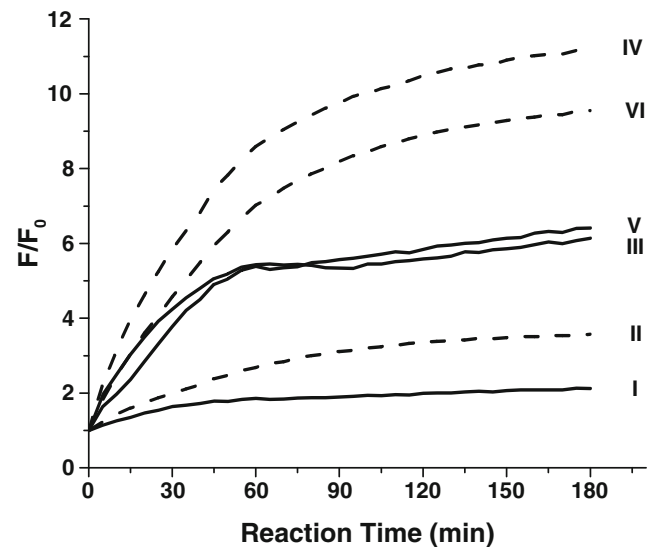


Fig. 6 Luminescence of supernatants of cancer cell lines before and after spiking with diacetyl. Solid lines (I, III and V) represent unspiked and dashed lines (II, IV and VI) represent spiked samples of SW620 (I and II), LS174 (III and IV) and SW837 (V and VI), respectively. A 560 nm (exc.)/590 nm (em) filter combination was used

Conclusion

The fluorescent probe RBH is introduced for determination of diacetyl. To our knowledge, this is the first time that RBH was used as a probe for diacetyl. It shows good selectivity to diacetyl compared to other carbonyls of physiological relevance. A preliminary test for detection of cell-released diacetyl in cell medium in $\mu\text{mol/L}$ concentrations was successful. Further investigation of this probe for detection of diacetyl in cancer cell culture is now in progress. We think that the present derivatization reaction may offer a convenient way for detection of diacetyl in vitro, and thus to detect cancerous cells at an early stage.

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