Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 854 (2007) 239–244
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of propylthiouracil and methylthiouracil in human serum using high-performance liquid chromatography with chemiluminescence detection

Yue Wei, Zhu-Jun Zhang *, Yan-Tu Zhang, Yong-Hua Sun

Department of Chemistry, Shaanxi Normal University, Xi'an 710062, China

Received 23 October 2006; accepted 22 April 2007
Available online 1 May 2007

Abstract

Based on the sensitizing effect of formaldehyde on the chemiluminescence (CL) reaction of propylthiouracil (PTU) and methylthiouracil (MTU) with acidic potassium permanganate and the combination technique of high-performance liquid chromatography (HPLC), a sensitive, selective and simple post-column CL detection method for determining PTU and MTU is described. The optimal conditions for the CL detection and HPLC separation were carried out. The linear ranges were 0.1–20 $\mu g\,mL^{-1}$ for MTU and 0.1–10 $\mu g\,mL^{-1}$ for PTU, the detection limits were 0.03 $\mu g\,mL^{-1}$ for PTU, 0.03 $\mu g\,mL^{-1}$ for MTU and the quantification limits were 0.1 $\mu g\,mL^{-1}$ for PTU, 0.1 $\mu g\,mL^{-1}$ for MTU. The method has been satisfactorily applied for the determination of MTU and PTU in human serum samples.

Keywords: HPLC; Chemiluminescence; Propylthiouracil; Methylthiouracil

1. Introduction

PTU (Fig. 1A) and MTU (Fig. 1B) are the drugs of thiouracil antithyroid family which block thyroid hormone synthesis and they are commonly used to treat hyperthyroidism in pregnancy and liver disease. On the other hand, PTU and MTU have been applied illegally to animals to obtain a higher live weight gain in some regions. The first effect gives a fraudulous higher weight ("water instead of meat") and the uncontrolled introduction of this and other thyreostats into the human food chain could have serious health implications. Consequently specific legislation has been promulgated within the European Union prohibiting the use of thyreostats in animal production [1,2]. Based on these reasons, the need for an analytical procedure capable of both identifying and quantifying PTU and MTU is very significative.

Several different analytical methods for the determination of PTU and MTU have been described such as Polarography [3,4], conductometric titration[5], potentiometric titration [5–7] and spectrophotometry [8,9], but these methods can only be applied in pharmaceutical preparations due to their lower sensitivity or lack of selectivity. In biological fluids, they have been determined by liquid chromatography [10–11], radioimmunoassay [12] and gas–liquid chromatography [13]. Although these methods have their respective advantages, but some different shortcomings also exist. A gas–liquid chromatographic technique, which involves the conversion of PTU to its salt form with tetrapropylammonium hydroxide is time consuming and technically difficult and radioimmunoassay are very laborious.

In recent years, chemiluminescence (CL) has become an attractive detection method for liquid chromatography due to its high sensitivity, selectivity and wide linear working ranges. And to our knowledge, the method of HPLC with chemiluminescence detection for determination of PTU and MTU has not been published until now.

Acidic potassium permanganate is one of the most important oxidants utilized in chemiluminescent reactions and considerable numbers of the investigations have been published [14–16]. In our study, we found that only weak CL signal was generated by the oxidation of PTU or MTU with acidic potassium permanganate, which can't be applied to the HPLC–CL because of its inferior sensitivity. However, in the presence of HCHO, the CL

* Corresponding author. Tel.: +86 29 85308748; fax: +86 29 85307774.
E-mail addresses: weiyue_2005@hotmail.com (Y. Wei), zzj18@hotmail.com (Z.-J. Zhang).

1570-0232/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2007.04.028

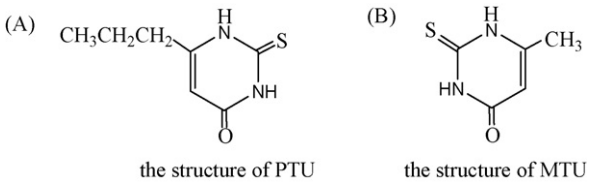

Fig. 1. The structure of (A) PTU and (B) MTU.

emission intensity can be enlarged to about 15 times; therefore, we established a new method based on the combination technique of HPLC with the CL reaction of MTU and PTU with acidified potassium permanganate and the sensitizing effect of formaldehyde on the CL system. And the method was validated and applied to determine MTU and PTU in biological samples successfully.

2. Experimental

2.1. Chemicals and solutions

All chemicals were of analytical reagent grade unless otherwise specified and distilled water was used throughout. Methanol (MeOH) and water (HPLC grade) were from Fisher Scientific (Fair Lawn, NJ, USA).

PTU and MTU were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Stock standard solutions of PTU and MTU were prepared in methanol at 2 mg mL^{-1}. The stock solutions were diluted with the mobile phase before use.

Potassium permanganate, formaldehyde and sulfuric acid were obtained from Xi'an Chemical Reagent Factory (Xi'an, China).

A stock standard solution of potassium permanganate 0.01 M was prepared daily by dissolving 158.0 mg of $KMnO_4$ in 100 mL of water; A formaldehyde stock solution (10%, v/v) and a sulfuric acid solution (1.0 M) were also prepared.

The HPLC mobile phases were prepared fresh daily, filtered through a 0.45 μm filter (Xinya, Shanghai, China), and then degassed prior to use.

2.2. Apparatus

Batch model IFFM-D luminescence analyzer (Ruimai Company, Xi'an, China) was employed to study the characteristics of the CL reaction. The flow manifold finally proposed for the HPLC–CL detection is schematically illustrated in Fig. 2. The HPLC system consisted of a model high-pressure pump (LC-6A, Shimadzu, Japan), a manual sampling valve injector with a 20 μL loop, an analytical column (Nucleosil C_{18}, 250 mm × 4.6 mm i.d., 5 μm, Macherey-Nagel, Germany). The CL detection was conducted on a flow injection chemiluminescence system comprising of a peristaltic pump (Ruimai Company, Xi'an, China), PTFE tubing (0.8 mm i.d.) which was used as connection material in the flow system and a glass spiral type flow cell. The change of CL signal in the flow cell was detected and recorded with a computerized IFFM-D luminescence analyzer. Data acquisition and treatment were performed with IFFM software running under Windows XP.

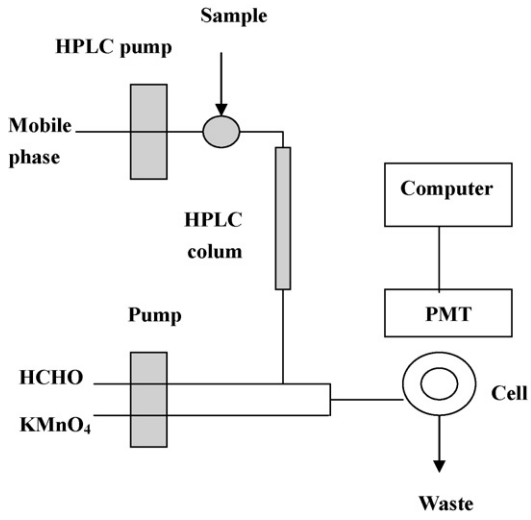

Fig. 2. Schematic flow diagram of HPLC–chemiluminescence detection system.

2.3. Sample preparation

The serum samples were collected from a patient, after he took orally 200 mg of propylthiouracil and 200 mg of methylthiouracil for some time, and the serum samples were collected in tubes. The extraction procedure, modified from the literature [11,17], consisted of addition of 4 mL of dichloromethane to 0.5 mL of serum (pH 6.0 adjusted with 10% HCl). Mixtures were then submitted to vortex-mixing for 10 min. After centrifugation at 3000 r min^{-1} for 10 min, the organic phase of 3.8 mL was carefully transferred to conical tubes and evaporated to dryness in a water bath at about 40 °C; the residue was dissolved in mobile phase. After mixing for 30 s, 20 μL aliquots of the solution were taken for chromatographic analysis directly. The samples used in the recovery studies were fortified by the addition of a known concentration of PTU (MTU) in the mobile phase (0.5, 1, 2 μg mL^{-1}).

2.4. Standard solutions and calibration

A standard stock solution, containing the two drugs, was prepared at a concentration of 1 mg mL^{-1} of each compound in methanol, and was kept stable at −20 °C. Several tubes were taken, different amounts of standard solution of PTU (MTU) were added to 0.5 mL drug-free serum in order to get the concentrations of 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 and 100 μg mL^{-1} of PTU (MTU), and then the above method was followed to cope with human samples. Volumes of 20 μL were then chromatographed as described. The calibration curve was obtained by linear regression of the peak–height ratio.

2.5. Procedure

Separation of thiouracil antithyroid drugs was carried out on a Nucleosil C_{18} column with an isocratic elution program at

a flow rate of 1 mL min^{-1}. The mobile phase was the mixture of methanol and water (40:60, v/v) and the volume of sample injected was 20 μL in all instances (blanks, standards and samples). As shown in Fig. 2, the solutions of HCHO first reacted with the effluent from the HPLC column and then mixed with solutions of $KMnO_4$ in a mixing coil in the flow cell by a peristaltic pump at a flow rate (per tube) of 2 mL min^{-1}. The emitted light was monitored by the photomultiplier tube (operated at −800 V). The quantitative determination was based on the net CL intensity $\Delta I = I_s - I_0$, where I_S was the CL intensity in the presence of antithyroid drugs and I_0 was the intensity in the absence of antithyroid drugs.

3. Results and discussion

3.1. Optimization of the separation system

As for HPLC–CL detection, the composition of the mobile phase and its flow rate were optimized as a compromise between the resolution and the CL intensity. In our experiment, acetonitrile and methanol have been examined as the organic part of the mobile phase. Although acetonitrile–water and methanol–water could almost completely separate PTU and MTU with an isocratic elution program, the use of mobile phase containing acetonitrile greatly quenched the CL signal and produced serious background noise while methanol produced a lower background emission and its baseline was fairly smooth, the results showed methanol–water was chosen as the mobile phase for the further study. The concentration of methanol in the mobile phase was initially optimized by varying the methanol–water mixture ratio in the range 25–50% at a constant flow rate of 1 mL min^{-1}, and 40% methanol was proved to be more effective on the separation.

3.2. Optimization of the CL system

TO obtain the maximal relative CL intensity, the concentration of $KMnO_4$, HCHO and sulfuric acid were investigated. We found that formaldehyde can enhance the CL emission intensity by the reaction of the studied PTU and MTU with $KMnO_4$ in sulfuric acid medium. It may be suggested that the HCHO accelerates the CL reaction between potassium permanganate and thiouracil antithyroid drug in acid medium, and increases the luminescence quantum efficiency [18].

The kinetic curve, CL, UV and the fluorescence spectra were examined in order to obtain more information about the enhanced CL mechanism.

The kinetic curve of the CL reaction was tested with a static system by using the reaction of PTU (MTU), $KMnO_4$ and HCHO in sulfuric acid solution. The typical CL kinetic curve was shown in Fig. 3. It can be seen from Fig. 3 that this CL reaction was so rapid that the CL intensity reached a maximum at 1.3 s. And it also indicated that the CL intensity of the reaction of acidic $KMnO_4$ with PTU (MTU) was enhanced in the presence of HCHO.

The CL spectrum generated from the reaction was examined by using a series of interference filters to obtain an idea about

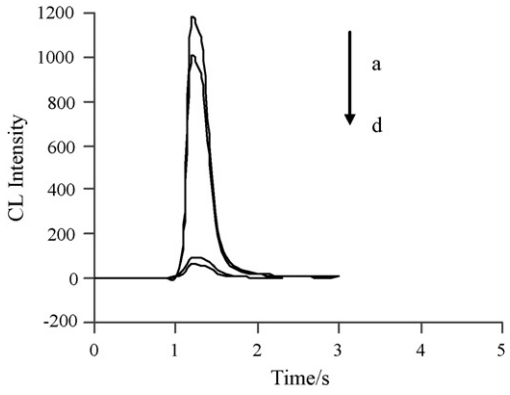

Fig. 3. Kinetic curves of chemiluminescence. (a) $KMnO_4$–H_2SO_4–PTU–HCHO, (b) $KMnO_4$–H_2SO_4–MTU–HCHO, (c) $KMnO_4$–H_2SO_4–H_2O–PTU, (d) $KMnO_4$–H_2SO_4–H_2O–MTU.

the reaction product and is shown in Fig. 4 which demonstrated that only formaldehyde enhanced the CL emission intensity and did not influence the CL spectra (λ_{max} = 630 nm), which revealed that the emitter is the same in presence and absence of formaldehyde. Therefore, it can be concluded that formaldehyde does not change the mechanism of the CL reaction.

The UV spectra of PTU and its oxidation products were measured and are shown in Fig. 5. For PTU, there are two characteristic absorption bands at 213 nm and 274 nm before addition of $KMnO_4$ (see Fig. 5a). However, the characteristic absorption bands at 213 nm and 274 nm are gradually shifted toward violet direction after addition of $KMnO_4$ and formaldehyde, at the same time, the absorption bands of $KMnO_4$ at 305 nm and 525 nm disappeared, which indicates that PTU is oxidized by $KMnO_4$ in the acidic solution.

The fluorescence spectra of PTU–H_2SO_4, $KMnO_4$–PTU–H_2SO_4 and $KMnO_4$–PTU–HCHO–H_2SO_4 were recorded in the range of 200–700 nm in static, respectively. But no fluorescence emissions were observed in the range of 250–700 nm. These results indicate that PTU and the product of oxidation of PTU by potassium permanganate are non-fluorescent compounds. It was shown that the enhanced CL did not result from oxidation of PTU by potassium permanganate and must be from the intermediate radical products.

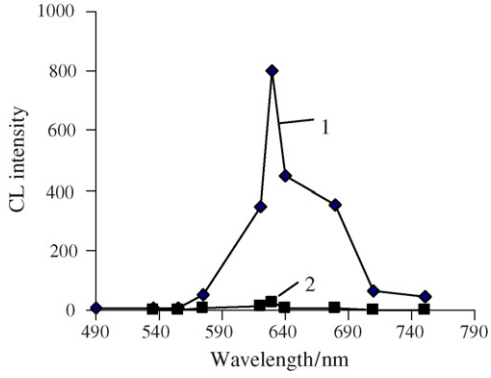

Fig. 4. The CL spectra curve (1): $KMnO_4$ 2.0×10^{-4} M, H_2SO_4 1 M, PTU 5.0×10^{-6} g mL^{-1}, HCHO 10% (v/v); (2): $KMnO_4$ 2.0×10^{-4} M, H_2SO_4 1 M, PTU 5.0×10^{-6} g mL^{-1}.

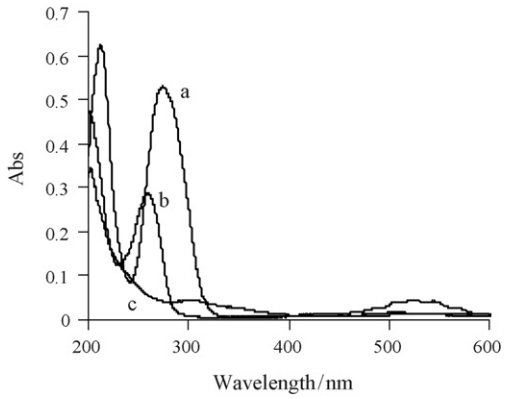

Fig. 5. The UV spectra curve (a) PTU5.0×10^{-6} g mL^{-1}, H_2SO_4 1 M; (b) $KMnO_4$ 2.0×10^{-4} M, H_2SO_4 1 M, PTU 5.0×10^{-6} g mL^{-1}, HCHO 10% (v/v); (c) $KMnO_4$ 2.0×10^{-4} M, H_2SO_4 1 M.

Because the peak of chemiluminescence spectrum is at 630–640 nm, which is very similar to that of singlet oxygen chemiluminescence (630–650 nm), we assumed that $KMnO_4$ could react with PTU in the presence of formaldehyde to produce $^1O_2{}^1O_2$ ($^1\Delta_g{}^1\Delta_g$), a complex oxygen molecule of single state, which could transform into $^3O_2$ ($3\Sigma_g$), a triplet state oxygen. During the transformation, it could produce CL and the formaldehyde could accelerate oxidation reaction rate [19,20]. The similar phenomenon (including CL kinetic response curve, CL, UV and the fluorescence spectra) was also found when PTU was replaced by MTU. This was attributed to the similarities of PTU with MTU in molecular structure. Therefore, the enhanced CL mechanism of PTU can be identical with that of MTU. And the possible reaction mechanism is suggested as following:

$$MnO_4^- + H^+ + \text{formaldehyde} + \text{PTU (MTU)} \rightarrow 1O_2(^1\Delta_g) + H_2O + \text{Mn(II)} + \text{products}$$

$$2^1O_2(^1\Delta_g) \rightarrow {}^1O_2{}^1O_2(^1\Delta_g{}^1\Delta_g)$$

$$^1O_2{}^1O_2(^1\Delta_g{}^1\Delta_g) \rightarrow 2\,^3O_2(^3\Sigma g) + h\nu\,(\lambda_{max} = 630\,\text{nm})$$

The effect of $KMnO_4$ concentration on the CL intensity was studied in the range of 1.0×10^{-5} to 2.0×10^{-4} M. As shown in Fig. 6, the CL intensity increased with the increase in $KMnO_4$ concentration up to 1.0×10^{-4} M and then decreased gradually

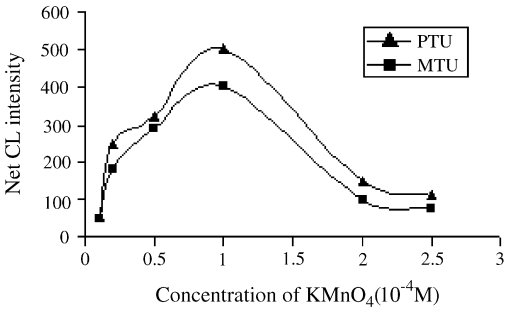

Fig. 6. Effect of $KMnO_4$ concentration on CL signal, $C_{H_2SO_4} = 1.0$ M; $C_{HCHO} = 10\%$ (v/v); flow rate = 2.0 mL min^{-1} for each flow line.

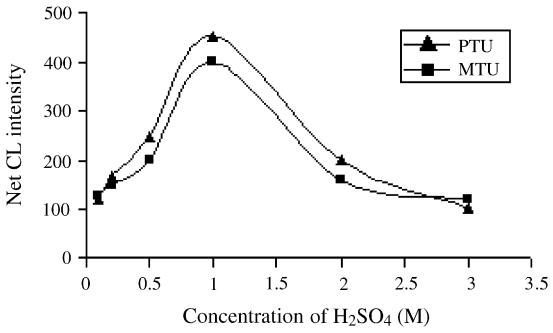

Fig. 7. Effect of sulfuric acid concentration on CL signal, $C_{KMnO_4} = 1.0 \times 10^{-4}$ M; $C_{HCHO} = 10\%$ (v/v); flow rate = 2.0 mL min^{-1} for each flow line.

with a continuous increase in the concentration. The decrease in the signal at higher concentration of $KMnO_4$ was owing to the adsorption of light emission by the intense color of the permanganate solution. Thus, the optimal concentration of $KMnO_4$ was 1.0×10^{-4} M.

Hydrochloric acid, phosphoric acid, polyphosphoric acid, sulfuric acid and nitric acid were used as the reaction media to study their effect on the CL intensity. The results showed that sulfuric acid was a more suitable medium for all analytes since it gave the strongest light intensity and the highest signal-to-noise ratio (S/N). The effect of sulfuric acid concentration in the range of 0.1–2 M was further studied and the results are shown in Fig. 7. Sulfuric acid solution 1.0 M was chosen as the optimum sulfuric concentration in $KMnO_4$ solution.

Fig. 8 shows the effect of HCHO concentration on CL intensity. The maximal relative CL intensity was achieved at 10%. As a result, 10% HCHO was chosen. The effect of flow rate on the CL intensity ranged from 0.8 to 2.5 mL min^{-1}. Under optimal total flow rate condition, the flow rate of HCHO and $KMnO_4$ was 2 mL min^{-1}.

3.3. Method validation

In the present work, the HPLC–CL method for the determination of PTU and MTU was validated by determining its performance characteristics regarding linearity, limit of detection (LOD), limit of quantification (LOQ), precision and accuracy.

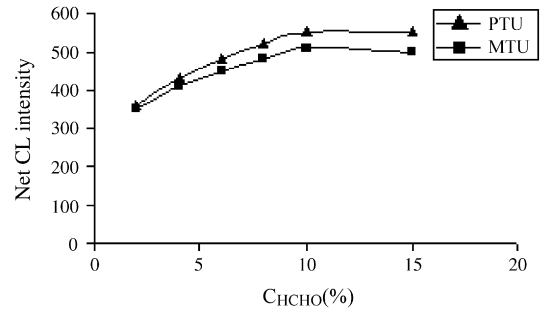

Fig. 8. Effect of formaldehyde concentration on CL signal, $C_{KMnO_4} = 1.0 \times 10^{-4}$ M (in 1.0 M H_2SO_4); flow rate = 2.0 mL min^{-1} for each flow line.

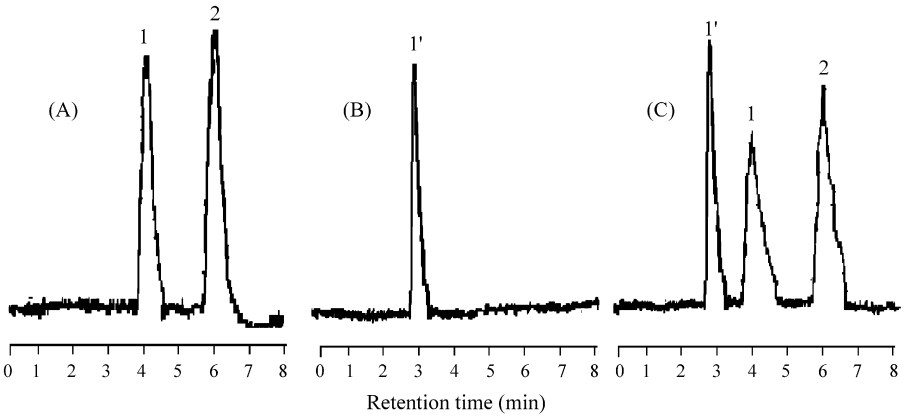

Fig. 9. Chromatograms obtained with (A) a standard mixture of MTU and PTU; (B) blank serum; (C) a serum sample at 1 h after oral administration of PTU and MTU, (1) MTU, (2) PTU, (1′) unknown substance in serum.

Table 1
Parameters of calibration graphs and precision values obtained with HPCL–CL

Compounds	Calibration equation ($\mu g\,mL^{-1}$)	Correlation coefficient	LOD ($\mu g\,mL^{-1}$)	LOQ ($\mu g\,mL^{-1}$)
PTU	$\Delta I = 8.0307C + 5.4862$	0.9963	0.03	0.1
MTU	$\Delta I = 4.131C + 4.0171$	0.9972	0.03	0.1

3.4. Linearity and sensitivity

Representative chromatograms are shown in Fig. 9. Under the chromatographic conditions described, endogenous components in human serum did not give any interfering peaks. Under the optimum conditions described in the previous section, the calibration curves were prepared over the range 0.1–20 $\mu g\,mL^{-1}$ and 0.1–10 $\mu g\,mL^{-1}$ for MTU and PTU, respectively. The figures of merit in the present $KMnO_4$–HCHO chemiluminescence detection method for MTU and PTU are summarized in Table 1. The regression coefficients (r) were greater than 0.9950 for all the curves, The limit of detection at a signal-to-noise ratio of three is 0.03 $\mu g\,mL^{-1}$ and the limit of quantification at a signal-to-noise ratio of ten is 0.1 $\mu g\,mL^{-1}$, which is valuable for detecting these compounds in biological samples in order to obtain therapeutic and toxic levels.

3.5. Precision and accuracy

The intra-day precision was tested with 11 repeated injections of PTU (MTU) sample solutions at three concentration levels (0.5, 2.5 and 5 $\mu g\,mL^{-1}$). The relative standard deviations (R.S.D.s) were 3.5% 3.8%, 3.2% (3.6%, 3.9%, 3.5%), respectively, the inter-day precision of the proposed method was studied by analyzing two identical samples (at 5 $\mu g\,mL^{-1}$) injected six times every day, on five consecutive days. The relative standard deviation was 4.0% for PTU and 4.8% for MTU. Accuracy was calculated as the percentage recovery. The precision (R.S.D.) were all less than 5%. These results indicated that the present method had a good precision and accuracy and would be acceptable for the quantitation of human plasma concentration in clinical use.

3.6. Stability

MTU and PTU were observed to be stable for at least 7 days at −20 °C and for at least 1 month at −80°C. In conclusion, the proposed HPLC method is sufficiently sensitive and selective for monitoring unchanged propylthiouracil in pharmacokinetic studies.

3.7. Application of the method

3.7.1. Determination of MTU and PTU in human serum samples

To test the applicability of the proposed HPLC method to real sample, the present $KMnO_4$–HCHO chemiluminescence

Table 2
Determination of propylthiouracil and methylthiouracil in serum samples

Compounds	Original ($\mu g\,mL^{-1}$)	Added ($\mu g\,mL^{-1}$)	Total ($\mu g\,mL^{-1}$)	Recovery (%)	R.S.D. (%) ($n = 5$)
Propylthiouracil	2.10	0.5	2.59	97	4.5
	2.16	1.0	3.12	96	4.0
	2.14	2.0	4.26	106	4.8
Methylthiouracil	2.08	0.5	2.56	96	5.0
	2.12	1.0	3.07	95	5.5
	2.14	2.0	4.16	101	4.7

detection method was applied for the determination of MTU and PTU in human serum, following the method described above; the results are summarized in Table 2. Fig. 9A shows a chromatogram with CL detection obtained from a standard mixture of MTU and PTU and as can be seen, the retention time of MTU and PTU is 4 min and 6 min, respectively. The detection limit was 0.03 $\mu g\,mL^{-1}$ and the linear response covers a wide range of PTU and MTU levels that may occur in serum of patients during variable stages of therapy and drug regimens. Fig. 9B and C shows a chromatogram with CL detection obtained from the drug-free serum and a patient serum sample at 1 h after oral administration of PTU and MTU. The method enables the determination of MTU and PTU in human serum without significant interference. The proposed method had a wider linear range and lower detection limit than those reported the HPLC methods using UV [10,11], therefore, the method offered an alternative, sensitive and simple approach for the determination of PTU and MTU in human serum samples.

4. Conclusion

Based on the sensitizing effect of formaldehyde on the CL reaction of thiouracil antithyroid drugs with acidified $KMnO_4$ and the combination technique of HPLC, a novel CL—post-column detection method has been established for the determination of PTU and MTU. Based the obtained results, the following conclusions can be drawn. The method has proved that the CL reaction is well compatible with the mobile phase of HPLC. The detection limits are acceptable for the determination of PTU and MTU in biological samples and the linear calibration range is wide enough for quality control of pharmaceuticals. Moreover, the method allowed for simultaneous sensitive detection of these antithyroid drugs in human serum.

Acknowledgement

This study was supported by the Natural Science Foundation of China (no. 30470886).

References

[1] Council of the European Communities Decision 602/1981/EC, Off. J. Eur. Commun. L 222/32.
[2] Council of the European Communities Decision 649/1986/EC, Off. J. Eur. Commun. L 275/36.
[3] N.B. Tadros, A. Besada, Y.A. Gawargions, Egypt J. Pharm. Sci. 30 (1989) 241.
[4] J.M. Lopez, P.C. Gonzale, P Sanz-Pedrero, Ann. R. Acad. Farm. 47 (1981) 161.
[5] K. Nikoli6, B. Stankovi6, M. Bogavac, J. Serb. Chem. Soc. 59 (1994) 385.
[6] M. Kucharski, H. Sikorska-Tomicka, T. Szumilo, Zesz. Nauk. Politech. Biolostockiej Mat. Fiz. Chem. 7 (1981) 81.
[7] S. Pinzauti, G. Papeschi, E. La Porta, J. Pharm. Biomed. Anal. 1 (1983) 47.
[8] E.M. Mohamed, S.M. Tawakkol, Y.H. Aboul-Enein, Chem. Biomed. Environ. Instrum. 11 (1981) 241.
[9] A. Besada, N.B. Tadros, Y.A. Gawargions, Egypt J. Pharm. Sci. 30 (1989) 251.
[10] C.G. Duarte, A.C.M. Polizello, A.I. Assis-Pandochi, A.C.C. Spadaro, J. Pharm. Biomed. Anal. 23 (2000) 237.
[11] M.T. Rosseel, R.A. Efebvre, J. Chromatogr. 507 (1990) 247.
[12] R. Halpern, D. Cooper, V. Kieffer, H.V. over Saxe, F. Maloof, E.C. Ridgway, Endocrinology 113 (1983) 915.
[13] P. Batjoens, Derabander Hubert, De Wasch Katia, J. Chromatogr. A. 750 (1996) 127.
[14] J.M. Lin, Chemiluminescence—Basic Principle and Applications, Chemical Industry Press, Beijing, 2004, p. 91.
[15] B.J. Hindson, N.W. Barnett, Anal. Chim. Acta 445 (2001) 1.
[16] P. Fletcher, K.N. Andrew, A.C. Calokerinos, S. Forbes, P.J. Worsfold, Luminescence 16 (2001) 1.
[17] G.R. Cannell, J.P. Williams, J. Chromatogr. 564 (1991) 311.
[18] S.L. Liao, X.P. Wu, Z.H. Xie, Anal. Chem. Acta 537 (2005) 189.
[19] G.N. Chen, F.X. Huang, X.P. Wu, Z.F. Zhao, J.P. Duan, Anal. Ioanal. Hem. 376 (2003) 73.
[20] M. Liu, Y.H. He, J.R. Lu, Fen xi Hua xue 33 (2005) 535.