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Short communication

Determination of thiopental in urine sample with high-performance liquid chromatography using iodine–azide reaction as a postcolumn detection system

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Abstract

The reaction between iodine and azide ions induced by thiopental was utilized as a postcolumn reaction for chromatographic determination of thiopental. The method is based on the separation of thiopental on an Nova-Pak® CN HP column with an acetonitrile–aqueous solution of sodium azide as a mobile phase, followed by spectrophotometric measurement of the residual iodine ($\lambda = 350$ nm) from the postcolumn iodine–azide reaction induced by thiopental after mixing an iodine solution containing iodide ions with the column effluent containing azide ions and thiopental. Chromatograms obtained for thiopental showed negative peaks as a result of the decrease in background absorbance. The detection limit (defined as S/N = 3) was 20 nM (0.4 pmol injected amount) for thiopental. Calibration graphs, plotted as peak area versus concentrations, were linear from 40 nM. The elaborated method was applied to determine thiopental in urine samples. The detection limit (defined as S/N = 3) was 0.025 nmol/ml urine. Calibration graphs, plotted as peak area versus concentrations, were linear from 0.05 nmol/ml urine. Authentic urine samples were analyzed, thiopental was determined at nmol/ml urine level.

Keywords: Iodine–azide reaction; Thiopental; HPLC

1. Introduction

Thiopental, 5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid is an ultrashort-acting intravenous anesthetic [1]. It is frequently used clinically in the treatment of intensive care patients suffering from severe head injuries and in the management of intracranial hypertension [2]. A fatal case involving mixtures of chemicals including thiopental [3], a suicide by thiopental injection [4,5] and drug-facilitated sexual assault [6] were reported. Monitoring its concentration in body fluids is important also for the optimization of pharmacotheraphy. Although thiopental is typically excreted in urine at high percentage in metabolized form, there remains about 1% of the unchanged drug which can be highly useful for screening and identification of the thiopental [7–9].

High-performance liquid chromatography has been well established as an analytical technique for determination of a wide range of drugs in variety of complex matrices. Several high-performance liquid chromatographic methods have already been reported for determination of thiopental in plasma or serum using UV detection around $\lambda = 280$ nm [10–16] or mass spectrometry [8]. Main practical problems in sample treatment prior to drug analysis in biological fluids are related with matrix elimination, which has to carried out as preclean chemistry protocol in order to avoid sticking the column by protein and to obtain low process blanks which demand mobile phase systems of high purity and good sample clean-up of the biological sample. The postcolumn photochemical reaction was also applied for determination of the drug but it cannot be detected with sufficient sensitivity

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[17]. The described photoreactor does not decrease column efficiency.

The iodine–azide reaction induced selectively by sulphur(II) compounds is widely used for their determination by varied analytical techniques such as measurement of nitrogen evolved during the reaction, coulometry, flow injection analysis, titrimetry, enthalpimetry, and kinetic methods with amperometric or spectrophotometric detection. Lately, the iodine–azide reaction has been applied as postcolumn reaction detection system [18,19]. The determination is based on the separation of sulphur(II) compounds with chromatographic column and then measurement ($\lambda = 350$ nm) of the unreacted iodine in the iodine–azide reaction. The reaction takes place only in the presence of a sulphur(II) compound (selective induction). When constant concentrations of iodine solution in a postcolumn reagent and azide ions in the mobile phase are supplied to the HPLC system, a constant absorbance is maintained and recorded as a background from iodine absorption. The signal decreases when a sulphur(II) compound appears in the sample, due to consumption of iodine in the iodine–azide reaction. The induction activity is detected as a negative peak photometrically at $\lambda = 350$ nm. The peak area are proportional to the amount of the sulphur(II) compound.

For the determination of thiopental in urine samples, it is necessary to use a selective and sensitive detection system such as iodine–azide reaction because of the low level of thiopental present in urine and also to prevent interference from the complex sample matrix. In this study, we describe a simple, rapid, reliable and specific method for determination of thiopental (as a standard solution and in urine sample) based on high-performance liquid chromatography and iodine–azide reaction as the detection system which combines both the specificity and selectivity of chromatographic separation and iodine–azide reaction as the postcolumn detection system. The sample preparation we present has two advantages: it is a one step process and no internal standard is required.

2. Experimental

2.1. Chromatographic system

A flow diagram of the chromatographic system used in this study is shown in Fig. 1. The analytical column was a Nova-Pak® CN HP (150 mm × 3.9 mm i.d., 5 μm, Waters). The flow-rate was 1 ml/min at ambient temperature. Eluate from the Postcolumn Reaction Module (Waters) was monitored at a wavelength of $\lambda = 350$ nm using a variable wavelength LC spectrophotometer (Waters 2487 Dual λ). The mobile phase delivery system was a Multisolvent Delivery System Model 600E (Waters). Test samples were applied to the HPLC column with a Rheodyne 7725i injector. Reagent Manager (Waters) was used as a single-piston, pulse-dampened pumping system for postcolumn reagent delivery to the Postcolumn Reaction Module (the reaction tube, 4 m × 0.25 mm i.d.) (Waters). The temperature of the postcolumn iodine–azide reaction was controlled by temperature control system (Waters). The chromatographic system and recorder were connected with busSaT/In Module (Waters). The chromatograms were integrated with Millennium^{32} software (Waters).

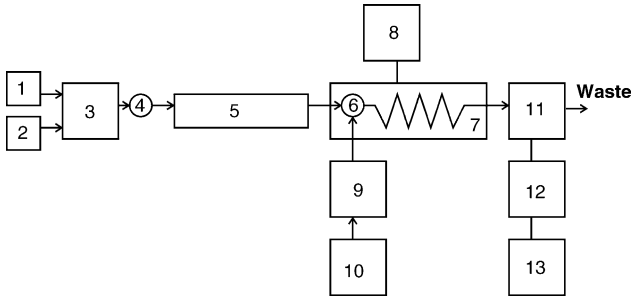

Fig. 1. Flow diagram system with iodine–azide procedure detection. (1): sodium azide solution, (2): acetonitrile, (3): pump, (4): injector valve, (5): analytical column, (6): mixing tee, (7): postcolumn reaction module, (8): temperature control system, (9): pump, (10): iodine solution in potassium iodide solution, (11): LC spectrophotometer, (12): busSaT/In module and (13): computer.

2.2. Chemicals and solutions

Thiopental was purchased from Biochemie (Kundl, Austria). Sodium azide, hydrochloric acid, arsenic(III) oxide, iodine, potassium iodide, ethylenediaminetetraacetic acid disodium salt (EDTA), HPLC-grade methanol and acetonitrile were obtained from Aldrich (Steinheim, Germany) or LAB-SCAN Analytical Sciences (Dublin, Ireland).

All the solutions were freshly prepared daily. The water used in preparation of solutions was triple distilled and then degassed.

2.2.1. Solution

A stock thiopental solution: 100 μmol thiopental was dissolved in 1 ml 1 M sodium hydroxide solution and diluted to 100 ml with water. Working standard thiopental solutions (20 nM–1 μM) were prepared by appropriate serial dilution of stock solution with water. A sodium azide solution: 1.5 g sodium azide was dissolved in water and hydrochloric acid was added to obtained pH 7.8 and then was adjusted to 0.5 l with water. An iodine solution: 6.3 g iodine and 20 g potassium iodide were dissolved and were adjusted with water to 0.5 l. To 200 μl of the solution mentioned above 0.657 g of potassium iodide was added and diluted with water to 0.2 l.

2.3. Sample collection of urine and processing

To 4 ml of urine, 0.75 ml 0.1 M EDTA and specified amount of thiopental were added and then diluted with methanol to 10 ml. The sample was processed further as in Section 2.4.

The calibration curve was prepared by adding the known amount of thiopental (within the range 0.2–10 nmol) to thiopental-free urine sample and assaying in the manner described in Section 2.4. The peak area of thiopental is plotted against urine concentration of thiopental.

0.75 ml 0.1 M EDTA was added to a real-world urine sample and diluted with methanol to 10 ml. If necessary, the precipitation was filtered. The filtrate was processed further as in Section 2.4. The volume of urine sample was adjusted as the thiopental peak high was ca. 0.1 AU. Aliquots of the urine sample were taken, and 0.5, 0.75 and 1 nmol of thiopental were added to aliquots. Proper dilution and quantities of thiopental added was read from the calibration curve. The samples were assayed in the way described in Section 2.4. The thiopental peak area is plotted against amount of added thiopental into urine samples. The relationship between peak area and thiopental concentration added was linear and was extended backward. The amount of thiopental was read using standard addition technique.

2.4. Recommended procedure

2.4.1. Iodine–azide procedure detection

A 0.3% sodium azide solution buffered to pH 7.8 was mixed with acetonitrile (84:16) and then was pumped as an eluent at flow-rate of 1.0 ml/min. A 20 μl aliquot of a thiopental standard solution or a thiopental urine sample solution was injected into a separating column. The effluent from the column was mixed with 0.2 mM iodine solution in 20 mM potassium iodide solution pumped with flow-rate of 0.3 ml/min. The mixture obtained was passed through the postcolumn reaction module maintained at 30 °C, in which iodine–azide reaction induced by thiopental took place. The absorbance of the residual iodine was measured at λ = 350 nm.

2.4.2. Spectrophotometric detection

Spectrophotometric detection was performed using chromatographic conditions described in Section 2.4.1. The effluent from the column was monitored by an ultraviolet detector set at λ = 275 nm. Thiopental has an UV–vis absorption maximum at this wavelength in studied chromatographic conditions.

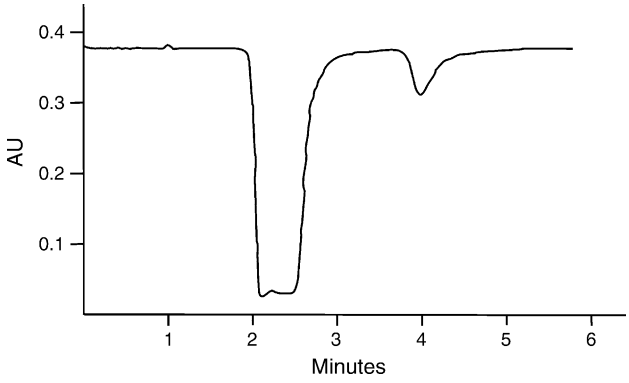

Fig. 2. Chromatogram obtained for thiopental in patients' urine sample (3.49 nmol/ml urine) in urine with iodine–azide reaction procedure detection method (for chromatographic conditions see text).

3. Results and discussion

3.1. Validation

A standard solution (20 μl) containing known amount of thiopental was treated as described in Section 2.4. No interference was observed in the region of interest where the analyte was eluted, as shown in the thiopental in real word urine (1 ml) sample chromatograph Fig. 2. The results are reported in Table 1.

The relationship between detector response and thiopental concentration was continuous and reproducible and was demonstrated using nine-point calibration curves (the amount of thiopental versus peak area). The calibration curves were linear in the range 40–1000 nM (for 20 μl loop) for standard solution sample and 0.05–2.5 nmol/ml urine. This cal-

Table 1
Determination of thiopental with iodine–azide procedure detection ($n = 5$)

	Taken [pmol]	Found [pmol] $\bar{x} \pm t_{0,95}\bar{s}$	R.S.D. [%]	Recovery [%]
Thiopental standard solution	0.800	0.765 ± 0.063	6.7	96
	1.20	1.11 ± 0.06	4.2	92
	1.60	1.54 ± 0.07	3.7	96
	2.00	1.98 ± 0.07	2.9	99
	4.00	4.04 ± 0.08	1.7	101
	8.00	8.06 ± 0.10	1.1	101
	12.0	12.3 ± 0.1	0.6	102
	16.0	16.0 ± 0.2	1.3	100
	20.0	19.8 ± 0.3	1.1	99
Thiopental in urine	0.800	0.926 ± 0.040	3.5	116
	1.20	1.09 ± 0.03	2.2	90
	1.60	1.56 ± 0.04	2.1	97
	2.00	1.90 ± 0.16	6.9	95
	6.00	6.15 ± 0.23	2.9	102
	20.0	20.0 ± 0.5	1.9	100

ibration range can be easily extended upwards if required. The equations for the linear regression line and coefficient of correlation for the peak area ratios (mAUs) was $y = 0.2010x - 0.0015$, $r^2 = 0.9996$ for standard solution sample (pmol) and $y = 0.1957x - 0.0311$, $r^2 = 0.9998$ for urine sample (pmol).

The detection limit, established as the concentration required to generate a signal-to-noise ratio of 3, was estimated by analyzing solutions of decreasing concentration of thiopental. Before analyzing each sample, water was processed in such way. It was confirmed that there were no contaminants. The values obtained were 20 nM (0.4 pmol on-column) and 0.025 nmol/ml urine. The lower limit of quantitation is reported as the analyte amount required to generate a signal that is six times the standard deviation of the background signal. At 40 nM (0.8 pmol on-column) for both standard solution and 0.05 nmol/ml urine the percentage deviation from the nominal concentration and the R.S.D. were 6.7% for standard solution and 3.5% for urine solution and these concentration levels were recognized as the lower quantitation limits.

Intra-run imprecision and inaccuracy for the assay of thiopental – expressed in RSD were determined. Suitable values are shown in Table 1.

To show the advantage of the iodine–azide reaction as the postcolumn detection system, we checked UV detection of thiopental ($\lambda = 275$ nm) in our HPLC system. Thiopental sample was injected using the same chromatographic conditions for postcolumn detection system, but iodine solution was not supplied to the HPLC system. A calibration curve was linear in the range 3–20 μM of thiopental. The detection limit using UV detection was 0.6 μM. At this concentration the signal-to-noise ratio was 3. At 1 μM the percentage deviation from the nominal concentration and R.S.D. were both lower than 3% and this level of concentration was recognized as lower limit of quantitation in UV detection. Comparing the iodine–azide detection system with UV detection method, one may conclude that the linear range of thiopental concentration is much more wide and lower using iodine–azide reaction as a postcolumn detection system. The primary advantage of the postcolumn method using iodine–azide detection procedure is ca. 300-fold reduction in the detection limit and determination limit when compared to UV detection.

Developed procedure was applied to determination of thiopental in real-word urine samples on the basis of three patients' examples. The details concerning the determination is shown in Table 2. There is some relationship between the dose value and determined thiopental concentration in authentic urine sample but exhaustive studies of the matter were not the object of our research.

Table 2
Determination (nmol/ml urine) of thiopental in real-word urine samples

Dose [mg/h]	Sex	Found	R.S.D. [%]
50	Female	0.36 ± 0.03	2.9
140	Male	0.77 ± 0.02	1.3
200	Male	3.49 ± 0.03	3.6

3.2. Influence of various factors on determination of thiopental

There are several parameters common to HPLC with postcolumn detection system which control the optimum performance of determination. Some of them influence only the separation process (i.e. stationary phase), others influence only iodine–azide detection procedure (i.e. concentration of iodine and iodide ions, flow-rate of iodine in potassium iodide solution, temperature of postcolumn reaction module), but most of them influence both (i.e. composition and pH of mobile phase, flow-rate of mobile phase). These parameters were separately evaluated to develop optimized determination conditions. To optimize postcolumn reaction, the conditions should lead to the highest consumption of iodine in the induced reaction.

3.2.1. Types of stationary and mobile phases

Three columns were investigated (CN, Ph and C_{18}) to obtain optimal separation conditions. The CN-column was chosen as it gave higher peaks than the ones obtained with the Ph- and C_{18}-column. The retention times obtained with three columns significantly depend on the type of the stationary phases. They are 4, 20 and 27 min. for CN-, Ph- and C_{18} -column respectively. The CN-column was chosen as the retention data is smaller and the resolution of thiopental peak from urine peak is satisfactory.

The effect of the composition of the mobile phase on the induction of iodine–azide reaction was investigated. An addition of organic solvent into the mobile phase lowers the consumption of iodine in the induced reaction. The consumption is higher in the case of acetonitrile added to the mobile phase than when methanol is used (data not shown). Acetonitrile was chosen as an organic cosolvent so as to obtain the lowest detection system. Using CN-column with acetonitrile within the range 14–18% gives good separation of thiopental and the other peak in the urine sample.

3.2.2. Concentration of acetonitrile in mobile phase

The influence of the acetonitrile concentration in the mobile phase was investigated. The optimal conditions were achieved when acetonitrile and sodium azide solution were mixed in the ratio 16:84 (v/v) when CN-column was used. In these conditions peaks are shaped well and the peak widths are smaller than 0.4 min.

The retention times for thiopental peaks do not change considerably within the range of acetonitrile 5–20% using CN-column (the range of retention time is 3.7–4.8 min).

The consumption of iodine in an induction reaction is higher when the concentration of acetonitrile in the reaction medium decreases (data not shown). To obtain lower detection limit, the CN-column was chosen and acetonitrile as an

organic modifier mobile phase. Applying this chromatogram system leads also to the shortest analysis time.

The influence of the concentration of acetonitrile in the mobile phase on the postcolumn reaction course was investigated maintaining the constant concentration of sodium azide. The studies were performed in the system shown in Fig. 1, but the separating column was withdrawn. The optimal conditions were achieved when the percentage of acetonitrile in the mobile phase was 16. Oddly, some addition of organic solvent increases the iodine consumption in iodine–azide reaction. Similar effect was observed in the case the iodine–azide reaction induced by Vitamin B_1 in methanol–water mixture [20].

3.2.3. Concentration of sodium azide in mobile phase

Sodium azide in a neutral pH buffer is widely used to prevent bacterial growth. It does not interact with proteins or change their chromatographic behaviour. It cannot be used in ion exchange chromatography since it binds to anion exchangers and blocks binding sites. Sodium azide cannot be added into a mobile phase applied to the determination of sulphur anions [18,19]. It was supplied together with iodine as a postcolumn reagent and diluted into the postcolumn reaction tube with the mobile phase. It leads to the employment of high concentration of sodium azide (10%) to complete the postcolumn reaction [18,19]. Because of its poisonous properties it should be used in very small amounts. To prevent the dilution of sodium azide (leading to smaller consumption of iodine in the induced reaction) and also to avoid the need to change the buffer for separation process and postcolumn detection system, sodium azide was used as a buffer and was passed through the separating column in our research.

To measure the effect of the azide solution concentration on thiopental peak area, azide solutions in the range of concentration 0.1–1.5% were employed. The optimal concentration of sodium azide was chosen to be 0.3%.

The retention times of the thiopental peak (standard solution and in urine sample) and those of thiols (in urine) do not change considerably within the range of sodium azide concentration 0.1–1.5% using CN-column with acetonitrile as a modifier of the mobile phase.

3.2.4. The pH of sodium azide solution

Sodium azide solution pH is the factor which influences both separation and the course of iodine–azide reaction. The consumption of iodine in the induced reaction has been found to be dependent on pH reagent solution [21]. The use of solution with a pH lower than 5.5 is not recommended because of the emission of the poisonous, volatile hydrazoic acid. Above pH 8.0 the catalytic reaction does not proceed since iodine forms iodate(I), which is not a reagent in iodine–azide reaction. In the case of thiopental, peak area increases with increasing pH value within the range 5.5–7.7 and decrease with increasing pH value within the range 7.8–8.0. The same relationship is observed in the case of the consumption of iodine in the induced reaction measured in aqueous medium [21]. Considering the influence of all parameters on the course of the iodine–azide reaction and chromatographic separation, pH in the range 7.7–7.8 was chosen as the most favourable pH for the determination of thiopental.

The retention times for thiopental peaks do not change within the range of pH of sodium azide solution 5.5–8.0 using CN-column with acetonitrile as a modifier of the mobile phase.

3.2.5. The flow-rate of mobile phase and iodine solution as the key to optimise the reaction time of postcolumn reaction

The contact time between the eluate (containing thiopental and azide ion) and the postcolumn reaction solution (containing iodine solution) is very important for the reaction to proceed sufficiently. The flow injection analysis showed a decrease in the peak area with an increase in mobile phase flow rate within the rate 0.5–2.2 ml/min. This relationship confirms that the contact time mentioned above is lower than the induction time of iodine–azide reaction induced by thiopental. It is necessary to optimize the reaction time as this depends on the mobile phase, iodine solution flow-rate and the distance from the column tip to the detector cell. A study of the flow rate of the carrier streams was conducted.

Dependence of the thiopental peak area on the mobile phase flow-rate was examined in the range 0.5–1.5 ml/min. The retention time changed within range 7.2 min (for the flow-rate 0.5 ml/min)–2.2 min (for the flow rate 1.5 ml/min). The highest peak was obtained when the mobile phase was pumped with the flow-rate 1 ml/min. The retention time obtained in these conditions was 3.6 min. The resolution of thiopental and thiols (in urine sample) does not depend on the flow rate and it value is within the range 1.5–1.8 ml/min.

The results showed a decrease in the peak area with its increase in iodine solution flow rate over 0.5 ml/min. It means that thiopental has not enough time for induction of the iodine–azide system (the reaction is incomplete). The pump applied to the iodine solution gives some background noise on the chromatogram. Applying high flow-rate of iodine solution gives the noise peak area, so it was necessary to measure the dependence of detection limit on the ratio of thiopental peak area and noise peak area. The highest signal-to-noise ratio was obtained when the iodine solution was pumped with a flow-rate of 0.3 ml/min.

3.2.6. The concentration of iodine and iodide ions

The concentrations of iodine and iodide ions are factors which depend only on the postcolumn reaction. The iodine in potassium iodide solution is supplied to the system after the separation step (Fig. 1.). The concentration of iodine solution does not influence peak area for thiopental in the range 0.1–0.4 mM. The concentration of iodine solution of 0.2 mM [with flow-rate 0.3 ml/min and c(KI) = 20 mM] was chosen, then the absorbance was ca. 0.5 AU.

In our research iodide ions influence only the course of iodine–azide reaction. To measure the effect of the concen-

tration of iodide ions on the area of the thiopental peak, potassium iodide solutions in the range of concentration 3–50 mM were employed. At the concentration of potassium iodide within the range 0.2–20 mM, the peak area increase since the concentration of triiodide ions is increased as a result of a further shift of the equilibrium iodine/iodide ions to the right. It gives higher absorbance with a constant concentration of iodine. The peak area stays constant within the range 20–50 mM and it means that iodide ions within this range do not influence the course of iodine–azide reaction induced by thiopental. The optimal concentration of potassium iodide was chosen 20 mM. Higher concentrations were not checked against to volumetric titration data [22].

3.2.7. The temperature of the postcolumn reaction module

The system response was studied by varying the temperature of the postcolumn reaction module within the range 25–50 °C. The peak area increased with an increase in temperature up to 30 °C due to a faster reaction and decreased above 30–50 °C. The temperature applied to postcolumn reaction module was maintained at 30 °C.

3.3. Stability of thiopental in urine

Thiopental in urine sample was monitored at ambient temperature for 3 h. The decay of thiopental in urine was observed after 1 h. This decay appears to be due to poor stability of thiopental in urine resulting from great susceptibility to oxidative reactions. The product of the reaction was not an inductor of the postcolumn reaction and the peak area decreased with time. Addition of 750 μl of 0.1 M EDTA into urine sample causes no significant changes in peak area for 5 h.

3.4. Interferences

A shift of wavelength detection from the UV region (corresponding to thiopental absorption) to the vis region ($\lambda = 350$ nm, iodine absorption) allows to avoid matrix interference. Compounds which do not contain sulphur(II) are not visible in a chromatogram obtained with iodine–azide reaction as a postcolumn detection system. Sulphur(II) compounds present in urine such as cysteine, cystine, homocysteine, glutathione, methionine, thiocyanate and as well as the other compounds which react with iodine e.g. ascorbic acids appear on a chromatogram but they elute with the front of the mobile phase in RP-HPLC mode. Only sulphur(II) compounds which coelute with thiopental, can interfere with its detection.

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