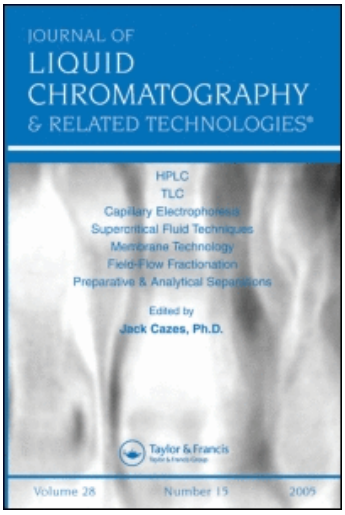

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High Performance Liquid Chromatography Coupled with Post-Column Iodine-Azide Reaction for the Determination of Benzylthiouracil in Urine

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Abstract: The reaction between iodine and azide ions induced by benzylthiouracil was applied as a post-column detection system for determination of benzylthiouracil in urine. Neither extraction, nor preconcentration of the sample are necessary. The reproducibility, linearity, and recovery were evaluated under the optimum conditions. The benzylthiouracil standards added to normal urine show that the response of the detector, set at 350 nm (corresponding to unreacted iodine in the post-column iodine-azide reaction), was linear within the concentration range of 0.4-4.5 $nmol \cdot mL^{-1}$ urine. Recovery and the relative standard deviation values for precision within the calibration range were from 94 to 104% and from 1 to 4.5%, respectively. Lower limits of detection (LLD) and quantitation (LLQ) were 0.3 and 0.4 $nmol \cdot mL^{-1}$ urine, respectively.

Keywords: Benzylthiouracil, High performance liquid chromatography, Iodine-azide reaction, Post-column reaction, Urine

INTRODUCTION

Benzylthiouracil (6-benzyl-2-thiouracil; BTU) is applied in the treatment of Grave's disease, which is a form of autoimmune thyroiditis.[1] The action of BTU is similar to other antihydroid drugs.[2] However, this drug

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may cause vasculitis associated to antineutrophil cytoplasmic antibodies (ANCA).[3,4]

This compound has been determined previously by the potentiometric titration with mercury (II)[5] and iodine in alkaline solution.[6,7] Other studies concern the pulsepolarographic[8] and cathodic stripping voltammetric[9] method. Gas chromatography,[10] high performance liquid chromatography,[11,12] and thin-layer chromatography[13–16] have been also applied for the determination in biological samples[10,11,13,17] or in feeds.[14,15]

BTU can also be determined by the measurement of the amount of iodine consumed in the induced iodine-azide reaction:

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

Iodine, which is consumed in the reaction is proportional to the amount of BTU present in the sample. The quantity of the compound can be determined using various techniques, e.g., back titration of excess of iodine,[18] dead stop[19] and kinetic stop flow method,[20] and TLC.[16] Considering this, the application of above mention iodine-azide reaction, selectively induced by sulfur (II) compounds, provides the possibility for a sensitive and selective determination and detection method.

In this paper, induced reaction between iodine and azide ion was applied as a post-column detection system in HPLC for determination of BTU in urine. In the system of post-column reaction, the stream of eluant from an HPLC column (consists of sodium azide and separated BTU) is mixed with a stream of iodine in a potassium iodide solution. The obtained mixture is moved through a coil to ensure sufficient time (maintained by flow rates of mobile phase and iodine solution) for the completion of chemical reactions. The final step is the passage of the mixed streams into the UV/VIS absorbance detector ($\lambda = 350\,\text{nm}$). So far, the iodine-azide reaction as a postcolumn reaction was applied for determination of sulfide,[21] thiosulfate,[21,22] thionates[22] with ion or ion-pair chromatography, as well as 2-thiobarbituric acid,[23] and thiopental[24] with reversed phased chromatography. As a result of the sodium azide buffer activity towards anion exchangers and blocks binding sites, this compound is usually excluded from being applied to ion exchanger chromatography. In addition, buffer sodium azide has no application in the determination of sulfur anions determination as a part of a mobile phase.[21,22] In the case of this research, sodium azide was incorporated into the mobile phase as one of the reagents in the iodine-azide reaction. It substantially made the system of the BTU determination procedure simpler. To get the achievement of a post-column reaction, a higher concentration of sodium azide solution

was introduced in ion or ion-pair chromatography. Due to the poisonous properties of sodium azide, it is advisable to use it in rather small amounts. In the presented study, sodium azide acted as a buffer that was pumped through the separation column. The explanation for this procedure was based on two main cases. Firstly, it was essential to stop sodium azide dilution, which can lead to a decrease in iodine consumption in the induced reaction. Secondly, using sodium azide solution as a constituent of the mobile phase, it was possible to use only one buffer solution for the separation process and for the post-column detection system.

EXPERIMENTAL

Chemicals, Reagents, Standard Solution Preparation

All chemicals were of analytical or HPLC grade. BTU, sodium azide, hydrochloric acid, sodium hydroxide, iodine, potassium iodide, and acetonitrile were obtained from Aldrich (Steinheim, Germany), LAB-SCAN Analytical Sciences (Dublin, Ireland) or POCH (Gliwice, Poland).

All the solutions were freshly prepared daily. Deionized water with subsequent 15 min helium sparging was used to prepare the solutions.

Working standard BTU (100 μmol) was dissolved in 1 mL 1 $mol \cdot L^{-1}$ sodium hydroxide solution and diluted to 100 mL with water to produce a stock solution containing 1 $mmol \cdot L^{-1}$. Appropriate serial dilutions of the stock solution with mobile phase were performed to prepare working standard BTU solutions (10 $\mu mol \cdot L^{-1}$).

A mobile phase solution: 12.5 g sodium azide was dissolved in water and hydrochloric acid was added to obtain pH 5.5, then the solution was adjusted to 0.5 L with water. The mobile phase was consisted of a mixture of acetonitrile, sodium azide solution (pH 5.5; 2.5% w/v) and water (30:50:20, v/v/v) and was mixed with the HPLC pump.

A post-column reagent solution: 6.3 g iodine and 20 g potassium iodide were dissolved and adjusted with water to 0.5 l. One mL of the solution mentioned above was added to 0.416 g of potassium iodide and diluted with water to 0.25 L.

Preparation of the urine calibration standards involved 1 mL portion of BTU-free urine was spiked with increasing amounts of the working standard solution of BTU and then diluted with water to 10 mL to give concentrations given in Tables 1 and 2.

The sodium azide solution pH was adjusted by potentiometric titration. The calibration of the titration system was carried out with standard pH solutions.

Table 1. Determination of BTU with iodine-azide procedure detection (n = 4)

Spiked concentration (nmol · mL^{-1} urine)	Measured concentration $x \pm t_{0,95} \cdot \bar{s}$ (nmol · mL^{-1} urine)	RSD (%)	Recovery (%)
Inter-day			
0.400	0.400 ± 0.007	1.4	100.1
0.600	0.610 ± 0.011	1.4	101.7
0.700	0.727 ± 0.009	1.0	103.9
0.800	0.834 ± 0.018	1.7	104.2
0.900	0.899 ± 0.022	2.0	99.9
1.500	1.517 ± 0.051	2.7	101.2
2.000	1.876 ± 0.039	1.7	93.8
2.500	2.489 ± 0.077	2.5	99.6
3.000	3.006 ± 0.045	1.2	100.2
3.500	3.624 ± 0.202	4.5	103.5
4.000	3.941 ± 0.092	1.9	98.5
4.500	4.511 ± 0.119	2.1	100.2
Intra-day			
0.400	0.411 ± 0.007	3.6	102.7
1.500	1.512 ± 0.021	2.9	100.8
4.500	4.657 ± 0.067	3.1	103.5

Instruments

The chromatographic separation was performed on a Waters liquid chromatographic system equipped with Multisolvent Delivery System

Table 2. Determination of BTU with UV procedure detection (n = 4)

Spiked concentration (nmol · mL^{-1} urine)	Measured concentration $x \pm t_{0,95} \cdot \bar{s}$ (nmol · mL^{-1} urine)	RSD (%)	Recovery (%)
30.00	28.75 ± 0.13	0.35	96
40.00	40.15 ± 1.98	3.84	100
50.00	50.61 ± 0.63	0.97	101
70.00	69.92 ± 1.51	1.68	100
80.00	80.07 ± 0.51	0.50	100
90.00	88.91 ± 0.43	0.38	99
100.0	93.26 ± 0.57	0.48	93
125.0	127.6 ± 2.5	1.49	102
150.0	151.2 ± 1.4	0.71	101
175.0	173.4 ± 1.1	0.51	99
200.0	199.3 ± 0.6	0.23	100

Model 600E, 717plus autosampler, and a variable wavelength LC spectrophotometer (2487 Dual λ). An analytical column, Symmetry C_{18} (150 mm × 3.9 mm i.d., 5 mm, Waters) was used for chromatographic separation at ambient temperature. The mobile phase consisting of a mixture of acetonitrile 2.5% sodium azide; pH 5.5- water (30:50:20, v/v/v) was delivered at a flow rate of $0.7\,mL \cdot min^{-1}$ at ambient temperature. The iodine-azide post-column reaction was carried out on a Waters system provided with a Reagent Manager as a single-piston, a pulse dampened pumping system for post-column reagent (mixture of $0.4\,mmol \cdot L^{-1}$ iodine solution in $10\,mmol \cdot L^{-1}$ potassium iodide) delivery at a flow rate of $0.3\,mL \cdot min^{-1}$ to the Post-column Reaction Module (the reaction tube, 6 m × 0.46 mm i.d.) with Temperature Control System. The chromatograms were integrated with a EmpowerTM software (Waters). The injection volume was 10 µL and the detection was performed at a wavelength of 350 nm.

Spectrophotometric Detection

Spectrophotometric detection was performed using chromatographic conditions described previously. The effluent from the column was monitored by an ultraviolet detector set at $\lambda = 275$ nm. BTU has an UV–vis absorption maximum at this wavelength in studied chromatographic conditions.

Assay Validation

Lower limits of detection (LLD) and quantitation (LLQ) limit were calculated on the basis of signal-to-noise ratios of 3:1 and 10:1, respectively. To estimate these values (LLD and LLQ), solutions of decreasing concentration of BTU in urine sample were analyzed. Any contamination to the analyte was excluded by processing water according to the procedure before analyzing samples.

The construction of calibration curves involved the preparation of fourteen different calibration standards of BTU according to the procedure described above. These curves were plotted using the integrated peak area of BTU versus standard BTU concentration. Linearity was established on the basis of the equation $A = aC + b$ where A is the peak area ($mV \cdot s$) and C ($nmol \cdot mL^{-1}$ urine) is the concentration of BTU.

Intra-day accuracy and precision were calculated by analyzing four replicates of quality control (QC) samples at different concentration levels during the same day. Inter-day accuracy and precision were determined by QC samples on three separated occasions in replicate ($n = 4$).

QC samples were analyzed against the calibration curves. The applied criterion for precision and accuracy evaluation required the relative standard deviation and mean value to be less than 10%.

Stability of BTU in Urine and Tested Interferences

For the stability test of BTU, a solution of 1 $nmol \cdot mL^{-1}$ urine concentration was prepared by spiking the urine sample with an appropriate amount of the drug. The described procedure was applied in the next step at 4 (autosampler stability) and 37°C (in body conditions) for 5 hours.

To establish possible interferences the following compounds were evaluated: cysteine, cystine, homocysteine, glutathione, methionine, thiocyanate, ascorbic acid, and thiouracils (methyl-, propylthiouracil). The procedure involved the preparation of a 1 $nmol \cdot mL^{-1}$ urine drug solution further spiked with the appropriate amount of chosen substance to give a final concentration of 10 $nmol \cdot mL^{-1}$ urine for each compound.

RESULTS AND DISCUSSION

In the present study, a straightforward and accurate HPLC method with post-column iodine-azide reaction as a detection system for determination of BTU in urine samples was applied. In this part of the article the most relevant points of the study are present.

Optimization of the HPLC with Post-Column Reaction System

To establish optimal conditions for HPLC determination with the post-column iodine-azide reaction, a wide range of parameters, which can lead to the highest iodine consumption needed to be considered. These parameters include iodine in the potassium iodide solution concentration and its flow rate as well as the Post-column Reaction Module temperature. However, there are also a number of factors with an impact on the separation and detection process alike, which include flow rate of the mobile phase, pH, and concentration of the sodium azide solution. Only the type of the column has an impact on the separation process.

The BTU samples were successfully separated on the Symmetry C_{18} analytical column. Several mobile phases consisting of different concentrations of acetonitrile and sodium azide (varied with concentration, pH) with different volume ratios were tested.

For chromatographic separation with iodine-azide post-column reaction as a detection system, the composition of the mobile phase and its

flow rate, as well as concentration and pH of the sodium azide solution, were optimized as a compromise between the resolution and the detection system intensity.

The effect of sodium azide buffer pH is mainly found in iodine consumption. There are some restrictions applied to pH when it comes to sodium azide solution. At solutions of pH lower than 5.5, the emission of poisonous, volatile hydrazoic acid is detectable. Above pH of 8.0 the formation of iodate (I) prevents the iodine-azide reaction from occurring. Consequently, a pH range 5.5–8.0 was chosen for BTU analysis. According to the results obtained, peak area decreases with increasing pH value within the range 5.5–8.0. Thus, the value of 5.5 was selected as the optimal pH and gave the best results. It was shown that volumetric titration[18,19] in aqueous medium demonstrated similar results.

To conduct the iodine-azide reaction properly, the contact time between eluate (containing BTU and azide ion) and the post-column reaction reagent (containing iodine solution) should be long enough in order to complete the reaction. These results confirm the suggestion that the contact time was long enough to complete the iodine-azide reaction induced by BTU when the mobile phase was delivered at $0.7\,mL \cdot min^{-1}$, the maximum iodine consumption was recorded.

Acetonitrile: 2.5% sodium azide solution (pH 5.5): water (30:50:20, v/v/v) at a flow rate of $0.7\,mL \cdot min^{-1}$ was found to be an appropriate mobile phase for the separation of the tested compound within a run time of 6.6 minutes in isocratic mode with reasonable bandwidth (less than 0.5 min.). Several sources of urine sample were checked and in each case the BTU peak was well separated from blank urine peak (Figure 1).

In the case of sulfide ion determination, high potassium iodide concentration hampers the course of the iodine-azide reaction.[21] Potassium iodide solution concentration within the range $0.2–50\,mmol \cdot L^{-1}$ was applied to establish the influence of the iodide concentration on the area of BTU peak. The experiments were performed under constant concentration and a flow rate of iodine solution. An increase in the peak area was observed in the range $0.2–10\,mmol \cdot L^{-1}$. This was attributed to a further shift in the equilibrium iodine/iodide ions to the right, which in consequence led to the increase in the triiodide ions concentration. The recorded absorbance was higher with a constant iodine concentration. There was no change in the peak area within the range 10–50 of $mmol \cdot L^{-1}$. This proved that the course of the iodine-azide reaction is not under the effect of iodide ions when this range is concerned. As a consequence, the value of $10\,mmol \cdot L^{-1}$ for potassium iodide was found to be optimal for further research.

The flow rate of iodine solution in the range $0.1 - 0.6\,mL \cdot min^{-1}$ was assessed with constant iodine and iodide ions concentration. The oxidation of BTU in the iodimetric reaction was quicker at high amounts of

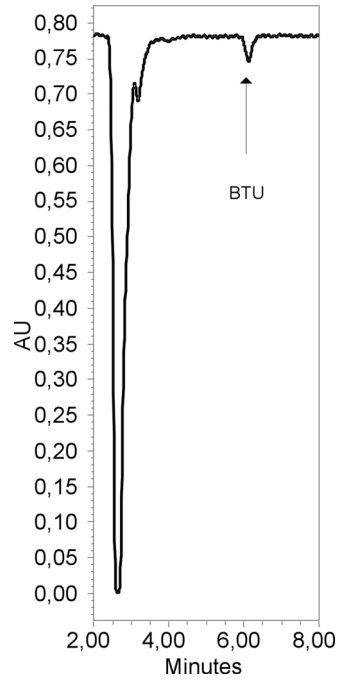

Figure 1. Chromatogram obtained for BTU in spiked urine sample (0.4 $nmol \cdot mL^{-1}$ urine) with iodine-azide reaction procedure detection (for chromatographic and postcolumn reaction conditions see text).

iodine; thus the reaction took part in the induced reaction for a shorter time with a decrease of iodine consumption (increased detection limit). A flow rate of $0.3\,mL \cdot min^{-1}$ was chosen as the iodine consumption in the iodine-azide reaction induced by BTU, which was highest at this flow-rate (highest peak area).

There was no impact of the iodine solution concentration on the BTU peak area in the range of $0.1–0.5\,mmol \cdot L^{-1}$. It was noted that an iodine concentration of 20–30% of the initial quantity gave the highest iodine consumption in the iodine-azide reaction.[18] Applying lower concentration of iodine led to the higher rates of reaction[25] and lower detection limit. When lower iodine concentration was checked the higher reaction rate was obtained that led to a lower detection limit. However, very low concentrations of iodine resulted in complete consumption of iodine in the induced reaction. This caused the absorbance decreased level of ca. 0 AU at which there was no proportional relation between the peak area and BTU amount. Since iodine concentration did not have an impact on BTU peak area, then iodine solution concentration

(applying $10\,\text{mmol}\cdot\text{L}^{-1}$ potassium iodide solution concentration and postcolumn reagent rate of $0.3\,\text{mL}\cdot\text{min}^{-1}$) should be maintained in order to achieve absorbance that is within the range 0.7–0.8 AU. The optimal concentration of iodine solution was found to be $c(\text{I}) = 0.4\,\text{mmol}\cdot\text{L}^{-1}$.

Iodine solution concentration of $0.4\,\text{mmol}\cdot\text{L}^{-1}$ in $10\,\text{mmol}\cdot\text{L}^{-1}$ potassium iodide solution with flow rate of $0.3\,\text{mL}\cdot\text{min}^{-1}$ at controlled temperature of 35°C was found to be an appropriate postcolumn reagent for the iodine-azide reaction induced by BTU.

Validation

Method validation was based on the following parameters: linearity, precision, detection, quantitation limit, and solution stability, as well as accuracy.

To establish detector response linearity, fourteen standard samples within the range 0.4–4.5 $\text{nmol}\cdot\text{mL}^{-1}$ urine were fixed, injected, and replicated four times. Under established conditions there were no interfering peaks, which disturbed BTU in the urine chromatogram. Intra and inter assay precision and accuracy based on peak area ratios are presented in Table 1. Good recoveries were also obtained (Table 1). No significant differences were observed between amounts of BTU added and the amounts found.

Standard curves consistently gave r^2 value above 0.995 within a calibration range of the analyte. The equation obtained by least squared regression was $A = 785.9c + 61.2$, where A is the peak area [$\text{mV}\cdot\text{s}$] and c is the concentration of BTU ($\text{nmol}\cdot\text{mL}^{-1}$ urine). The calibration range can easily be extended upwards if required.

The value of 0.3 $\text{nmol}\cdot\text{mL}^{-1}$ urine was found to be the estimated limit. The lower limit of quantitation (LLQ) was found to be 0.4 $\text{nmol}\cdot\text{mL}^{-1}$ urine at relative standard deviation (RSD) of 1.4% ($n = 4$).

To show the advantage of the iodine–azide reaction as the postcolumn detection system, UV detection of BTU ($\lambda = 275\,\text{nm}$) was checked in the HPLC system. The BTU sample was injected using the same chromatographic conditions for postcolumn detection system, but the iodine solution was not supplied to the HPLC system (Figure 2). A calibration curve was linear in the range $30 - 200\ \text{nmol}\cdot\text{mL}^{-1}$ urine (Table 2). The detection limit using UV detection was 20 $\text{nmol}\cdot\text{mL}^{-1}$ urine. At 30 $\text{nmol}\cdot\text{mL}^{-1}$ urine the percentage deviation from the nominal concentration and R.S.D. were both lower than 3%, and this level of concentration was recognized as the lower limit of quantitation in UV detection. Comparing the iodine–azide detection system with the UV detection method, one may conclude that the linear range of BTU concentration is much wider and lower using the iodine–azide reaction as a postcolumn

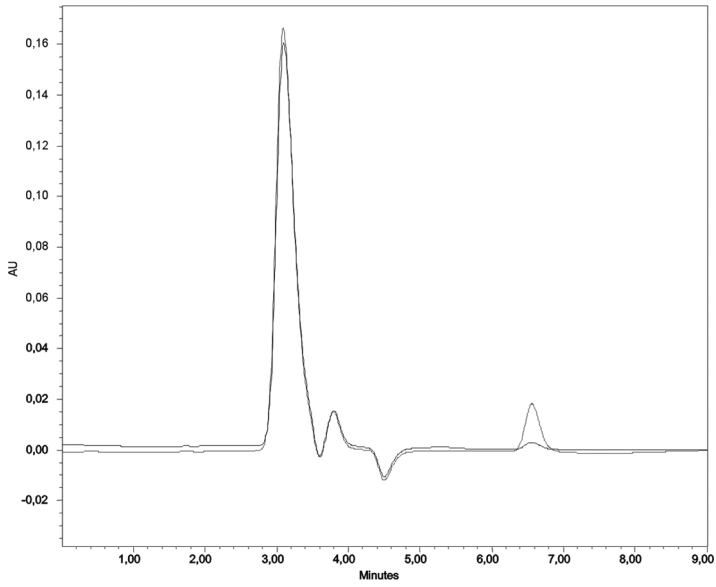

Figure 2. Chromatogram obtained for BTU in spiked urine samples (30 and 200 $nmol \cdot mL^{-1}$ urine) with UV procedure detection (for chromatographic conditions see text).

detection system. The primary advantage of the postcolumn method using the iodine–azide detection procedure is ca. 75-fold reduction in the lower limits of and quantitation when compared to UV detection.

Interferences and Stability

It was possible to eliminate matrix interferences by shifting the detection wavelength from the $\lambda = 275\,nm$ (corresponding to BTU absorption) to the $\lambda = 350\,nm$ (iodine adsorption). In general, applying iodine-azide detection system allows only sulfur (II) compounds to be visible on chromatograms. However, four groups of additional peaks may be found due to certain compounds: iodine-azide reaction inductors (e.g., cysteine or cystine), which generate negative peaks, compounds that react with iodine under experimental conditions (e.g., ascorbic acid), which generate negative peaks, compounds which react with iodide ions (e.g., bromate (V), iodate (V), nitrate (III)) that generate positive peaks, and compounds that absorb at 350 nm (e.g., sulfasalazine).

Although there are particular sulfur (II) compounds which can be found in urine samples such as cysteine, cystine, homocysteine,

glutathione, methionine, thiocyanate, ascorbic acids, but they eluate in the front of the mobile phase in RP-HPLC mode. The thiouracil derivatives have different retention times then the studied compounds. Possible interference may appear only if the retention times are similar then the studied compounds, which is not the case in this procedure.

BTU in the urine sample was monitored at 4°C (in analysis conditions) and 37°C (in body conditions) for 5 hours. The decay of BTU in urine was not observed within the studied time.

CONCLUSION

Operating an HPLC system with a postcolumn iodine-azide reaction system can be as routine as regular LC. The benefits from this LC/postcolumn combination include greatly improved sensitivity and enhanced selectivity for BTU that would normally be much more difficult to detect with other techniques. The sample preparation presented has two advantages: minimal sample pretreatment (one step process of dilution) and no internal standard are required.

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REFERENCES

1. Jorgensen, E.C. Thyroid hormones and antithyroid drugs. In *Medicinal Chemistry*, Burger, A., Ed.; Wiley-Interscience: New York, 1970; 838–858.
2. Burrow, G.N. Thyroid function and hyperfunction during gestation. Endocr. Rev. **1993**, *14*, 194–202.
3. Hachicha, M.; Kammoun, T.; Romdhane, W.B.; Abdallah, R.B.; Mahfoundh, A.; Kammoun, K.; Hachicha, J.; Triki, A. Vasculitis with renal involvement and antineutrophil cytoplasmic antibodies (ANCA) in a child receiving benzylthiouracil. Nephrol. Therapeut. **2007**, *3*, 147–151.
4. Jarraya, F.; Abid, M.; Jlidi, R.; Mkaouar, K.; Mnif, M.; Kharrat, M.; Charfeddine, K.; Kammoun, K.; Hmida, M.B.; Hachicha, J. Myeloperoxidase-antineutrophil cytoplasmic antibody-positive crescentic glomerulonephritis associated with benzylthiouracil therapy: Report of the first case. Nephrol. Dial. Transplant. **2003**, *18*, 2421–2423.
5. Pinzauti, S.; Dal Piaz, V.; La Porte, E. Potentiometric determination of antithyroid drugs in tablets. Farmaco Edizione Practica. **1973**, *28*, 396–402.

6. Ciesielski, W.; Zakrzewski, R. Potentiometric titration of 2-thiouracils. Arch. Pharm. Pharm. Med. Chem. **1998**, *331*, 371–372.
7. Ciesielski, W.; Zakrzewski, R. Iodimetric determination of 2-thiouracils. Chem. Anal. **2000**, *45*, 135–144.
8. Ciesielski, W.; Kasprzak, M. Pulsepolarographic determination of 6-benzyl-2-thiouracil. Acta Polon. Pharm. **2000**, *57*, 337–339.
9. Kasprzak, M.; Ciesielski, W.; Skrzypek, S. Cathodic stripping voltammetry of 2-thiouracils. Collect. Czech. Chem. Commun. **2005**, *70*, 188–197.
10. Watson, D.G.; Bates, C.D.; Skellern, G.G.; Mairs, R.; Martin, S. The analysis of thiocarbamides by gas chromatography/negative-ion chemical-ionization mass spectrometry. Rapid Comm. MS. **1991**, *5*, 141–142.
11. Moretti, G.; Amici, M.; Cammarata, D. Determination of methylthiouracil and analogous thyrostatics in animal tissue by high-performance liquid chromatography after solid-phase purification. Riv. Soc. Ital. Sci. Aliment. **1986**, *15*, 35–40.
12. Zatón, A.; Martinez, A.; de Gandaris, J.M. Study of binding of benzylthiouracil to human serum albumin by gel filtration chromatography. J. Liq. Chromatogr. **1987**, *10*, 899–908.
13. Moretti, G.; Amici, M.; Cammarata, P.; Fracassi, F. Identification of thyrostatic drug residue in animal thyroids by high-performance thin-layer chromatography and fluorescence reaction detection. J. Chromatogr. A **1988**, *442*, 459–463.
14. Giannessi, P. Examination of derivatives of thiouracil in feed. Ann. Staz. Chim. Agrar. Sper. Roma. Ser III **1961**, *190*, 6–10; Chem. Abstr. 1963, *58*, 3830g.
15. Carbonell, M.G.; Saldana, M.L.; Nanez, O.P. Simultaneous separation and identification method for nine compounds with antithyroid properties in mixed feeds and premix for steers. An. Inst. Nac. Inrest. Agrar. Ser. Ganad. **1984**, *21*, 134–141.
16. Zakrzewski, R.; Ciesielski, W. Application of improved iodine-azide procedure for the detection of thiouracils in blood serum and urine with planar chromatography. J. Chromatogr. B **2003**, *784*, 283–290.
17. Sánchez, Ballesteros, J. Analysis of antithyroid substance in cattle. Acta Cientifica Venezolana. **1982**, *33*, 152–157.
18. Kurzawa, J. The iodine-azide reaction induced by mercaptopyrimidines and its application in chemical analysis. Chem. Anal. **1987**, *32*, 875–890.
19. Kurzawa, J. Determination of micro- and nanogramme amounts of 2-thiouracil and its 6-substituted derivatives by induced iodine-azide reaction. Quim. Anal. **1985**, *4*, 117–128.
20. Kurzawa, J.; Wiśniewska, A.; Janowicz, K. Stopped-flow determination of 2-mercaptopyrimidines a inductors of the iodine-azide reaction. Anal. Chim. Acta **2006**, *567*, 286–292.
21. Miura, Y.; Fukasawa, K.; Koh, T. Determination of sulfur anions at the ppb level by ion chromatography utilizing their catalytic effects on the postcolumn reaction of iodine with azide. J. Chromatogr. A **1998**, *804*, 143–150.

22. Miura, Y.; Watanabe, M. Ion-pair chromatography of polythionates and thiosulfate with detection based on their catalytic effects on the postcolumn azide iodine reaction. J. Chromatogr. A **2001**, *920*, 163–171.
23. Zakrzewski, R.; Ciesielski, W. Application of iodine-azide reaction as postcolumn reaction in HPLC for determination of 2-thiobarbituric acid. Chromatogr. **2004**, *59*, 683–689.
24. Zakrzewski, R.; Ciesielski, W. Determination of thiopental in urine sample with high-performance liquid chromatography using iodine-azide reaction as a postcolumn detection system. J. Chromatogr. B **2005**, *824*, 327–332.
25. Kurzawa, J. Determination of micro- and nonogram amounts of sulfur (II) compounds by flow continuous analysis with application of the induced iodine-azide reaction. Chem. Anal. **1988**, *33*, 771–777.

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