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Spectrofluorometric Determination of Alpha Fetoprotein in Different Serum Samples of Liver Cancer by Tb-acetyl Acetone Complex Embedded in Polymethylmethacrylate Optical Sensor

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A SIMPLE, precise and sensitive method in which, Tb-acetyl acetone (Tb-ACAC) complex embedded in polymethylmethacrylate (PMMA) is used for the early diagnosis of liver cancer. The diagnosis process depends on the assessment of the concentration of alpha fetoprotein (AFP) in the serum samples of different liver patients. The Tb-acetyl acetone (Tb-ACAC) embedded in PMMA has strong emission band at 545 nm after excitation at 350 nm in ethanol. The assessment of alpha fetoprotein (AFP) depends on the quenching of the emission band at 545 nm in ethanol by the alpha fetoprotein (AFP). The calibration plot was achieved over the concentration 1 - 550 ng/mL with a correlation coefficient of 0.99 and a detection limit of 0.5 ng/mL. The method was used satisfactorily for the diagnosis of liver cancer in a number of serum samples collected from various patients and health state; healthy ($\leq$ 10 ng/mL) and HCC (400–550 ng/mL).

Keywords: Alpha fetoprotein; Polymethylmethacrylate, Tb-Acetylacetone, Optical sensor; Liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is one of the commonly encountered malignant neoplasms in the world. Almost 80% of HCC cases are due to underlying chronic hepatitis B and C virus infection. In patients with chronic liver diseases the early detection of HCC is very important in controlling this disease. The primary tumor marker for HCC is a single polypeptide chain glycoprotein namely α -fetoprotein (AFP) [1]. AFP is measured in nanograms per milliliter (ng/mL). An AFP level of less than 10 ng/mL is normal for adults. An extremely high level of AFP in your blood—greater than 400 ng/mL—could be a sign of liver tumors [2]. In some cases, at the low level of α–fetoprotein the combination between serum AFP levels and ultra-sonography sometimes misses HCC [3]. Therefore, improving the sensitivity towards measuring the serum AFP by effective and low cost optical sensor remains an active area of research [3]. Different spectrometric methods have been reported for the determination of the α-fetoprotein (AFP) in serum [4]. These methods are limited by their long incubation time (30–60 min) for the sample and reagent blanks. α-fetoprotein (AFP) was also determined by spectrofluorimetric methods [5], electrochemical immunosensor [6]. Some of these methods are unselective, require careful experimental conditions, considerably time consuming and not compatible to detect the alpha fetoprotein (AFP) at the early stage of diseases. Luminescent optical sensors lanthanide complexes have more advantages over the present ones; (1) the optical

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sensor has high stability and durability, (2) the sensor can provide constant signal response for 2 years which is a 24-fold better stability compared to the life time warranted for the chromatographic and colorimetric methods [7-11], (3) sensor is stable over all measurements which prevent the source of error in the measurement process and it gives a low standard deviation values. In this paper the determination of AFP in different serum samples of different patients with liver cancer is achieved. The determination process depends on the quenching of the luminescence intensity of Tb-ACAC embedded in PMMA in ethanol at 545 nm by different concentration of AFP.

Experimental

Apparatus

All fluorescence measurements were recorded with a Meslo-PN (222–263000) Thermo Scientific Lumina fluorescence Spectrometer in the range (190–900 nm). The absorption spectra are recorded with Thremo UV–Visible double beam spectrophotometer. All pH measurements are made with a pHs-janway3040 ion analyzer.

Materials and reagents

Acetyl acetone, CEA, PSA, CA-125, hCG, hLH, hTSH, hPRL were purchased from (Sigma-Aldrich). Alpha fetoprotein was purchased from Sigma. Ethanol and pure grade solvents (Aldrich) were used for the preparation of solutions. $Tb(NO_3)_3$ and polymethylmethacrylate were purchased from sigma. The Tb-acetyl acetone (Tb-ACAC) complex was prepared and characterized according to the previous work by the research group of M. S. Attia at Ain Shams University [12]. The luminescence intensity was measured at $\lambda_{ex}/\lambda_{em}$ = 350/545 nm. Stock and working solutions were stored at 0 – 4 °C when not in use. In all experiments, clean and sterilized volumetric flasks (10 mL) were used. Stock solutions of 1 and 550 ng/mL alpha fetoprotein were prepared by dissolving the content of the one vial alpha fetoprotein of each one in 1.0 mL of H_2O. Human serum samples were obtained from the New Al-Kasr El-Aini teaching Hospital Cairo University and Ain Shams Specialized Hospital, Ain shams University, Cairo, Egypt in accordance with WHO (World Health Organization) approved the protocol for human specimen collection and for the use of this material and related clinical information for research purposes. [All patients are consented and approved the using of their clinical samples in the research work]. A stock solution (1.0×10^{-2} mol L^{-1}) of the prepared optical sensor was prepared by dissolving the required weight of the Tb-acetyl acetone (Tb-ACAC) in ethanol. The working standard solution (5.0×10^{-4} mol L^{-1}) of the optical sensor was prepared by appropriate dilution of the stock with ethanol. Stock solution (600 ng/mL) of alpha fetoprotein was prepared in distilled water. More diluted solutions (1–550 ng/mL) of Alpha Fetoprotein were prepared by diluting the stock solution with ethanol. The luminescence intensity was measured at $\lambda_{ex}/\lambda_{em}$ = 350/545 nm. Stock and working solutions were stored at 0 – 4 °C when not in use.

General procedures

Preparation of Tb-acetyl acetone (Tb-ACAC) complex embedded in PMMA [13-18].

(I) 0.12 g of Tb-acetyl acetone (Tb-ACAC) complex was dissolved in 10 mL ethanol
(II) 5 g of PMMA was dissolved in 25 mL chloroform
(III) 5 mL of solution (i) was mixed well with continuous stirring for 5 min. with 5 mL of solution (ii) in 10 mL measuring flask.
(IV) Solution (iii) was poured in a clean petri dish (petri dish was wished by acetone, n-propanol, ethanol, chloroform) to dry and obtain the PMMA film embedded Tb-acetyl acetone (Tb-ACAC) complex
(V) The photophysical properties of the PMMA film were measured (absorbance and fluorescence measurements).

Recommended procedure

An appropriate amount (150 µL) of various standard concentrations of the alpha fetoprotein in ethanol was mixed with the optical sensor Tb-acetyl acetone (Tb-ACAC) embedded in PMMA in the cell. The luminescence spectra were then recorded at the excitation wavelength. The optical sensor was rinsed with ethanol after each measurement and the calibration plot was constructed by plotting the luminescence intensity at λ_{em} = 545 nm on the y axis against the reciprocal of alpha fetoprotein concentration on the x axis and all data were compared by data obtained by standard method.

Standard method

AFP in serum was determined using an electrochemiluminescence kit (Roche Company, Switzerland) on an Elecsys 2010 electro- chemiluminescence instrument (Roche Company).

Analytical application

The optical sensor was immediately mixed with an accurate volume (50 µL) of plasma / serum samples of HCC patients (5 persons) and healthy control (5 persons) and pH was adjusted at 5 by using the phosphate buffer. The volume was completed to 3.0 mL with ethanol then the optical sensor Tb-acetyl acetone (Tb-ACAC) complex embedded in PMMA was immersed in each solution in the measuring cell and the emission intensity at 545 nm was measured against the reagent blank.

Results and Discussion

Absorption and emission spectra

The absorption spectrum of 3 x 10^{-4} mol L^{-1} ACAC in the PMMA shows a band at 276 nm due to π - π* transition (Fig. 1). Upon the addition of 1 x 10^{-4} mol L^{-1} of the Tb^{3+} ion into ACAC in the PMMA matrix, a red shift was observed in the band by 6 nm; this indicates complex formation between Tb^{3+} and ACAC [18-26]. The luminescence emission spectra of Tb^{3+} in different concentrations of ACAC in the PMMA matrix are shown in Fig. 2. From curve (1) in Fig. 2, it can be seen that a single Tb^{3+} ion in the PMMA matrix has nearly no peak. After the addition of ACAC to the Tb^{3+} ion in the PMMA matrix, the characteristic peaks of the Tb^{3+} ion ($^5D_4 \rightarrow {}^7F_6$, $^5D_4 \rightarrow {}^7F_5$, and $^5D_4 \rightarrow F_4$) appeared (see curves 2 and 1 in Fig. 2) [27-31]. Comparing curve 4 with curves 2 and 3 in Fig. 2, it can be seen that the characteristic peak of Tb^{3+} at 544 nm has been enhanced after the addition of ACAC, which indicates a good energy transfer from ACAC to Tb^{3+} in the complex. Fig. 3 shows the effect of solvent on the luminescence intensity of the Tb–ACAC sensor embedded in the PMMA matrix [32-38]. The results show that the highest luminescence intensity for the optical sensor is obtained in ethanol. Therefore, ethanol is used in all measurements. Upon the addition of different concentrations of AFP (1-550 ng/mL) in ethanol to the Tb–ACAC complex embedded in PMMA matrix, the quenching of all of the characteristic peaks of the Tb ion in the Tb–ACAC complex occurred (Fig. 4); especially of the electrical band at 544 nm, which is very sensitive for the chemical environment around the optical sensor. Therefore, the Tb–ACAC complex can be used as a sensor for AFP in ethanol.

Selectivity

Selectivity of the developed method was tested by studying the influence of a series of interfering species e.g. NaCl (10 µg mL^{-1}), total proteins (1.0 µg mL^{-1}), CEA (10 µg mL−1), hCG (1000 IU mL^{-1}), hLH (10 IU mL^{-1}), hTSH (100 mIU mL^{-1}), urea (100 µg mL^{-1}) on the luminescence spectrum of Tb–ACAC complex embedded in PMMA matrix after addition of [AFP], (500 ng/mL). The tolerable limit was defined as the concentration of the added species individually causing a deviation less than 3% of the luminescence intensity at the optimum conditions of the optical sensor Tb–ACAC complex. The results indicated no significant effect on the luminescence intensity of the Tb–ACAC complex embedded in PMMA matrix.

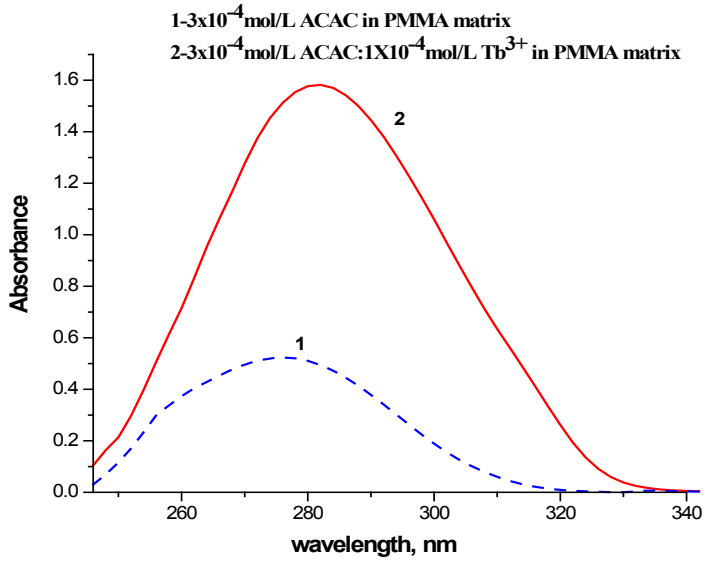

Fig.1. Absorption spectra of ACAC and Tb-ACAC embedded in PMMA

Analytical performance

The effect of the concentration of the AFP on the luminescence intensity of the optical sensor Tb–ACAC complex embedded in PMMA matrix is shown in Fig. 4. The validation of the proposed method for measuring the AFP concentration under the optimized experimental conditions was determined via the limit of detection (LOD), limit of quantification (LOQ), linear dynamic range (Table 2), repeatability, and recovery (Table 3). The luminescence intensity of Tb–ACAC complex embedded in PMMA matrix was recorded at various concentrations of [AFP]. The plot of the measured signal by the developed procedure versus 1/[AFP] concentration was found linear over the concentration range 1- 550 ng/mL with a correlation coefficient of 0.99 (Figure 5. The values of detection limits were calculated according to ICH guidelines [39] using the formulae LOD = 3.3S/b and LOQ = 10S/b (where S is the standard deviation of the blank luminescence intensity values, and b is the slope of the calibration plot), and are also presented in Table 1. The comparison of the proposed Tb–ACAC complex embedded in PMMA matrix for the determination of AFP with other published methods [7-11] indicates that the developed method has a good stability, lower limit of detection (0.5 ng/mL) and wide linear range of application (1-550 ng/mL).

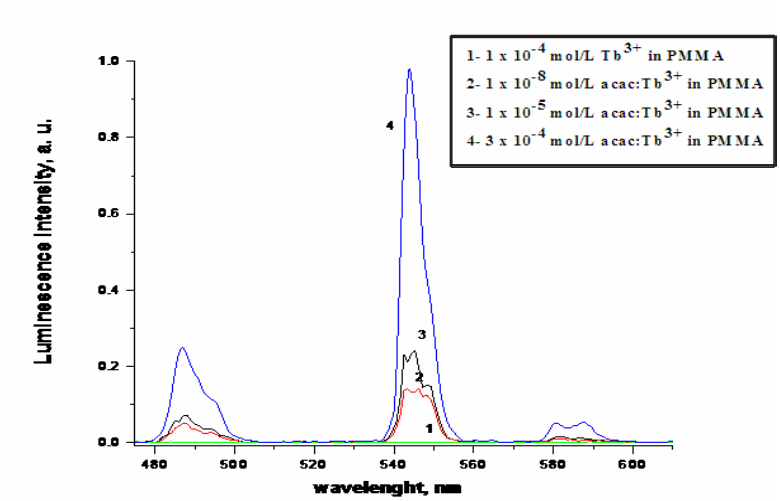

Fig. 2. Luminescence emission spectra of Tb3+ and Tb with different concentrations of ACAC embedded in PMMA at λex= 350.

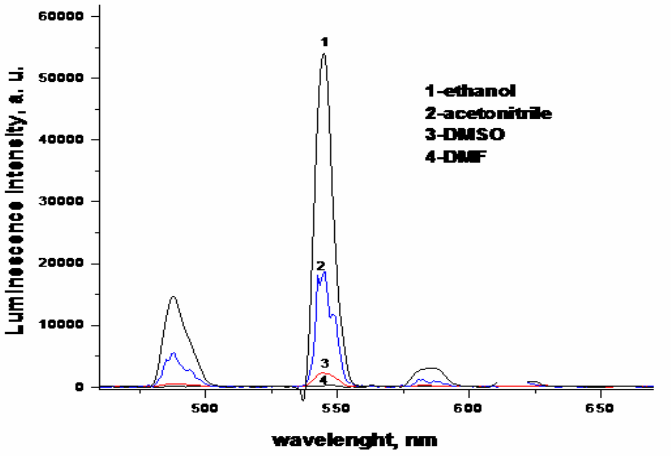

Fig. 3. Luminescence emission spectra of Tb3+-ACAC embedded in PMMA in different solvents at λex= 350.

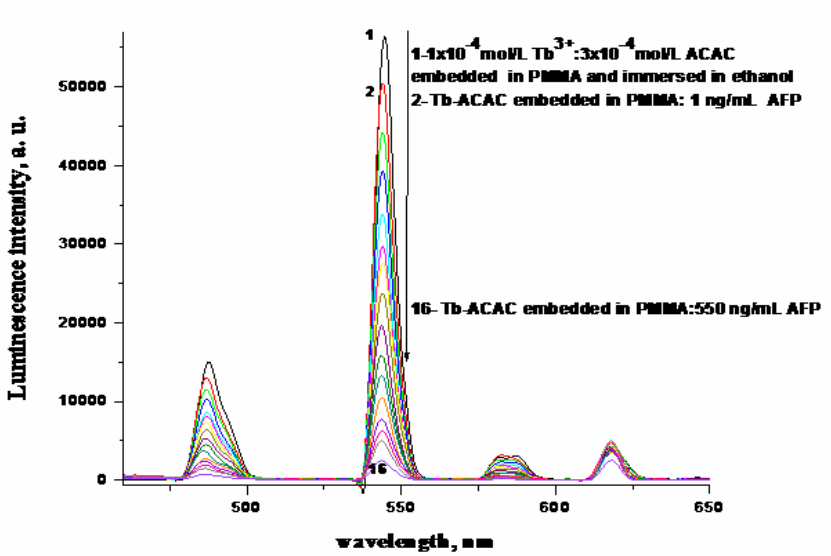

Fig. 4. Luminescence emission spectra of Tb3+-ACAC embedded in PMMA in different AFP concentrations at λex= 350.

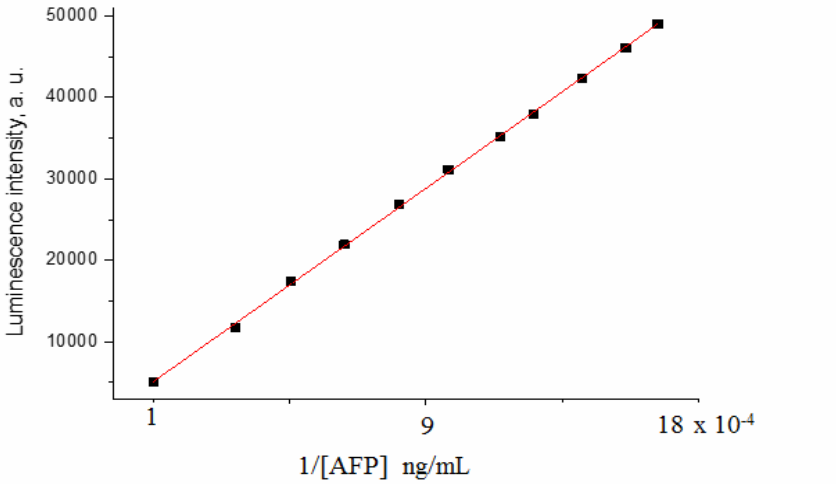

Fig. 5. Linear relationship between the luminescence intensity of Tb-ACAC embedded in PMMA and 1/[AFP] in ethanol at λex= 350.

TABLE 1. Sensitivity and regression parameters for optical sensor

Parameter	Method
λ_{em}, nm	545
Linear range, ng/mL	1-550
Limit of detection (LOD), ng/mL	0.5
Limit of quantification (LOQ), ng/mL	1.5
Intercept (a)	2526
Slope (b) x 10^6	2.9
Standard deviation	0.045
Variance (Sa^2)	$2.5x10^{-5}$
Regression coefficient (r)	0.99

Response time

Response time is an important analytical feature of any optical sensor. The response time is the time required for the analyte to transfer from the bulk of the solution to the membrane interface and react with the optical sensor. The response time was tested by recording the luminescence intensity change of the optical sensor at λ_{em} = 545 nm. It was observed that the proposed sensor reached about 95% of the steady-state response within 10–20 s, depending on the concentration of AFP.

Reproducibility, short-term stability and lifetime.

Reproducibility is considered an important characteristic feature for any optical sensor. The reproducibility was examined by preparing 5 different concentrations of the sensor Tb–ACAC complex embedded in PMMA matrix and measuring the luminescence intensity of each concentration at $\lambda_{ex}/\lambda_{em}$ = 350/545 (nm) (each measurement repeated three times) in ethanol. The resulting coefficient of variation was found to be 1.22%. The results indicate that the reproducibility is satisfactory. The short term stability of the optical sensor was studied over a period of 10 h. From the luminescence intensity measurements, after every 5 min (n = 3), it was found that the response was almost complete with only a 0.50% change in the luminescence intensity after 10 h of monitoring. In addition, it was found that the optical sensor could be stored without any measurable changes in its luminescence intensity for at least 2 years, which implies that the sensor is quite stable in the PMMA matrix.

Accuracy and precision of the method.

To evaluate accuracy and precision, the assays described under general procedures were repeated three times within one day to determine the repeatability (intra-day precision) and three times on different days to determine the intermediate precision (inter-day precision) of the method. These assays were performed for ten healthy state samples. The results of this study are summarized in Table 2. The percentage relative standard deviation (%RSD) values were =1.60–2.80% (intra-day) and 1.50–3.30% (inter-day). The inter-day values indicate the high precision of the method. Accuracy was evaluated as percentage relative error (RE) between the measured mean concentrations and the obtained concentrations of AFP. Bias (bias% = [(concentration found - known concentration) X 100/known concentration]) was calculated at each concentration and these results are also presented in Table 2. Percent relative error (%RE) values of = 0.10–1.24% (intra-day) and 0.19–1.34% (inter-day) demonstrate the high accuracy of the proposed method.

TABLE 2. Analytical results of the serum samples of healthy and patients analyzed by the standard and the developed methods and statistical comparison of the results with the reference method.

Sample	Standard method	Intra-day accuracy and precision (proposed method) (n = 3)			Inter-day accuracy and precision (proposed method) (n = 3)		
	Average (ng/mL) ± %RSD	Average Found* (ng/mL) ± CL	%RE	%RSD	Average found* (ng/mL) ± CL	%RE	%RSD
Healthy (1)	9 ± 1.9	8.7 ± 1.5	1.72	3.3	9.2 ± 0.6	2.2	2.7
Healthy (2)	4 ± 1.8	4.1 ± 1.7	0.83	2.5	4.2 ± 0.7	5.0	1.9
Healthy (3)	3.3 ± 1.5	3.5 ± 1.0	2.38	6.0	3.35 ± 1.0	1.5	2.1
Healthy (4)	11 ± 2.7	10.9 ± 2.9	6.89	0.9	11.6 ± 1.1	5.4	2.3
Healthy (5)	5.3 ± 2.1	5.5 ± 1.4	2.00	3.7	5.7 ± 1.2	7.5	2.1
Patient (1)	392 ± 1.5	391 ± 1.5	1.08	0.2	392.8 ± 1.5	0.2	2.3
Patient (2)	421 ± 1.6	419 ± 2.2	1.65	0.4	422.7 ± 1.6	0.4	1.7
Patient (3)	433 ± 3.1	430 ± 1.4	2.25	0.6	434.8 ± 1.0	0.4	3.6
Patient (4)	524 ± 1.2	522 ± 1.9	0.89	0.3	523.1 ± 1.3	0.1	2.4
Patient (5)	562 ± 2.4	564 ± 1.8	1.23	0.3	564.6 ± 1.1	0.4	2.1

* The average value for three readings, %RE: Percent relative error, %RSD: Relative standard deviation and CL: Confidence limits. (CL = ± ts/√n., t = 4.303, at the 95% confidence level s = standard deviation and n = number of measurements).

Analytical applications

The analytical utility of the proposed method was tested by measuring the concentration of the AFP for 10 serum samples of healthy and patients are repeated with the new method. Good agreement between the average values obtained by the developed procedure (6.30 ± 2.24 ng/mL) and the standard method (6.60 ± 2.0 ng/mL) for health state and no significant differences between two methods. A comparison between the values of the mean for the patient samples using the standard method (466.40 ± 1.56 ng/mL) with that obtained by the developed method (465.20 ± 1.77 ng/mL) revealed no significant differences between the two methods, Table 2. In HCC samples (5), the average value obtained by the standard method (426.95 ± 1.94 ng/mL) was found also quite close to the mean value (424.23 ± 1.52 ng/mL) obtained by the developed method with a standard error in the range 0.90–4.90 confirming the accuracy of the proposed procedure.

Conclusion

The developed method provides an excellent approach for early diagnosis of different liver diseases. The method depends on the measuring the concentration of the AFP compared to other reported methods. The method is sensitive and provides a wide linear dynamic range of AFP concentrations by measuring the luminescence intensity of Tb–ACAC complex embedded in PMMA matrix under the optimal conditions. A detection limit of 0.5 ng/mL was achieved.

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التقدير الفلوروسيني لالالفا فيتوبروتين في عينات الدم المختلفة لمرض سرطان الكبد باستخدام المجس الضوئي متراكب التربيم اسيتيل اسيتون المغموس في وسط البولي ميسيل مساكريلايت

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[٢]قسم الفيزياء - كلية العلوم - جامعة المنيا - المنيا - مصر

[٣]قسم الفيزياء - كلية العلوم - جامعة عين شمس - القاهرة - مصر

[٤]قسم الكيمياء - كلية العلوم - جامعة عين شمس - القاهرة - مصر

يتضمن البحث طريقة جديدة سهلة ودقيقة والتي فيها يستخدم مركب تربيوم-الأسيتيل اسيتون المتضمن في البولي ميثيل ميثااكريلات للتشخيص المبكر لسرطان الكبد. تعتمد طريقة التشخيص على تقدير تركيز الالفا فيتوبروتين في عدة عينات لمرضى سرطان الكبد. والجدير بالذكر أن متراكب التربيوم-الأسيتيل اسيتون المغموس في البولي ميثيل ميثااكريلات له حزمة ضوئية ذات شدة انبعاث ضوئي عند الطول الموجي ٥٤٥ نانومتر بعد اثارته في الايثانول عند ٣٥٠ نانومتر. كما أن تقييم الالفافيتوبروتينيعتمد على عملية التثبيط لطيف الانبعاث الحادث في الايثانول عند ٥٤٥ نانومتر بواسطة الالفا فيتوبروتين. ولقد تم عمل منحنى المعايرة مع تركيزات (550-1 ng/mL) بمعامل ارتباط مقداره ٠,٩٩ وحد كشف مقداره 0.5ng/mL. وقد استخدمت هذه الطريقة برضا تام لتشخيص سرطان الكبد في عدد من العينات المأخوذة من بعض المرضى وقد تبين أن المعافى يكون > 10 والمريض (400-550) mg/mL.