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ORIGINAL PAPER

Sensitive Phosphorimetric Determination of Bumetanide in Human Urine with Its Inhibition Effect on the Formation of [Fe-morin] $^{3+}$ Complex

Li-Qing Zeng · Jia-Ming Liu · Fei-Ming Li · Xiao-Mei Huang · Li-Ping Lin · Xin-Xing Wang · Li-Hong Zhang · Chang-Qing Lin

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Abstract A novel solid substrate-room temperature phosphorimetry (SS-RTP) was developed for determination of bumetanide (BMTN). It was validated by determining selectivity, linearity, accuracy, precision, and signal to noise ratio (S/N) for analysis. And all the experiments presented in this work were based on that BMTN inhibited the formation of [Fe-morin]$^{3+}$ ([FeR]$^{3+}$) complex by the reaction between Fe^{3+} and R, which led to severe quenching of room temperature phosphorescence (RTP) signal. The rate constant of the reaction (k) was 2.44×10^{-4} s^{-1}, the activation energy (E) was 21.39 kJ mol^{-1}. Detection limit of this method (LD, 5.0 ag $spot^{-1}$, corresponding concentration was 1.2×10^{-14} g mL^{-1}) was evaluated and compared with other methods, indicating better sensitivity for BMTN determination using this technique. And due to the high sensitivity of the method, it has been successfully applied to determine BMTN in human urine samples. The linear range was from 0.040 pg mL^{-1} to 4.0 pg mL^{-1}, allowing wide determined range of BMTN. Meanwhile, the mechanism of this method was also discussed.

L.-Q. Zeng · J.-M. Liu (✉) · F.-M. Li · X.-M. Huang · L.-P. Lin · X.-X. Wang
Department of Chemistry and Environmental Science,
Zhangzhou Normal College,
Zhangzhou 363000, People's Republic of China
e-mail: zzsyliujiaming@163.com

X.-M. Huang · L.-H. Zhang · C.-Q. Lin
Department of Food and Biological Engineering,
Zhangzhou Institute of Technology,
Zhangzhou 363000, People's Republic of China

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Introduction

BMTN, 3-(butylamino)-4-phenoxy-5-sulfamoylbenzoic acid, is a strong diuretic agent that widely offered for the prevention of acute renal failure and the treatment of oedema desease, high blood pressure, and acute drug intoxication [1]. However, the massive-dose or long-term use of BMTN would cause hypokalemia, low chlorine alkalosis, and hyperuricemia [2]. Meanwhile, BMTN, which would be used to reduce weight in heavyweight items (such as weightlifting, boxing, etc.) or to mask other banned drugs in urine, is also one of the diuretic drugs banned by the International Olympic Committee [3]. And to rely on taking BMTN to improve athletic performance launches a serious challenge to the Olympic spirit and the "fair competition" criteria. Moreover, BMTN is a prohibited chemical substance which is often unlawfully adulterated with Chinese patent drugs and health products because of its accelerated effects on drug efficiency and weight loss [4]. That seriously impacts on medicine and health care products market, but also causes great harm to human health.

Therefore, the trace analysis of BMTN with high sensitivity and selectivity is very desirable in monitoring its concentrations in the human urine samples and its quality of pharmaceutical formulations or biological fluids. Many analytical methods, such as capillary electrophoresis-amperometric detection (LD=1.5 ng mL^{-1}) [5], high performance liquid chromatography-fluorescence spectro-

photometry (LD=0.22 ng mL^{-1}) [6], gas chromatographic-mass spectrometry (LD=0.70 ng mL^{-1}) [7], automated flow injection fluorescence spectrophotometry (LD=0.70 ng mL^{-1}) [8], liquid chromatography-tandem mass spectrometry (LD= 10.0 ng mL^{-1}) [9], capillary electrophoresis-amperometric detection [10] and in-situ analysis by optic fiber sensor [11], have been developed for the determination of BMTN in tablets and human urine samples. Unfortunately, some of these methods lack adequate detect ability (require large amounts of sample), some are time-consuming or costly, and others just have low sensitivity and long run times which are not suitable in all conditions.

In our prior study, we successfully determined the content of clenbuterol hydrochloride [12] in human serum, which showed the high sensitivity and selectivity of catalytic SS-RTP. Thus, in the present work we propose a new ultra-sensitive method of inhibition SS-RTP for the determination of trace BMTN has been established based on the reaction between Fe^{3+} and R at pH 3.26, whose formation procedure was inhibited by BMTN and therefore led to the severe quenching of RTP signal. The experimental results indicate that the quenched RTP intensity was proportional to the concentration of BMTN over a wide range and LD was at femtogram level. Various parameters for the determination of BMTN have been evaluated and conditions have been optimized for its determination in human urine samples. Additionally, the establishment of the new method and the application and study of interaction mechanism could drive the study process of exhilarant detection to a certain extent, and had important research value and broad application prospects to the maintenance of equity of sports matches and the study of rudimental analysis.

Experimental

Apparatus

Phosphorescent measurements were carried out on a Perkin-Elmer LS-55 luminescence spectrophotometer with a solid surface analysis apparatus (Norwalk, CT 06859-0243, USA). The instruments' main parameters were as follows: delay time: 0.1 ms; gate time: 20 ms; cycle time: 20 ms; flash count: 1.0; Ex. Slit: 10 nm; Em. Slit: 10 nm; scan speed: 1,500 nm min^{-1}. The acidity of all the systems was measured by the pHS-3B precision acidometer and 80-2 centrifuge (Shanghai Surgical Instruments Factory) was adopted for sample treatment. All the materials were weighed by AE240 electron analytical balance (Mettler Toledo Instruments Company). A 0.50-μL flat head micro-injector (± 0.01 μL, Shanghai Medical Laser Instrument Plant) was used to introduce solution.

Reagents

Stock solution of BMTN (1.0 mg mL^{-1}) was prepared by dissolving 0.0100 g of BMTN standard (Sigma Aldrich, Germany) in anhydrous ethanol solution and was diluted to 10.0 mL. The solutions remained stable for at least one week if kept refrigerated and protected from light. Working standard solutions with BMTN concentrations ranging from 0.010 pg mL^{-1} to 10.0 pg mL^{-1} were daily prepared by the appropriate dilution of stock solutions with the same solvent. A 1.0×10^{-2} mol L^{-1} stock standard solution of R was made by dissolving 0.6765 g of the compound (Merck, China) in 95% ethanol, and then diluted to 200 mL with 95% ethanol

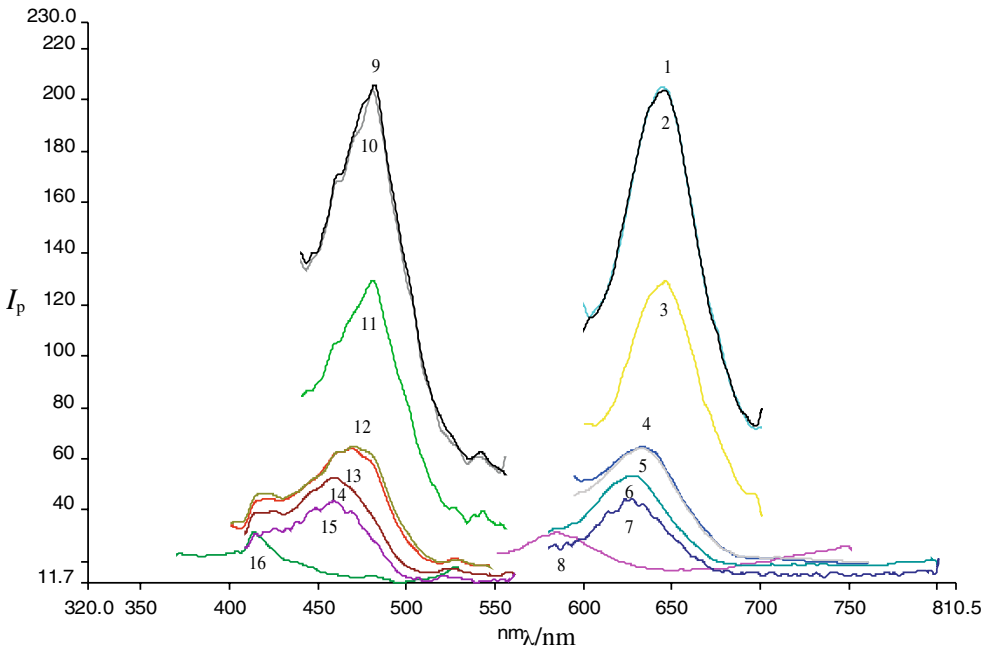


Fig. 1 RTP spectra for the R-$FeCl_3$-PA-HCl buffer-BMTN system

Table 1 RTP characteristics of R-$FeCl_3$-PA-HCl buffer-BMTN system

Curves of the system	λ_{ex}^{max} (nm)	λ_{em}^{max} (nm)	$\Delta\lambda$ (nm)	I_p	ΔI_p
1.1' 4.4' + 1.00 mL $FeCl_3$	481.3	643.6 (λ_2)	10.7 (λ_2–λ_1)	204.8 (I_{p2})	
2.2' 1.1 + 1.00 pg BMTN	480.0	645.0		203.2 (I_{p1})	1.6 (I_{p2}–I_{p1})
3.3' 1.1' + 100.0 pg BMTN	480.3	646.2		129.3 (I_{p1})	75.5 (I_{p2}–I_{p1})
4.4' 2.00 mL R + 1.50 mL PA-HCl	469.3	632.9 (λ_1)		64.6 (I_{p3})	140.2 (I_{p2}–I_{p3})
5.5' 2.00 mL R+ 1.50 mL PA-HCl + 100.0 pg BMTN	468.3	632.0		64.2	
6.6' 1.50 mL PA-HCl + 100.0 pg BMTN	458.7	627.9		53.3	
7.7' 6.6' + 1.00 mL 1×10^{-4} mol L^{-1} $FeCl_3$	457.8	626.9		44.5	
8.8' Paper	413.3	584.3		31.3	

$\Delta\lambda$ is the change of emission wavelength after the formation of $[FeR]^{3+}$. $\Delta\lambda=\lambda_2-\lambda_1$. ΔI_p is the change of the RTP intensity. $\Delta Ip=I_{p2}-I_{p1}$ or $\Delta I_p=I_{p2}-I_{p3}$

in a calibrated flask. 0.2705 g $FeCl_3\cdot6H_2O$ was dissolved in appropriate amount of dilute hydrochloric acid solution (hydrochloric acid: water=1:40) and was diluted to 100 mL. Then the solution was mixed homogeneously and was filtered through a G-4 funnel to collect the filtrate and a 1.00×10^{-2} mol L^{-1} $FeCl_3$ solution was obtained. The 0.050 mol L^{-1} phthalic acid-hydrochloric acid buffer (PA-HCl, pH 3.2) was prepared by dissolving 10.21 g phthalic acid in about 250 mL of water, adjusting the pH to 3.2 with 0.2 mol L^{-1} HCl, and completing the volume to 1,000 mL with water. And 1.0 mol L^{-1} I^- solution was also used as the ion perturber. All the reagents are A.R. grade except that BMTN is primary standard regent. All solutions were prepared in water purified by thrice quartz sub-boiling distillation.

Filter paper was purchased from Xinhua Paper Corporation (Hangzhou, China); polyamide membrane (PAM), acetylcellulose membrane (ACM) and nitric acid cellulose membrane (NCM) were purchased from Luqiaosijia Biochemical Plastic Plant. The paper sheets were pre-cut into wafers (Φ=1.5 cm) and a ring indentation was made at the center of the strip with a standard pinhole plotter (Φ= 4.0 mm) for used.

Preparation of human urine samples

Ten healthy male volunteers, age at 20.5±1.2 years and weight of 57.6±4.6 kg and no history of low blood pressure, heart, liver or kidney diseases, were banned from alcohol and tobacco, did not use any drugs before and during tests. All of them took 1 mg BMTN (purchased from Beijing Yanjing Pharmaceutical Co., Ltd., the batch number was 090523. All administrations were according to the principle of Public Health Bureau of China) with 100 mL warm water. They could drink and eat after medication for 2 h and 4 h respectively. Urine samples were collected before the medication (0 h) and after the medication for 0.25 h, 0.5 h, 0.75 h, 1.0 h, 1.25 h, 1.50 h, 1.75 h, 2.0 h, 3.0 h, 4.0 h, 6.0 h, 8.0 h, 10.0, 12.0, 24.0 h (each sample was 5 mL). And then they were stored in the refrigerator at 0 °C. Before analysis, samples were thawed at room temperature and centrifuged for 15 min at 4,000 r/min to remove protein and other impurity deposits. The solution was diluted to 10^3 times before use.

Experimental methods

To a 25 mL colorimetric tube, a certain amount of BMTN working solution, 2.00 mL R, 1.50 mL PA-HCl buffer and 1.00 mL $FeCl_3$ were added, diluted to 25 mL with water, and then mixed homogeneously. The colorimetric tube was heated at 100 °C for 15 min, and then cooled by flowing water for 5 min. The paper prepared was immersed in 1.0 mol L^{-1} I^- solution for 10 s and then dried at 90±1 °C for 2 min. 0.40 μL of test solution was suspended onto the center of paper by a 0.50-μL flat head micro-injector and

Table 2 The optimization of basic instrumental parameters

Basic instrumental parameters	Conditions	The ΔI_p in R-$FeCl_3$-buffer solution-BMTN system	Optimal
Delay time (ms) RSD (%)	0.050, 0.10, 0.30, 0.50, 0.70	22.4, 38.9, 29.3, 20.5, 15.7 1.5, 1.0, 1.2, 1.8, 2.3	0.10
Gate time (ms) RSD (%)	1.0, 1.5, 2.0, 2.5, 3.0	15.8, 23.9, 38.6, 27.5, 16.2 2.4, 1.4, 1.1, 1.6, 2.2	2.0
Cycle time (ms) RSD (%)	10, 15, 20, 25, 30	13.2, 20.6, 37.9, 23.5, 17.0 2.5, 1. 9, 1.0, 1.6, 2.1	20
Flash count (ms) RSD (%)	1.0, 1.5, 2.0, 2.5, 3.0	38.3, 29.0, 20.7, 14.6, 8.9 1.1, 1.2, 1.8, 2.4, 4.1	1.0

RSD Relative standard deviation

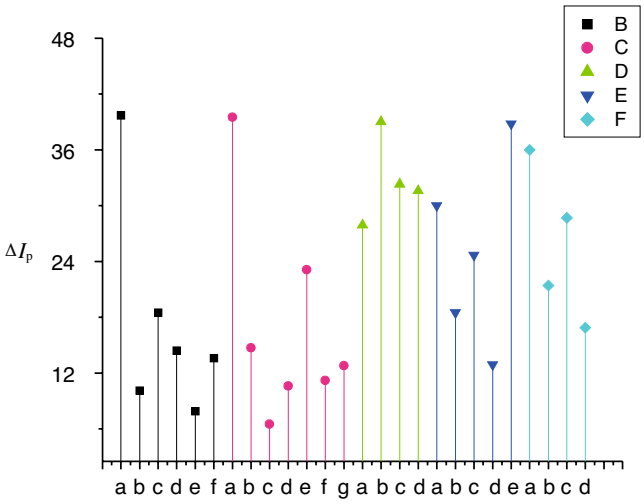


Fig. 2 The optimization of the system

then the paper was dried at 90±1 °C for 2.5 min. At the same time, a blank test was also conducted. The phosphorescence intensity of test solution (I_{p1} of R-$FeCl_3$-PA-HCl-BMTN system) and reagent blank (I_{p2} of R-$FeCl_3$-PA-HCl system) were directly measured at 480/646 nm (λ_{ex}^{max}/λ_{em}^{max}). Then ΔI_p (= I_{p2}–I_{p1}) was calculated.

Sample preparation and the infrared spectra analysis

To both of two 25-mL colorimetric tubes, 2.00 mL R and 1.50 mL PA-HCl were added, meanwhile 1.00 mL $FeCl_3$ was added to one of them, diluted with water. Then, the two tubes were heated in water bath at 100 °C for 15 min, cooled by flowing water for 5 min. Transferred to separatory funnels, R and $[FeR]^{3+}$ were respectively extracted with 9.00 mL ethanol for three times. The extracts were collected, evaporated, dried in vacuum, finally R and $[FeR]^{3+}$ complex powders were obtained.

To another two 25-mL colorimetric tubes, 100.0 pg BMTN, 1.50 mL PA-HCl and 1.00 mL $FeCl_3$ were added, diluted with water. One of the tubes was heated in water bath at 100 °C for 15 min, cooled by flowing water for 5 min. Then, both of the two solutions were respectively extracted according to the measure described above. The extracts were collected, evaporated, dried in vacuum, finally BMTN and BMTN' powders were obtained.

Table 3 Optimization of measurement condition

Reagents	Conditions	The ΔIp in R-$FeCl_3$-buffer solution-BMTN system	Optimal
R (mol L^{-1}) RSD (%)	10^{-1}, 8×10^{-2}, 4×10^{-2}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}	32.7, 35.0, 36.2, 38.8, 30.9, 22.5, 16.3 1.5, 1.4, 1.2, 1.0, 1.2, 1.5, 2.2	10^{-2} mol L^{-1}
(mL) RSD (%)	0.10, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00	14.1, 20.5, 27.6, 33.7, 39.4, 36.3, 31.2 3.0, 2.1, 1.8, 1.3, 1.1, 0.9, 1.9	2.00 mL
$FeCl_3$ (mol L^{-1}) RSD (%)	10^{-3}, 5×10^{-4}, 10^{-4}, 10^{-5}, 10^{-6}	29.8, 34.3, 36.4, 28.1, 19.5 2.2, 1.4, 1.1, 1.2, 1.8	10^{-4} mol L^{-1}
(mL) RSD (%)	0.10, 0.50, 0.70, 1.00, 1.50, 2.00	13.0, 22.3, 30.7, 36.9, 34.1, 31.5 2.9, 1.9, 1.0,1.8, 2.1, 2.7	1.00 mL
I^-(mol L^{-1}) RSD (%)	0.050, 0.10, 0.50, 1.00, 1.50	8.2, 15.7, 23.8, 37.5, 32.6 4.2, 2.6, 2.1, 1.2, 1.3	1.00 mol L^{-1}
pH RSD (%)	2.85, 3.26, 3.43, 3.85, 4.52, 6.30, 7.56	29.9, 38.1, 37.8, 37.6, 37.3, 25.4, 18.0 1.3, 1.1, 1.2, 1.1, 1.3, 1.4, 1.9	3.26–4.52
PA-HCl (mL) RSD (%)	0.50, 1.00, 1.50, 2.00, 2.50	18.7, 25.9, 35.7, 33.4, 29.1 1.6, 1.3, 1.1, 1.4, 1.8	1.50 mL
DTEM (°C) RSD (%)	80, 85, 90, 95	18.9, 35.1, 37.4, 33.5 1.5, 1.3, 1.2, 1.4	90 °C
DT (min) RSD (%)	0.5, 1.0, 1.5, 2.0, 2.5, 3.0	24.7, 32.0, 34.1, 36.2, 37.8, 37.0 1.7, 1.5, 1.6, 1.2, 1.1, 1.3	2.5 min
RTEM (°C) RSD (%)	50, 60, 70, 75, 80, 85, 90, 95, 100	17.6, 20.1, 24.4, 27.2, 30.0, 31.9, 33.7, 35.8, 38.5 2.3, 2.0, 1.8, 1.5, 1.6, 1.3, 1.4, 1.1, 1.2	100 °C
RT (min) RSD (%)	5, 7, 10, 12, 15, 20, 25	15.8, 19.2, 29.5, 35.6, 40.0, 36.9, 32.2 2.4, 2.1, 1.8, 1.5, 1.0, 1.4, 2.4	15 min
PT of N_2 (min) RSD (%)	5, 10, 20, 30	37.0, 37.3, 37.2, 37.5 1.2, 1.1, 1.3, 1.0	10 min
No PT of N_2 (min)	5, 10, 20, 30	32.4, 28.6, 30.1, 25.3	
RSD (%)		1.5, 1.7, 1.6, 1.7	
ST (min) RSD (%)	5, 10, 20, 30, 40, 50, 60	39.2, 38.9, 38.5, 38.0, 37.6, 25.7, 16.1 1.0, 1.1, 1.2, 1.1, 1.2, 1.7, 2.4	5–40 min

Samples were prepared by the potassium bromide disk method, and the infrared spectra (IR) of R, $[FeR]^{3+}$ complex, BMTN and BMTN' were scanned.

Results and discussion

Phosphorescence spectra

The phosphorescence spectra of R-$FeCl_3$-PA-HCl buffer-BMTN system were scanned by experimental method (Fig. 1). Results show that after being heated at 100 °C for 15 min, R could only emit weak RTP ($\lambda_{ex}^{max}/\lambda_{em}^{max}$=469.3/632.9 nm, I_p=64.6) on the filter paper with 1.00 mol L^{-1} I^- as the ion perturber. However, in the presence of Fe^{3+}, the system's RTP signal was enhanced strongly ($\lambda_{ex}^{max}/\lambda_{em}^{max}$= 481.3/643.6 nm, I_p=204.8, ΔI_p=140.2) with the red shift for 10.7 nm, indicating that R might complex with Fe^{3+} to form $[FeR]^{3+}$. And when 100.0 pg BMTN was added, the RTP of the system quenched sharply ($\lambda_{ex}^{max}/\lambda_{em}^{max}$=480.0/645.5 nm, I_p=129.3, ΔI_p=75.5). It implies that BMTN was oxidized to BMTN' by Fe^{3+} while Fe^{3+} was reduced to Fe^{2+}, which inhibited the complex reaction between R and Fe^{3+}. The inhibition effect of BMTN led to the change of RTP intensity (ΔI_p), providing the possibility for determining BMTN by inhibited SS-RTP. In this procedure, $\lambda_{ex}^{max}/\lambda_{em}^{max}$ of the complex $[FeR]^{3+}$ remained unchanged, so 480/646 nm was chosen as the working wavelength (Table 1).

Optimum condition

Instrumental parameters

For the system containing 0.80 fg BMTN $spot^{-1}$, the effects of instrumental parameters (delay time, gate time, cycle time, and flash count) on the ΔI_p of the system were studied and listed in Table 2. And we can see that the value of ΔI_p reached the maximum when the instrumental parameters were delay time 0.10 ms, gate time 2.0 ms, cycle time 20 ms, and flash count 1.0 ms.

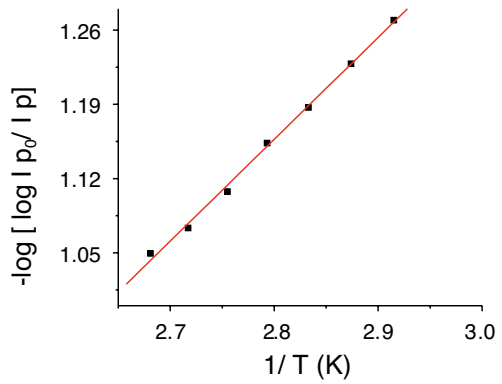


Fig. 3 The correlation of $-\log[\log I_{p0}/I_p]$ and 1/T

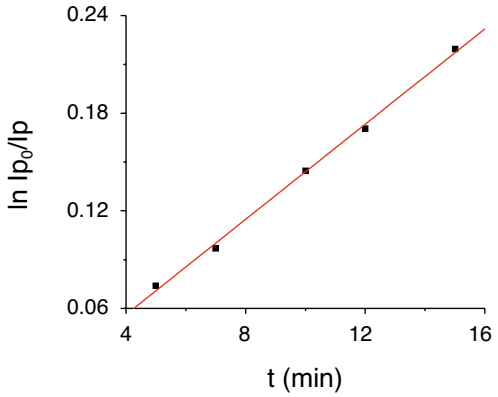


Fig. 4 The correlation of ln I_{p0}/I_p and t

Optimization of working condition

For the system containing 0.80 fg BMTN $spot^{-1}$, the optimization of luminescent substances, oxidants, ion perturbers and solid substrates were done in the experiment (Fig. 2), besides, the effects of the volumes and concentrations of reagents, reaction pH, time and temperature for reaction and desiccation, oxygen and stable time on the ΔI_p of the system were also studied, respectively (Table 3). And it was seen that the ΔI_p of the system was the maximum when 2.00 mL 1.0×10^{-2} mol L^{-1} R, 1.00 mL 1.0×10^{-4} mol L^{-1} $FeCl_3$, 1.50 mL pH 3.26 PA-HCl, 1.00 mol L^{-1} I^-, paper were selected, and the reaction temperature and time were 100 °C and 15 min, the test samples were desiccated at 90 °C for 2.5 min and the passing time of desiccated N_2 was 5–30 min. Under the optimal conditions above, the ΔI_p of the system almost stayed invariable and had good repeatability within 40 minutes after being cooled by flowing water for 5 min in the system.

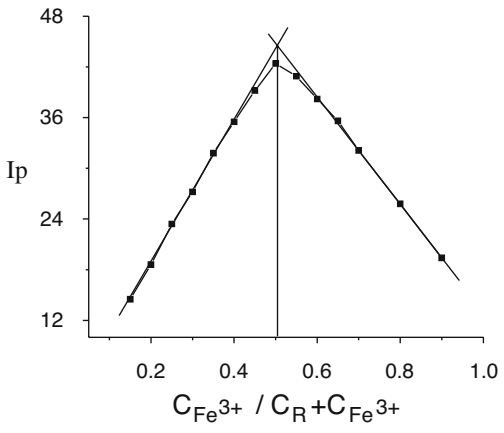


Fig. 5 Equimolar series method

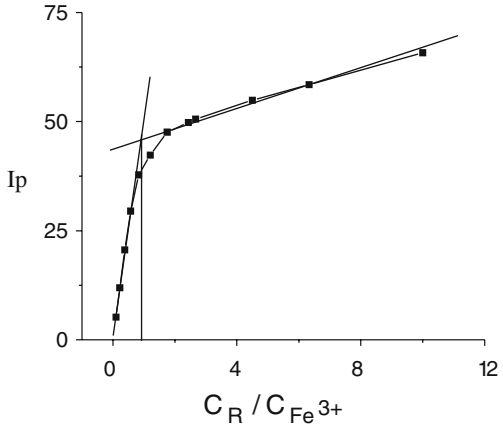


Fig. 6 Molar ratio method

Kinetic constants

For the system containing 0.80 fg BMTN spot^{-1}, 1/T was positively correlated with $-\log[\log I_p/I_{p0}]$ in the range of 35–100 °C (Fig. 3), and the regression equation was $-\log\left[\log I_p/I_{p0}\right] = -1.530 + 0.9597 \times 1000/\mathrm{T}$, R^2 was 0.9972, when T was 373 K, E was 21.39 kJ mol^{-1} calculated by k× (1/ T)×1000×8.314. Meanwhile, t was linear with $\ln I_{p0}/I_p$ in the range of 5–15 min (Fig. 4), and the regression equation was $\ln I_{p0}/I_p = -0.002350 + 0.01464$ t (min), R^2 was 0.9976, and when t was 15 min, k was 2.44×10^{-4} s^{-1} calculated by $1/t\times\ln I_p/ I_{p0}$.

The component of the complex

Under the optimum conditions described above, the component of the complex was determined by the equi-molar ratio continuous variation method and the molar ratio method [13] (Figs. 5 and 6). Results show that the value of I_p increased gradually along with increasing C_{Fe3+} / (C_R + C_{Fe}^{3+}). When it reached the maximum, the value of I_p reduced gradually along with increasing C_{Fe3+} /(C_R + C_{Fe}^{3+}). According to the coordinate of intersection point, the ratio for C_{Fe}^{3+} /(C_R + C_{Fe}^{3+}) was 0.5 (Fig. 5). The value of I_p increased gradually along with increasing C_{Fe}^{3+} / C_R, and then it tended to stability. According to the coordinate of intersection point, we could see that the ratio for C_{Fe}^{3+} / C_R was 1 (Fig. 6). Results indicate that the mole ratio in the complex for Fe^{3+} : R was 1 : 1, so the complex could be presumed as $[FeR]^{3+}$, which was coincident with Ref. [14].

Working curve, linear range and LD

The linear range, the regression equation of working curve, correlation coefficient (r), RSD% (eight fold replicate measurements for 0.016 fg and 1.6 fg BMTN spot^{-1}) and LD (calculated by 3Sb/k, which refers to the quotient between triple of the blank reagent's standard deviation and the slope of the working curve, Sb=0.074, n=11) were compared with Ref. [5, 6, 8]. Results are listed in Table 4.

Results show that in comparison to previous studies, the LD of this method was brought down to pg mL^{-1} level, thus achieved a great improvement. The precision of the method was checked and calculated to be less than 4.2%, which was very satisfactory. All these indicate that this method was better than Ref. [5, 6, 8], indicating its applicability for the determination of ultra-trace BMTN.

Interference study

Under the optimum experimental conditions, the effects of coexisted materials on the determination of 2.0 pg BMTN mL^{-1} were studied in this method. When the recovery was 100±5%, the allowed concentration of coexistence ions (matter) compared with Ref. [8] (20 μg BMTN mL^{-1}) is listed in the Table 5. The results reveal that none of the common excipients shown in Table 5 caused any serious interference with the proposed methods, suggested that BMTN could be successfully detected in real urine samples without the interference of the common excipients, high salt, proteins and endogenetic compounds. And the tolerable concentration of this method was higher than that of

Table 4 Analysis parameters

Method	Linear range	Regression equation	r	RSD (%)	LD (gmL^{-1})	QD (gmL^{-1})	Ref
This method	0.040–4.0 (pg mL^{-1})	ΔI_p=1.851+44.35 m_{BMTN} (fg spot^{-1}) (n=6)	0.9953	0.77–4.2	1.2×10^{-14}	4.0×10^{-14}	
CE-AD	0.0050–12.9 (μg mL^{-1})	y=0.812x+0.0761 (n=5)	0.9999	2.0	1.5×10^{-9}	5.0×10^{-9} (3.33×LD)	[5]
HPLC-FS	16.75–335.00 (μg mL^{-1})	y=1.3889x−0.0196	0.9997	0.49	2.2×10^{-10}	7.3×10^{-9} (3.33×LD)	[6]
AFIFD	0.05–10.0 (μg mL^{-1})	y=95.09x+4.65	0.9997	0.46	1.0×10^{-8}	3.3×10^{-8} (3.33×LD)	[8]

I_p is phosphorescent intensity. fg spot^{-1} is fg BMTN in per spot. y is absorbance, x is concentration. CE-AD is capillary electrophoresis-amperometric detection. HPLC-FS is high performance liquid chromatography-fluorescence spectrophotometry. AFIFD is automated flow injection fluorimetric determination

Table 5 Effects of excipients

Interference	Concentration of coexistent materials (μg mL^{-1})	This method recovery (%)	Er (%)	Method of Ref. [8] concentration of coexistent materials (μg mL^{-1})	Recovery (%)
Aerosil	4800	102.3	2.17	4200	103.3
Agar	4400	101.9	1.79	4000	103.2
Avicel	4500	104.5	4.49	4000	103.0
Lactose	4400	100.7	0.70	4000	102.8
Magnesium stearate	4400	97.6	–2.40	4000	100.8
Maize starch	4600	100.5	0.49	4000	98.9
Polyvinylpyrrolidone	4800	101.4	0.39	4200	99.3
Talcum	4500	98.2	–1.80	4000	100.6
Titanium dioxide	5000	103.9	3.9	4400	103.3
Tween 80	3300	102.7	2.70	2600	100.2
High salt	2500	100.5	0.49	–	–
Proteins	3600	98.4	–1.60	–	–
Endogenetic compounds	2800	101.7	1.70	–	–

"–" represents no results detected

Ref. [8], indicating higher selectivity of the inhibition reaction of this method.

Analytical application

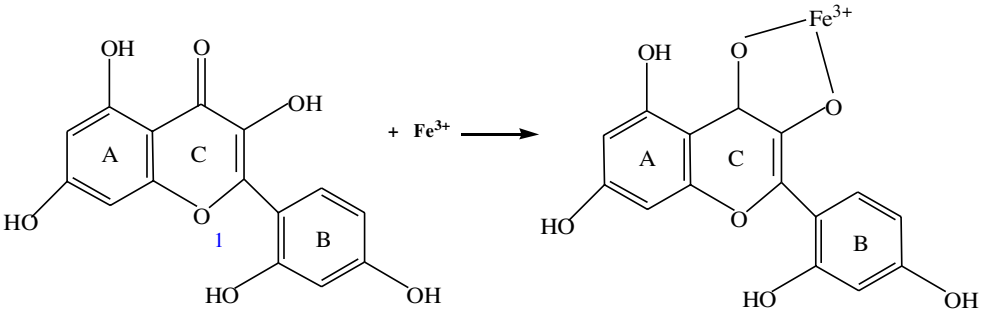
The proposed method was applied for the determination of BMTN in the 1.00 mL solution prepared in section "Preparation of human urine samples". The results showed in Table 6 reveals that they are in good agreement with those obtained by UV spectrophotometry [15] (measured at 328 nm, LD was 0.47 ng mL^{-1} according to the absorption coefficient of BMTN was 98.5).

From Table 6, we can see that the results of this method coincided with those of UV spectrophotometry with Er in the range of −2.7–4.5 (%) and RSDs within 0.2–3.4 (%), indicating that this method has higher accuracy and precision. In addition, it has been reported that metabolic half life of BMTN in human plasma was 1 h [16], so if plasma samples were used as the testing samples, the procedure would be limited by time. As can also be seen

Table 6 Determination of BMTN in the human urine samples (n=5, Time for adminstration: TAA)

TAA (h)	This method						UV spectrophotometry	
	Found (ngmL^{-1})	Added (ngmL^{-1})	Obtained (ngmL^{-1})	Recovery (%)	RSD (%)	Er (%)	Found (ngmL^{-1})	RSD (%)
0	0	4.00	3.94	98.5	0	0	0	0
0.25	0.90	3.00	3.92	100.5	3.2	3.3	0.87	−1.3
0.5	1.21	2.50	3.67	98.9	2.8	−1.6	1.23	2.5
0.75	1.86	2.00	3.85	99.7	3.4	1.1	1.84	1.9
1.0	2.20	1.50	3.77	101.9	2.4	4.5	2.10	2.7
1.25	3.32	1.00	4.29	99.3	2.9	−2.7	3.41	−2.1
1.50	2.94	0.50	3.43	99.7	1.1	−1.7	2.99	1.7
1.75	2.05	0.10	2.08	96.7	3.9	−2.4	2.10	1.2
2.0	1.52	2.50	4.00	99.5	1.7	−2.0	1.55	2.2
3.0	1.08	2.50	3.57	99.7	2.7	−0.93	1.09	1.8
4.0	0.80	2.00	2.81	100.3	2.1	−2.5	0.82	−2.8
6.0	0.60	1.50	2.13	101.4	2.7	1.7	0.59	−1.1
8.0	0.48	1.00	1.45	98.0	1.1	2.1	0.47	1.5
10.0	0.21	0.50	0.67	94.4	2.4	–	–	1.9
12.0	0.11	0.50	0.59	96.7	1.3	–	–	2.3
24.0	0.079	0.50	0.55	95.0	0.2	–	–	−1.9

Fig. 7 The formation of $[FeR]^{3+}$ complex



632.9 nm 643.6 nm

from the table, the content of BMTN in urine sample can still be accurately detected in 24 h. Therefore, that contributes more to the identification of BMTN in urine, but also detects the ultra-trace BMTN accurately, which is of great significance for the maintenance of the fair competition and human health.

The mechanism of inhibited SS-RTP

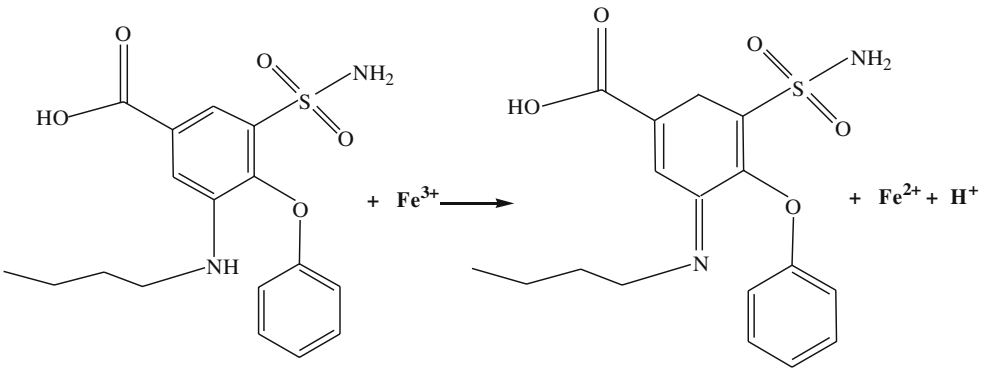
When the system was heated at 100 °C for 15 min, R emitted weak RTP signal on the surface of paper using 1.0 mol L^{-1} I^- solution as the ion perturber (Fig. 1, Cur. 2.2'). In the presence of BMTN, RTP of R just changed slightly (Fig. 1, Cur. 5.5'), indicating no reaction occurred between BMTN and R. However, the system could emit strong and stable RTP signal when Fe^{3+} was added (Fig. 1, Cur. 1.1'), even the $\lambda_{em}{}^{max}$ had a red shift for 10.7 nm, indicating a new substance formed. According to the conclusion of section "The component of the complex", the new substance was $[FeR]^{3+}$ and the formation reaction could be expressed as follows (Fig. 7) [14]:

From the main IR characteristic absorption peaks (cm^{-1}): R (υ O-H 3272, υ C = O 1658, υ C-C 1620, υ C-O-C 1168, υ C-OH 1369,δ C-H 832), $[FeR]^{3+}$ complex (υ O-H 3272, υ C-O 1632, υ C-C 1610, υ C-O-C 1168, υ C-OH 1362, δ C-H 832), it can be seen that stretching vibration absorption of C-O-C bond of R was at 1,168 cm^{-1} without any change, excluding the possibility of Fe^{3+} coordinating with O_1. While the stretching vibration absorption peak of carbonyl at 1,658 cm^{-1} disappeared, instead the stretching vibration absorption peak of C-O bond appeared at 1,632 cm^{-1}, showing coordination role of carbonyl. And all of these further proved the possibility of the $[FeR]^{3+}$ complex formed by R and Fe^{3+}.

When 100.0 pg BMTN was added to the system, the RTP signal of the system quenched ($\lambda_{ex}{}^{max}/\lambda_{em}{}^{max}$=480.0/645.5 nm, I_p=129.3), it might be explained that Fe^{3+} was reduced into Fe^{2+} while it oxidized BMTN to BMTN'[17]. As a result, the decreasing of the content of Fe^{3+} inhibited the formation of $[FeR]^{3+}$complex, leading to the quenching of RTP signal of the system (Fig. 8).

The main IR characteristic absorption peaks (cm^{-1}) of BMTN (υ COOH 1720, υ N-H 1620, υ NH_2 1580, υ C_6H_5-1500, υ CH 1340, υ COC 1225, υ CN 1160), and BMTN' (υ -C=N-2200, υ COOH 1720, υ C=C 1630, υ -NH_2 1580, υ -CH_2 1465, υ COC 1225) can be seen, υ NH 1620 and υ C_6H_5-1500 of BMTN disappeared, but 2200 cm^{-1}—C=N—, 1,630 cm^{-1} C = C and 1,465 cm^{-1}—CH_2 appeared, thus they show the further evidence of the benzene ring on the carbon being oxidized. This experiment corroborated the fact that BMTN could be oxidized to BMTN' by Fe^{3+}. Therefore, BMTN can be determined by the inhibited SS-RTP.

Fig. 8 The oxidant reaction between Fe^{3+} and BMTN



BMTN BMTN'

Conclusion

A ultra-sensitive, accurate and selective SSRTP was established for determination of BMTN with its inhibition effect on $[FeR]^{3+}$ complex. This method was suitable for the residue analysis of trace BMTN in human body for its convenience and rapidness, providing new detection technique for maintaining human health and also driving the research progress of exhilarant detection technique and SS-RTP.

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