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High-performance liquid chromatographic determination of propylthiouracil in human urine by post-column iodine–azide reaction

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ABSTRACT

A sensitive, selective and simple post-column detection method for the determination of propylthiouracil (PTU) based on its sensitizing induction on iodine–azide reaction and the combination technique of high-performance liquid chromatography has been presented. The analysis was conducted in the optimum conditions for iodine–azide detection system and HPLC separation. The linear range, the lower limit of detection and quantification for PTU in urine were established at the levels of 0.4–1.0 nmol/ml urine, 0.3 nmol/ml urine and 0.4 nmol/ml urine, respectively.

1. Introduction

Propylthiouracil (PTU) is a recognized drug belonging to the thionamide class, and one of the crucial agents in the therapy of thyrotoxicosis [1]. Its biochemical effect in the production of thyroid hormones is well defined, as it inhibits the activity of thyroid gland peroxidase, and in consequence, blocks iodine organification in the gland. This action is also common for other thionamides like methimazole and carbimazole [2]. Another characteristic of PTU is its ability to block the conversion of tetraiodothyronine (T4) to triiodothyronine (T3) in peripheral tissues. Due to this additional feature, the application of PTU is preferred to other thionamides. The treatment of thyroid diseases with the help of PTU involves autoimmune processes and is usually associated with suppressive or stimulatory events of the immune response [3].

Since the kidneys play an important role in body homeostasis, PTU content in urine can be used as an important indicator, in order to provide information on the balance between intake and output and also to recognize possible intoxication. This is due to PTU excretion in urine in the non-metabolized form. The frequently chosen way for the analysis and estimation of bioavailability of drugs is urinary recovery, mostly for its accessibility and non-invasive method collection.

The detection and determination of PTU in urine [4–6], plasma [7–13], serum [13–16], milk [5–17] and tissues [16,18,19] have already been investigated with the application of a variety of separation methods, e.g. GC–MS [5,6], HPLC–MS [4,18], HPLC with ultraviolet [7,11,13,17,18], electrochemical [18] and post-column chemiluminescence [15] detection, and TLC [20,21]. Additional requirements needed for successful results comprise the selective isolation of PTU and preconcentration steps, such as extraction including liquid–liquid extraction [10–13,15,16,18,19] solid-phase dispersion [5] or clean-up procedure with derivatization step [4,6].

Yet, the methods developed so far, have not comprised the application of liquid chromatography with iodine–azide reaction for the determination of PTU in urine. An expected advantage of the suggested detection technique is its better selectiveness than in case of the UV detection in analyses of complex matrices.

A selective and sensitive detection of thiols was provided by sulphur(II) (e.g. PTU) compounds, which induced the reaction between azide and iodine:

$$2N_3^- + I_2 \xrightarrow{PTU} 3N_2 + 2I^-$$

The suggested procedure is based on the separation of analyte on chromatographic column and on the subsequent measurement of the unreacted iodine in the iodine–azide reaction [22,23]. The observation covers also the selective induction of PTU. The presence of PTU in the chromatographic band is accompanied by the decrease in the iodine consumption in the iodine–azide reaction and determined in the form of a negative peak at $\lambda = 350$ nm. The quantitative distinction of the peaks is affected by the amount of

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the thiol. This modification has an immediate consequence in the significant improvement of detection sensitivity to the pmol level.

2. Experimental

2.1. Chemicals, reagents, standard solution preparation

The grade of all chemicals used was either analytical or HPLC. PTU, sodium azide, hydrochloric acid, sodium hydroxide, iodine, potassium iodide and acetonitrile were all supplied by Aldrich (Steinheim, Germany), LAB-SCAN Analytical Sciences (Dublin, Ireland) and POCH (Gliwice, Poland). The preparation of all solutions was conducted daily. The water used for solutions preparation was deionized with subsequent 15 min helium sparging.

The preparation of stock PTU solution comprised the dissolution of 100 μmol PTU in 1 ml of 1 M sodium hydroxide solution and its subsequent dilution to 100 ml with water. To prepare working standard of PTU solutions (0.1 μM), appropriate serial dilutions of the stock solution with water were administered.

The preparation of a mobile phase solution included the dissolution of 12.5 g sodium azide in water and the addition of hydrochloric acid to obtain pH 5.5. In the next step, the solution was adjusted to 0.5 l with water. The mobile phase was prepared by mixing acetonitrile, sodium azide solution of the given concentration and pH (pH 5.5; 2.5% (w/v)) and water (24:50:26, v/v/v) with HPLC pump, as shown in Fig. 1.

The procedure for the preparation of a post-column reagent solution comprised the dissolution of 6.3 g iodine and 20 g potassium iodide with water to 0.5 l. 1 ml of the solution mentioned was then supplemented with 0.832 g of potassium iodide and diluted with water to 0.25 l.

2.2. Calibration standards and sample preparation

Urine calibration standards were prepared with a 1-ml portion of PTU-free urine spiked with the increasing amounts of the working standard solution of PTU, which was formulated daily in the process of dissolution of standard solution with water. In the next step, the subsequent PTU in urine sample concentrations of 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 nmol/ml urine were diluted with water to 10 ml. Calibration standards were handled with the application of the recommended analytical procedure [20].

2.3. Analysis of patient's urine samples

Patient's urine (morning and at random time, mid-stream urine) was obtained from a volunteer (female, 50 years old) who took 50 mg of PTU per day. Freshly collected urine with PTU content was diluted with water to 10 ml. The urine sample volume was selected to adjust the PTU peak height of ca. 0.1 AU. The appropriateness of the dilution and quantities of PTU was consulted with the use of the calibration curve as a source of information. Afterwards, the procedure comprised the injection of 10 μl of the solution onto the chromatographic system. The subsequent amounts of 2.5, 5, 7.5 and 10 nmol of PTU were supplemented with urine sample aliquots. The amount of PTU was read using standard addition technique.

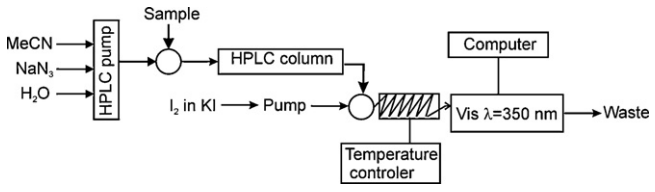

Fig. 1. Flow diagram system with iodine–azide detection procedure.

Table 1
Chromatographic and post-column reaction conditions applied in the determination of propylthiouracil

Parameter	Value of parameter
Column	C_{18}
Composition of the mobile phase	Sodium azide:water:acetonitrile (50:26:24, v/v/v)
Flow-rate of the mobile phase (ml/min)	1.4
Sodium azide solution concentration (%)	2.5
Sodium azide solution pH	5.5
Iodine solution concentration c(I) (mM)	0.4
Potassium iodide solution concentration c(KI) (mM)	20
Post-column reaction solution flow-rate (ml/min)	0.3
Post-column reaction module temperature (°C)	35
Detection wavelength (nm)	350
Injection volume (ml)	10

2.4. Instrumental

The study was conducted with the use of the following equipment for chromatographic separation: waters liquid chromatographic system equipped with Multisolvent Delivery System Model 600E, 717 plus autosampler, a variable wavelength LC spectrophotometer (2487 Dual λ). The separation was performed on an analytical column, symmetry C_{18} (150 mm × 3.9 mm i.d., 5 mm, waters) at an ambient temperature. The content of the mobile phase was delivered at an ambient temperature. As for the iodine–azide post-column reaction, it was administered with the use of Waters system supported by Reagent Manager as a single-piston, pulsedampened pumping system for post-column reagent. The delivery of the reagent was conducted to the post-column reaction module (the reaction tube, 6 m × 0.46 mm i.d.) with the simultaneous application of Temperature Control System. The integration of the chromatograms was administered with EmpowerTM software (Waters). Table 1 depicts the optimum conditions for the separation of PTU by HPLC and for its quantitation by the post-column reaction.

2.5. Assay validation

The attempt to determine the concentration of PTU in urine comprised the reference of the PTU peak area to standard curves of PTU in drug-free urine [20]. The attempt to estimate the values of lower limit of detection (LLD) (S/N = 3) and quantitation (LLQ) (S/N = 6) required the choice of solutions of decreasing concentration of PTU in urine samples for the analysis. Possible contaminations of the analyte were eliminated through the processing of water accordant with the given procedure before analyzing samples.

The calculation of intra-day accuracy and precision was conducted in the process of analysis of quadruplicate of QC samples at different concentration levels given in Table 2 during the same day. The determination of both inter-day accuracy and precision was administered with the use of QC samples (0.4, 0.7 and 1.0 nmol/ml) on three separated occasions in quadruplicate. The first occasion was the analysis of QC samples against calibration curves. The next two analyses comprised the evaluation of the applied criterion for precision and accuracy with the required relative standard deviation and mean value at the level lower than 6%. Recovery was calculated with the use of formula:

$$\text{Recovery}\,(\%) = \frac{\text{measured amount}}{\text{added amount}} \times 100$$

Table 2
Results of propylthiouracil determination with iodine–azide post-column detection system (inter-day); $n = 4$

Taken (nmol/ml urine)	Found $\bar{x} \pm t_{0.95}\bar{s}$ (nmol/ml urine)	R.S.D. (%)	Recovery (%)
0.400	0.399 ± 0.005	0.9	100
0.500	0.489 ± 0.002	0.2	98
0.600	0.575 ± 0.016	2.0	96
0.700	0.691 ± 0.012	1.3	99
0.800	0.801 ± 0.014	1.2	100
0.900	0.909 ± 0.009	0.7	101
1.00	1.02 ± 0.01	0.5	102

2.6. Stability of PTU in urine and tested interferences

In order to administer the stability test of PTU, urine samples were spiked with the appropriate amount of the drug to form the solution of 1 nmol/ml urine concentration. The same procedure was repeated in the next step at the temperature of 4 and 37 °C for 5 h.

The attempt to define possible interferences comprised analyses of the following compounds: cysteine, cystine, homocysteine, glutathione, methionine, thiocyanate, ascorbic acid and thiouracils (methyl-, benzyl-thiouracil). The subsequent analyses were conducted according to the transparent procedure: the prepared 1 nmol/ml urine drug solution was spiked with the appropriate amount of the chosen substance to obtain the final concentration of 10 nmol/ml urine for each compound to ensure enough sensitivity of interferences toward iodine–azide detection procedure.

2.7. Search for internal standard

Several thiol were tested as candidate internal standard. All these compounds were added to the spiked urine sample to achieve a final concentration of 1 nmol/ml, and the resultant mixture was subjected to HPLC separation under analysis conditions.

3. Results and discussion

The present study aimed at the improvement of an unambiguous and accurate HPLC method with post-column iodine–azide reaction that would serve as a detection system for the determination of PTU in urine samples. The most relevant points of the experiment are presented below.

3.1. Optimization of the HPLC with post-column reaction system

A wide range of parameters leading to the highest iodine consumption should be taken into consideration in order to establish optimum conditions for HPLC determination with post-column iodine–azide reaction. The following parameters can be enumerated for exemplification: iodine in potassium iodide solution concentration, its flow-rate, the post-column reaction module temperature. Still, there are factors which affect both separation and detection processes and for their dual activity they attract much attention as well. Here belong for instance: flow-rate of mobile phase, pH and concentration of sodium azide solution. The only parameter that influences the separation process is the type of a column.

The symmetry C_{18} analytical column served for the successful separation of the PTU samples. The study tested several mobile phases consisting of various concentrations of acetonitrile and sodium azide (varied with concentration, pH) with different volume ratios. Acetonitrile: 2.5% sodium azide solution (pH 5.5):water (24:50:26, v/v/v), was found to be the most appropriate mobile phase for the tested compound separation within a run-time of 3 min in isocratic mode. The test included checking the urine samples from several sources. Regardless of the source the PTU peak was separated well from blank urine peak.

3.1.1. Factors with an impact only on post-column detection process

The course of iodine–azide reaction is under the effect of iodide ions within the range of 1–50 mM when the iodine concentration and its flow-rate remained constant. The observed increase in the peak area occurred within the range of 1–20 mM. It is associated with the further shift in the equilibrium $I_2 + I^- \leftrightarrows I_3^-$ to the right that results in the increase in the triiodide ions concentration. The observed absorbance is higher with constant iodine concentration. The increase in the concentration of potassium iodide within the range of 20–50 mM was followed by the decrease in the peak area.

The test of the iodine solution flow-rate within the range of 0.1–0.6 ml/min was conducted with constant iodine and iodide ions concentrations (Fig. 2). As the oxidation of PTU in the iodimetric reaction is quicker at higher amount of iodine, the participation of the inductor in the induced reaction can be shortened, which results in the decrease of iodine consumption (increase of detection limit). The choice of the flow-rate level of 0.3 ml/min was justified by the highest iodine consumption in iodine–azide reaction induced by PTU and the biggest peak area.

The next analysed parameter was the flow-rate of iodine solution immediately influencing the levels of LLQ (maximum iodine consumption in the post-column reaction; maximum peak area) and LLD (maximum signal-to-noise ratio). The decrease in the peak area was observed with the increase in the iodine solution flow-rate above 0.3 ml/min (Fig. 2). This was due to the insufficient time of PTU induction of the iodine–azide reaction. As a result of this insufficiency, the reaction was not completed. The noise observed in the background originated from the pump applied to iodine solution. The noise peak area was detected with the high flow-rate of iodine solution. The selected optimum flow-rate for the iodine solution was that of 0.3 ml/min as it was accompanied by the highest signal-to-noise ratio.

The PTU peak area was not affected by the iodine solution concentration in the range of 0.1–0.5 mM. The highest iodine consumption in the iodine–azide reaction was observed with the iodine amount of 20–30% beside initial quantity [24]. The higher reaction rate followed by the lower detection limit resulted from the decrease in iodine concentration [25]. Nevertheless, the complete consumption of iodine in the induced reaction was noted when the concentration of iodine was too low. The immediate consequence was the absorbance level of ca. 0 AU at which no proportional relation between the peak area and PTU amount could be established.

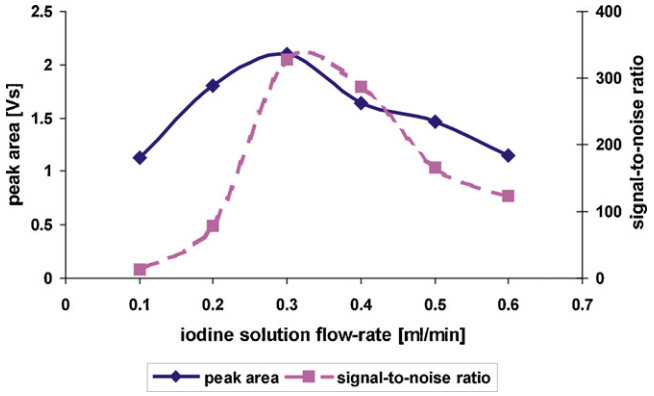

Fig. 2. The influence of iodine solution flow-rate on the PTU peak area and signal-to-noise ratio; $c_{PTU} = 2$ nmol/ml urine and for conditions see Table 1.

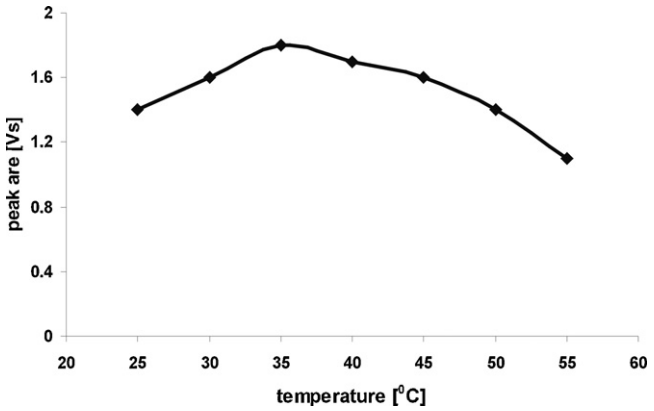

Fig. 3. The influence of post-column reaction module temperature on the peak area; c_{PTU} = 2 nmol/ml urine and for conditions see Table 1.

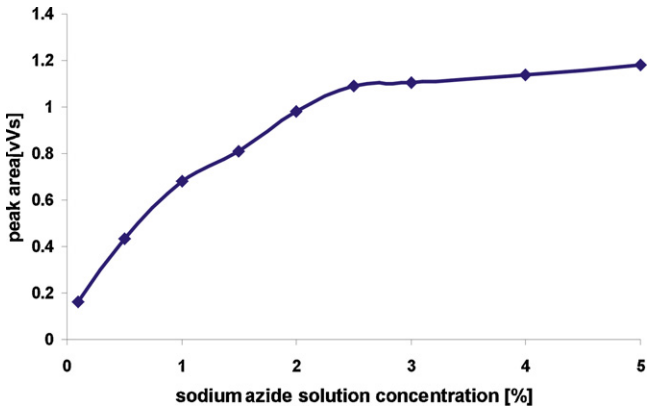

Fig. 4. The influence of sodium azide solution concentration on the peak area; c_{PTU} = 2 nmol/ml urine and for conditions see Table 1.

As there was no detectable impact of iodine concentration upon PTU peak area, it occurred necessary to maintain iodine solution concentration (applying 20 mM potassium iodide solution concentration and post-column reagent rate of 0.3 ml/min) to obtain the absorbance within the range of 0.4–0.6 AU. Therefore, the established optimum for iodine solution concentration was defined at the level of c(I) = 0.4 mM.

The study investigated the relation between the post-column reaction module temperature and the PTU peak area. The range selected for investigation was 25–40 °C under optimal conditions (Fig. 3). The constant level of 35 °C was maintained for the analysis in the post-column reaction module. The increase in temperature above 35 °C resulted in the decrease in the peak area. The explanation for this relation lies in the increase in the oxidation rate of an iodimetric reaction, in which a non-inducing compound is created from PTU. The results of the research proved the influence of the temperature increase upon the iodine consumption in the post-column iodine–azide reaction.

3.1.2. Factors with an impact both on separation and post-column detection process

Buffer sodium azide of neutral pH performs the function of a bacterial growth inhibitor. What is more, it does not evoke any modification of the proteins chromatographic performance as it shows no affinity with them. Eventually, sodium azide is frequently excluded from applying to ion exchanger chromatography due to its activity towards anion exchangers and binding sites blocking function. Additionally, it is not applied to sulphur anions determination as a part of a mobile phase [22]. Yet, in this research, in the RP HPLC mode, sodium azide was included in the mobile phase as one of the reagents of iodine–azide reaction, mainly because it significantly simplified the procedure of the PTU determination. In ion chromatography [22], a higher concentration of sodium azide solution was employed for the purpose of completing the post-column reaction. As sodium azide shows poisonous properties, it is suggested to apply it in small amounts. In the experiment, sodium azide solution was pumping through the separation column. The procedure was justified by two major facts. The first one was the necessity to cease sodium azide dilution as it led to the decrease in iodine consumption in the induced reaction. Secondly, the incorporation of sodium azide allowed for the use of only one buffer solution for separation process and for post-column detection system.

The analysis covered the range of 0.1–5% in order to establish the concentration of azide ions solution on the cause of iodine–azide reaction (Fig. 4). In general, the increase in the sodium azide concentration was accompanied by the increase in the PTU peak area. The value of 2.5% was finally selected for subsequent experiments, as further increase in sodium azide concentration does not result in any change in the peak area.

The iodine consumption is the main reflection of pH effect. Yet, it is essential to recognize the restrictions of pH referring to sodium azide solution. When pH of the solution is lower than 5.5, the poisonous, volatile hydrazoic acid is emitted. The increase of pH to the level above 8.0 results in the formation of iodate(I) that prevents iodine–azide reaction from its conduct. Eventually, the selected pH for PTU analysis was restricted to the range of 5.5–7.0. As can be seen from Fig. 5, the optimum value of 5.5 was chosen as providing the best results. Similar results were indicated by the following parameters: volumetric titration [24–26], spectrophotometric [27] measurements of the course of iodine–azide reaction in an aqueous medium.

The appropriate administration of the iodine–azide reaction requires sufficient contact time between eluate (containing PTU and azide ion) and the post-column reaction solution (containing iodine solution). As shown in the experiment results, the increase in the peak area is simultaneous with increasing flow-rate of mobile phase within the range 0.5–1.4 ml/min, while the increase in the flow-rate within the range 1.4–2.0 ml/min accompanies the decrease of the peak area. Therefore, the value of 1.4 ml/min occurred the optimum choice. The obtained results prove that the contact time for the completion of the iodine–azide reaction induced by PTU is sufficient.

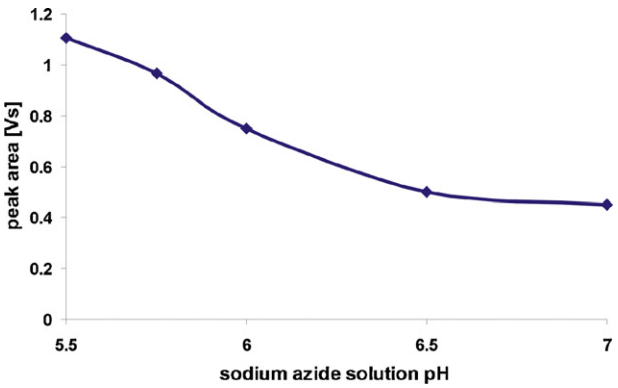

Fig. 5. The influence of sodium azide solution pH on the peak area; c_{PTU} =2 nmol/ml urine and for conditions see Table 1.

Table 3
Validation of intra-day assay; $n = 4$

Taken (nmol/ml urine)	Found $\bar{x} \pm t_{0.95}\bar{s}$ (nmol/ml urine)	R.S.D. (%)	Recovery (%)
0.400	0.407 ± 0.014	5.7	102
0.700	0.699 ± 0.023	5.1	100
1.00	1.00 ± 0.02	1.7	100

3.2. Validation

Tables 2 and 3 present the intra- and inter-assay precision and accuracy dependent on peak area ratios, with good recoveries obtained as well. The comparison of the amounts of PTU added and the amounts found showed no considerable differences.

The invariable result of standard curves was the r^2-value above 0.996 within a calibration range of the analyte. The equation derived from the least squared regression was $A = 1.47 \pm 0.06c$ where A is the peak area (Vs) and c is the PTU concentration (nmol/ml urine). The range of calibration can be expanded upwards, if necessary.

The lower limit of detection was estimated at the value of 0.3 nmol/ml urine. The relevant value for the lower limit of quantitation was established at 0.4 nmol/ml urine.

3.3. Interferences and stability

The process of elimination of matrix interferences was possible through the shift in the detection wavelength from the UV region (corresponding to PTU absorption) to vis region ($\lambda = 350$ nm, iodine adsorption). Generally, the application of iodine–azide detection system results in the visibility of sulphur(II) compounds only in chromatograms. Still, certain compounds may be responsible for four groups of additional peaks: iodine-azide reaction inductors (e.g. cysteine or cystine) generating negative peaks, compounds reacting with iodine under experimental conditions (e.g. ascorbic acid) that generate negative peaks, compounds reacting with iodide ions (e.g. bromate(V), iodate(V), nitrate(III)) that generate positive peaks, compounds that absorb at 350 nm (e.g. sulphasalazine).

Still, there is a number of sulphur(II) compounds that eluate in the front of the mobile phase in RP-HPLC mode and can be found in urine samples. Here belong: cysteine, cystine, homocysteine, glutathione, methionine, thiocyanate, ascorbic acids. The derivatives of thiouracils differ from the studied compound in their retention time. As the possible interference can be observed only with the significant similarity of retention times, this was not the case in the presented procedure.

Several sources of urine samples were checked and in each case the PTU peak was well separated from the blank urine peak.

Urine samples were monitored for PTU at 4 °C (in experimental conditions) and 37 °C (in physiological conditions) within 5 h. Within examined time, no decay of PTU in urine was reported (Table 4).

3.4. Internal standard

In order to minimize the contribution of sample preparation, injection and column deterioration to the final results, the internal standard was proposed. Benzylthiouracil, appearing as a symmetric peak, eluted near PTU (retention time 5.5 min), in the position free of other peaks. More over, the benzythiouracil has similar induction properties to PTU one [27].

Table 4
Stability results of PTU (1 nmol/ml urine) under various storage conditions

Storage conditions	RE (%)
Room temperature (5 h)	−1.0
Temperature of 37 °C (5 h)	−1.6

Table 5
Results of propylthiouracil determination in patient's urine samples

Dosage (mg/day)	Age (year)	Sex	Found $\bar{x} \pm t_{0.95}\bar{s}$ (nmol/ml)	R.S.D. (%)
			10.19 ± 0.64	4.9
			7.82 ± 0.41	4.1
50	50	Female	2.57 ± 0.07	2.0
			6.92 ± 0.12	1.4
			5.83 ± 0.36	4.8

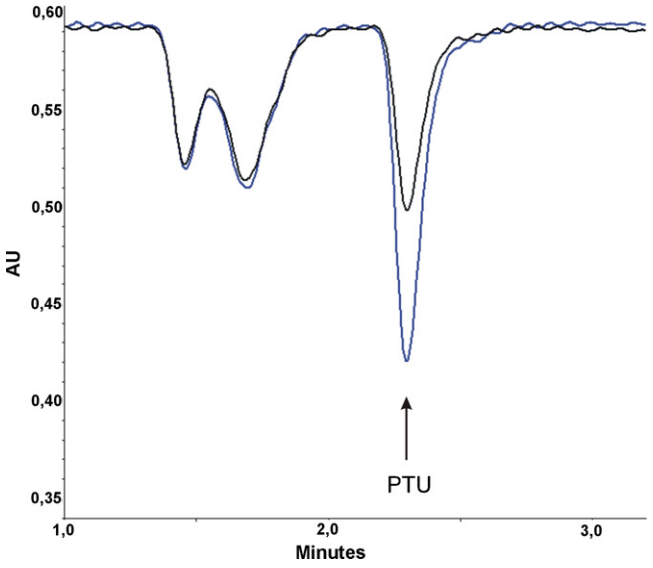

Fig. 6. Chromatogram obtained for propylthiouracil in patient's urine and 5 nmol PTU spiked patient's urine with iodine–azide reaction detection procedure (for chromatographic and post-column reaction conditions see Table 1).

3.5. Assay of PTU from urine samples

The established HPLC method of PTU determination with iodine–azide reaction system was applied to the real-world samples of urine (Table 5). Chromatogram obtained for propylthiouracil in patient's urine and PTU spiked (5 nmol/ml) patient's urine is depicted in Fig. 6. Various urine samples from one patient were taken at various times after the drug administration. Parameters like treatment period, collection time of sample, dose value, age, drug dosage, patients' weigh are related to PTU concentration in authentic urine sample.

4. Conclusion

There is as much of routine in operating the HPLC system with the post-column iodine–azide reaction system as in the regular LC. Yet, the LC/post-column combination ensures significant benefits: minimal sample pre-treatment, greatly improved sensitivity, and enhanced selectivity for PTU, the detection of which would normally be much more complicated with the application of other techniques. Previous studies on urine samples reached the levels of 0.5 μg/l [4], 016 μg/g [5], 50 ppb [6], respectively. The detection limits were obtained with execration, derivatization, clean-up and evaporation to dryness procedures with mass spectrometry as a detection system. The proposed method allows to decrease detection limit (0.3 nmol/ml urine; 50 ng/l urine sample solution)

in urine sample since the procedure require only dilution of urine samples.

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