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Simple and fast development and validation of high-performance liquid chromatography method with UV determination of thiopental in rat plasma

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Objective Nowadays old agent thiopental back to hot-topic of research as a new model of neuropharmacology study. Because of its simple, fast, and cost-benefit method of determination, this agent comes with an issue in pharmacokinetic and pharmacodynamics (PK–PD) area of research. Previous study was focused on human plasma and pilot study showed that there is differentiation between human and rat plasma.

Methods Separation was performed on a Nuleodur C18 ec HPLC column (250 × 4.0 × 5 µm), using a mixture of acetonitrile; potassium; dihydrogen phosphate buffer (10 mM, PH 2.7) as the mobile phase delivered at a flow rate of 1.2 mL/min, 280 nm and room temperature was selected for detection of thiopental and 1-naphtylamine as an internal standard. Plasma sample (100 µL) were treated with 180 µL precipitation solution and 20 µL of internal standard. After the mixture were vortexed and centrifuged at 1,000*g*, 40 µL of clear supernatant was directly injected into a 20-µL loop of HPLC apparatus. Calibration curve was fitted by peak area ratio of thiopental to internal standard. All the stages of blood collection were under supervision of Ahvaz ethical committee.

Results Thiopental and internal standard retention time were at 8.3 and 14.5 min, respectively. About 0.5 µg/mL was the level of limit of quantification of our method. The range of 99.4–100.3 was accuracy of the methods. Inter- and intra-day precisions were 4–19% and 6–8%, respectively. A good relationship in the form of the power was found ($r^2 = 0.999$).

Conclusion The presented simple, fast, and cost-benefit method was a great accurate, precise, and sensitive for determination of thiopental in plasma of rat.

Keywords chromatography, liquid, thiopental, plasma, rats

Introduction

Thiopental (5-ethyl-5-(1-methylbutyl)-2-thiobarbituric acid)[1] shown in Fig. 1 is an ultra-short-acting agent of anesthetic, which was used from 1934 to till now.[2] It is also indicated for hypoxic-ischemic injuries and status epilepticus in human.[1] The anesthetic effect of thiopental according to structural activity analysis study is back to malonyl urea ring.[1] There is a controversy about analgesic effect of this agent in pain model in laboratory animals. The recent study is accepting thiopental as cause hyperalgesia.[3] But there was no any pharmacokinetic–pharmacodynamics analysis modeling (PK–PD) that explains this issue with focus of effect by concentration in effect compartment. For a new study, need acceptable method of thiopental determination is important. This finding causes the old agent of anesthetic back to one of hot topic of pharmacology study. Although this drug is not new, but there is no method that is adjusted for determination of this agent in laboratory animal especially rat.

Materials and Methods

Chemicals and reagents

Thiopental (T120000 Sigma-Aldrich European pharmacopeia reference standard, USA) was used as internal standards. 1-Naphtylamine (8222910100 catalogue Num, Merck, Germany) was used as an internal standard. Chromatography grade of methanol and acetonitrile (Merck, Germany) were used as mobile phase.

Blood sample collection

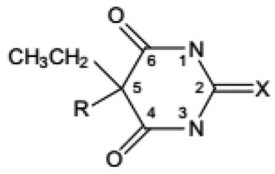
For blood sample collection, 10 male Wistar rats were moved to pharmacology animal house 1 week before the study. Animals were kept in a room with 12 cycles of light and dark. Rats were fed with special food that was adjusted in nutrition for lab animal (Khorak-Dam pars, Tehran, Iran). The water was available for Animal *ad libidum*.[4] Rats were taken to surgery in Department of Pharmacology. The surgery was catheterized with silicone catheter (SIL-C35 SOLOMON, USA). After the surgery, the blood sample was collected. The amount of blood collection was under Ahwaz ethical committee protocol. All types of study on animal parts were done under supervision of Ahwaz lab Animal ethical committee with registry code IR.AJUMS.REC.1395.137.

Preparation of standards samples

Thiopental and internal standard stocks solution were prepared separately by dissolving the accurately weighted standard compounds in methanol to give final concentrations of 1,000 and 70 µg/mL, respectively. The stock solutions were further diluted with mobile phase that mentioned below to achieve the spiking working standards. The spiking standard solutions (20 µL) were used to spike blank rat plasma samples (200 µL), for both calibration curves and QC samples during method validation study. All the working standards were kept at 4°C until the time of analysis.

Chromatography conditions

The chromatography system consisted of a Knaur® Smartline series HPLC pump, Smartline® series photo diode array wave



Compound	R	X
Thiopental racemate (*RS*)	CH_3-CH_2-CH_2-CH-CH_3	S
Thiopental *S*-(–)	H, CH_3-CH_2-CH_2-C*-, CH_3	S
Thiopental *R*-(+)	CH_3, CH_3-CH_2-CH_2-C*-, H	S
Thiopental isomer	CH_3-CH_2-CH-CH_2-CH_3	S
Thiopental carboxylic acid	COOH-CH_2-CH_2-CH-CH_3	S
Thiopental alcohol	CH_3-CHOH-CH_2-CH-CH_3	S

Fig. 1 **Thiopental and isomer structure formulae.**

length UV–visible detector and chromegate® software for data acquisition and integration (Knaur® Corporation, Germany). Chromatography was performed on a Nucleodur® C18 ec column (250 × 4.0 mm² i.d., 5 µm particle size). Thiopental and internal standard were detected at 280 nm. The mobile phase was an isocratic mixture of acetonitrile:methanol:potassium di-hydrogen phosphate buffer (10 mM, pH 2.7) (40:10:50). Flow rate was set at 1.2 mL/min. Chromatography was performed at ambient temperature.

Sample preparation

Plasma samples (100 µL) were treated by addition of 20 µL internal standard (70 µg/mL) and 180 µL modified precipitation solution. After vortex mixing and centrifugation at 10,000 *g*, 50 µL of the clear supernatant was directly injected into the chromatography column.

Results

Photo diode array analysis of sample showed 280 nm was optimized and high sensitive for detection of thiopental and naphtylamine. For better separation between spike wave of plasma and naphtylamine, and gained sharped and suitable shape of 25 cm C18 column was selected.

The selected internal standard (naphtylamine) is well separated from spike wave of plasma of rat and thiopental. In Fig. 2, rat blank plasma was shown and there was not any remarkable interface between thiopental and internal standard as shown in the figure. In Fig. 3, the final optimized condition was shown. Cause retention time of internal standard and thiopental recorded as 8.3 and 14.5, respectively.

For clean-up of plasma, direct precipitation method was used. The optimized solution for plasma clean-up in this technique was combination of methanol, TCA 10%, and zinc

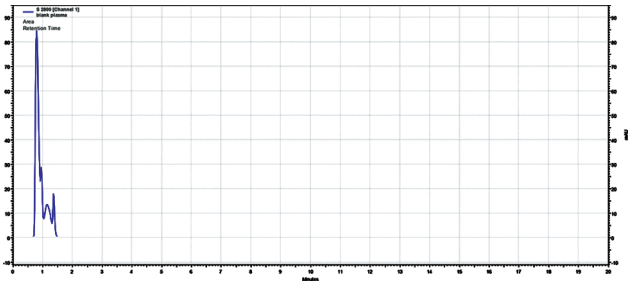


Fig. 2 **Rat blank plasma chromatogram.**

Table 1. **Parameters of the standard curve equations (PAR = a ×c^b)**

Parameter	PAR		
	Value	SE	*P*-value
A	12.2	0.04883	<0.0001
B	0.9889	0.0009	<0.0001
R^2	0.999	–	
Sy.x	2.054	–	
RMSE	2.027	–	
AICc	60.77	–	

sulphate. Our pilot study showed our optimized solution result in good recovery in comparison each of: methanol, TCA 10%, and acetonitrile. Regression analysis was shown that calibration curve was fit with power equation. The equation was $C = \frac{PAR}{12.2}^{\frac{1}{1.9889}}$ that from peak area ratio the amount of concentration is calculated. In the above equation, PAR is the peak area ratio of the external to internal standard, C is the concentration of the standard samples of thiopental sodium. The limit of detection and quantification was 0.001 and 0.05, respectively. Accuracy was in the range of 99.4–100.3 that is shown in Table 2. During the stability studies at different conditions (like three cycles of freeze and defrost) were within 15% of the initial nominal concentration. The method was stable and also instability was seen.

Discussion

All the reports are about to determination and quantification of thiopental in human plasma.[5–8] Gas chromatography (GC) is the main method for detection of thiopental in plasma.[9–11] Although pervious methods were differ in the level limit of quantification (LOQ) in human. Level of LOQ is important in pharmacokinetic (PK) study but in lab animal PK study, this importance is more. By accounting inter-individual variability in rat that cause different dose administration, the amount of dose was not transgression 300 µg (as bolus) per rat. Disposition of this drug is very fast in rats.[12]

In previous GC method, the LOQ was in the range of 5,[13] 10,[14] 25,[11] and 200 µg/L.[10,15]. In previous HPLC UV detection method by organic agent protein precipitation, the LOQ was 0.025–1 mg/L.[16–19] In our study, LOQ was 0.5 µg/mL and have many reasons, such as modified protein precipitation, using internal standard, etc.

Table 2. **Recovery, intra- and inter-day accuracy and precision results ($n = 5$)**

Nominal concentration (µg/mL)	Percision (%)		Accuracy (% ± SD)		Recovery (%±SD)
	Intra-day	Inter-day	Intra-day	Inter-day	
0.05	37	67	99.41 ± 0.54	99.11 ± 0.67	64 ± 7
5	16	11	100.30 ± 0.50	100.10 ± 0.11	85 ± 13
10	2	21	101 ± 0.42	100.30 ± 0.34	89 ± 21
70	9	5	100 ± 0.19	100.00 ± 0.05	88 ± 8
100	2	5	100.2 ± 0.19	100.20 ± 0.05	83 ± 6
Internal Standard (µg/mL)	–	–	–	–	96 ± 6

In human, the major problem is shape of the peak and clean-up procedure for report of determination of thiopental and modification is a major need. UV detection was used based on the presence of malonyl rings in the structure of the tested compound. UV detection is suitable but range of detection changeable, and it seem our methods is most suitable in rat determination of thiopental for pharmacologic study in recent days. The thiopental is present in many model of study in rat and mice. The range of study is more important in basic study because the dose that is administrated in rat or mice is low and pharmacokinetic of this is show fast disposition. A C18 column were used in pervious detection of thiopental in human as stationary phases. High lipophilicity of the thiopental led to either long retention times or broad and bad-shaped peaks on C18 columns. Use of long column and the optimized mobile phase resulted in elution of both the thiopental and the internal standard at reasonable retention times with good shaped peak. Different extraction procedures such as liquid–liquid extraction with ethyl acetate, dichloromethane, diethyl ether and direct protein precipitation with either methanol, TCA or acetonitrile were assessed. Among these methods of sample clean up, protein precipitation with methanol, zinc sulphate, and TCA was selected because of its simplicity, good shaped peak, and acceptable recovery of the analytes. It has been reported that MeOH alone in the ratio of 2:1 of MeOH to plasma sample could result in protein precipitation up to 91.4% of total plasma proteins.20. Several barbiturate such as phenobarbital and so on were tested to be used as internal standard and none of them was not suitable and finally naphtylamine [(Naphthalen-2-yl)amine] was selected due to well absorption at 280 nm. Multiphasic disposition kinetics was detected following the administration of thiopental to rats, which could be related to fast distribution nature of the thiopental.

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There is no conflict of interest.

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