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Short communication

Effects of intraoperative fluid infusions, sample storage time, and sample handling on unbound propofol assay in human blood plasma

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Abstract

Free drug assay in physiological fluids is getting more and more attention nowadays. The principal reason is the fact that the unbound drug form is responsible for the therapeutic or toxic effects of its application.

Unbound drug concentration significantly depends on the extent of its binding by plasma. This article describes the influence of different factors on unbound propofol concentration. These factors are presence of infusion fluids in blood, type of anticoagulant, sample storage time and plasma freezing. The following conclusions result from the experiments carried out:

1. The lowest free drug fractions are observed in samples containing carbohydrate infusion fluids. The free drug percentage is virtually independent of its total concentration in the range of clinically relevant concentrations.
2. There is no evident anticoagulant influence (heparin, citrate, EDTA and oxalate) on free propofol level in plasma samples.
3. Longer storage of plasma at 4 °C causes a slight rise of free propofol concentration in heparinised plasma and no evident changes in plasma containing citrate.
4. Plasma freezing induces the increase of free drug concentration both for citrate and heparin.

These findings are valuable both for clinicists and pharmacologists, and important for chemical analysts.

Keywords: Unbound drug; Propofol; Sample preparation; Infusion fluids; Anticoagulants; Plasma storage

1. Introduction

Drugs are transported by blood in two forms: bound (as complexes with proteins and blood cells) and free (solved in plasma). It is the unbound drug form that determines its therapeutic or toxic effects. As relying solely on total drug concentration measurements is risky in many clinical situations [1], free drug assay in blood is getting more and more attention nowadays. Moreover, the knowledge of free drug concentration in biological fluids helps elucidate drug transport mechanisms in humans.

Unbound drug concentration significantly depends on the extent of its binding by plasma proteins. While significant changes in free plasma drug concentration are not expected when its composition is relatively stable, changes can be more pronounced and significant for patients whose blood composition undergoes rapid changes. Such changes occur for instance during infusions of supplementary fluids administered intra- and postoperatively or in intensive care units. The clinically important question is whether the administration of commonly used infusion fluids can cause evident changes of unbound drug concentration.

The free drug concentration problem looks quite different from the analytical point of view. It can be expected that the type of anticoagulant used in blood sampling and blood sample handling can influence the final free drug concentration. Moreover, the storage of samples, necessitated by the long and tedious procedure of unbound drug determination, may also negatively influence the results.

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Propofol (2,6-diisopropylphenol) is one of the anaesthetics strongly bound with plasma proteins. It is a sterically hindered phenol used intravenously both for the induction [2] and maintenance [3] of anaesthesia. It is also used for sedation, as a supplement to regional anaesthetics, and in critically ill patients in intensive care units.

Propofol binds strongly with plasma proteins (97.4–99%) [4–6], and due to its effect on the central nervous system, is a potentially dangerous substance. For these reasons it is a good test substance for this research project.

The present article describes the influence of the following factors on the unbound drug concentration in plasma:

(1) intraoperative application of infusion fluids (glucose, hydroxyethylated starch, Ringer and polyelectrolyte solutions);
(2) anticoagulants used during blood sampling;
(3) sample storage time at 4 °C;
(4) sample pretreatment before storage (plasma separation directly after sampling followed by its storage in frozen state at −20 °C).

There are few publications available on free drug analysis and scarce information about factors influencing free drug concentration in blood. Hence, our results may prove interesting to clinicists, pharmacologists and analytical chemists alike [6].

2. Experimental

The measurement of unbound drug concentration is a two-stage procedure:

1. sample preparation by isolating unbound drug fraction and its processing prior to chromatographic analysis;
2. determination of unbound drug concentration.

2.1. Samples

After obtaining an approval from the University Ethics Committee and consent from the patients, blood samples were taken from radial artery of patients scheduled for elective neurosurgical procedures. Details of premedication, anaesthesia, propofol infusion, and patients' treatment were described in [7].

2.1.1. The effect of infusion fluid type on unbound drug fraction

The samples for this investigation were the solutions of human serum albumin of final concentration 5 g albumin per 100 ml in the following infusion fluids:

- Ringer solution;
- polyelectrolyte (PWE) solution;
- 5% glucose solution;
- 6% hydroxyethylated starch (HAES) solution.

2.1.2. The effect of anticoagulant on unbound drug fraction

The blood samples (4 × 36 ml) were taken from the radial artery of each patient into capped vials containing respectively:

- 320 μl of sodium heparin diluted with Ringer solution to 4 ml;
- 4 ml of trisodium citrate dihydrate (3.8%, w/v);
- 4 ml of disodium EDTA dihydrate (1.8%, w/v);
- 4 ml of potassium oxalate dihydrate (2%, w/v) and sodium fluoride (2.5%, w/v).

Therefore the volume of anticoagulant used was kept the same in all cases.

2.1.3. The effect of storage time on unbound drug fraction

Sixty millilitres of blood were taken from the radial artery of one patient (other than above). Plasma was isolated from the blood by its centrifugation (800 rpm, 20 min) in a constant rotor angle centrifuge MPW-341 (Mechanika Precyzyjna, Warsaw, Poland), and then stored at 4 °C. Aliquots of the plasma were assayed for propofol content on storage days 1, 4, 7, 14, 21, and 28.

2.1.4. The effect of plasma freezing on unbound propofol concentration

Three blood samples (18 ml each) were taken from three different patients into capped vials containing 2 ml of trisodium citrate dihydrate (3.8%, w/v). Another 18 ml of blood was additionally taken from patient 3 into a capped vial containing 160 μl of sodium heparin diluted with Ringer solution to 2 ml. Thus, the volume of anticoagulant used for all samples was the same. Plasma was then isolated from the blood by its centrifugation as above. Each plasma sample was then divided into two parts. One part was used for unbound propofol assay immediately; the other part was frozen, kept at −24 °C for 2 weeks, and assayed for free propofol after thawing.

2.2. Sample preparation

2.2.1. Unbound propofol isolation

Unbound propofol was isolated by ultrafiltration on Amicon MPS (Millipore, Bedford, MA, USA) units, utilising the YM-10 membranes (product no. 40424, Millipore, Bedford, MA, USA) of 10 kDa molecular mass cutoff. The ultrafiltration units were centrifuged in a constant rotor angle centrifuge MPW-341 (Mechanika Precyzyjna, Warsaw, Poland). One millilitre of each plasma sample was put into a sample compartment of the ultrafiltration unit. After the attachment of an ultrafiltrate collection container, the unit was centrifuged at 2500 rpm till 400 μl of ultrafiltrate was obtained.

2.3. Propofol assay

For propofol assay, to each sample of blood plasma (1 ml) or plasma ultrafiltrate (400 μl), thymol (internal standard), dihydrogen sodium phosphate (1 ml of 0.1 M NaH_2PO_4), and cyclohexane (5 ml for plasma and 3 ml for plasma ultrafiltrate) were added. The mixtures were vigorously shaken for 10 min at 200 rpm. After centrifugation (3000 rpm for 5 min), in order to separate the phases, an aliquot of the cyclohexane layer (4 or 2 ml, respectively) was transferred to a clean tube with TMAH solution (20 or 10 μl, respectively). The solvent was evaporated to dryness in a stream of nitrogen. The residue was re-dissolved in mobile phase and injected into the chromatographic column. Propofol quantitation limit in plasma was 143.2 ng ml^{-1} with coefficient of variation ($n = 3$) of 2.8% at 150 ng ml^{-1}, 2.3% at 750 ng ml^{-1} and 0.9% at 1500 ng ml^{-1}. The limit of propofol quantitation in plasma ultrafiltrate was 3.7 ng ml^{-1} with coefficient of variation ($n = 3$) of 11.1% at 5 ng ml^{-1}, 12.1% at 20 ng ml^{-1}, and 9.8% at 40 ng ml^{-1}. Other details of sample preparation and the analytical procedure can be found in [7,8].

2.4. Chromatographic equipment

The concentrations of propofol were measured by means of high performance liquid chromatography (HPLC) in plasma as well as in CSF. A Gilson liquid chromatograph (Middleton, WI, USA) consisting of a dual high-pressure pump (Model 122), integrated with a manometric module and a dynamic mixer, was employed for HPLC analysis. Propofol in plasma (high levels) was detected with an UV–vis variable wavelength detector working at 270 nm (Model 155), also from Gilson, whereas propofol in CSF (lower levels) was detected with a fluorescence detector (Jasco FP-920, Japan) set at excitation wavelength 276 nm and at emission wavelength 310 nm. Chromatographic separations were carried out using a 150 mm × 4.6 mm i.d. C_{18} silica gel column (Prodigy RP C_{18}, 5 μm, Phenomenex, USA) equipped with 0.5 μm pre-filter (Supelco, Bellefonte, PA, USA) and guard column ODS C_{18} (Alltech, Deerfield, IL, USA). The samples were injected into the column by a Model 7125 injection valve from Rheodyne (Cotati, CA, USA).

2.5. Drugs and infusion fluids

The following drugs were used: propofol (2,6-diisopropylphenol) in soybean oil emulsion for infusions (Diprivan; AstraZeneca, Caponago, Italy); diazepam (Relanium; Polfa, Warsaw, Poland); fentanyl (Fentanyl; Polfa, Warsaw, Poland); *cis*-atracurium (Nimbex; Glaxo Wellcome, Dartford, UK); neostigmine bromide (Polstigminum; Pliva, Krakow, Poland); atropine (Atropinum sulphuricum; Polfa, Warsaw, Poland). Glucose, Ringer solution and polyelectrolyte solution (PWE) were obtained from Polfa, Lublin, Poland. HAES solution was from Fresenius Kabi, Bad Homburg, Germany.

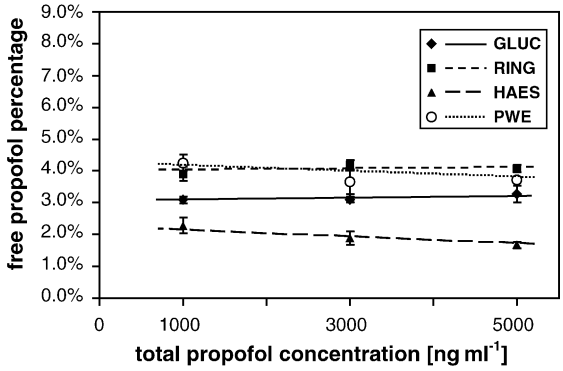

Fig. 1. Free propofol percent vs. total propofol concentration in HSA solution diluted with different infusion fluids (GLUC—glucose, RING—Ringer solution, HAES—hydroxyethylated starch, PWE—polyelectrolyte solution). Shown are mean values ± S.D., $n = 3$.

2.6. Reagents and solutions

All chemicals, except those separately mentioned, were obtained from the Polish Factory of Chemical Reagents-POCh (Gliwice, Poland) and were of analytical grade. A mixture composed of 75% methanol and 25% deionised Milli-Q water was used as mobile phase. Stock solutions of thymol and propofol in methanol (1 mg ml^{-1}) were each prepared and stored at 4 °C. Tetramethylammonium hydroxide (TMAH, 25% in methanol; Aldrich, Germany) was diluted with 2-propanol (3:37, v/v). Artificial plasma was prepared from human serum albumin (ZLB, Berno, Switzerland).

2.7. Statistical analysis

The data are expressed as mean value with standard deviation (S.D.) where applicable.

3. Results

As specified in Section 1, the research work had four aims. The results concerning the influence of the infusion fluid type on unbound propofol percentage are shown in Fig. 1. This investigation was performed using samples with propofol concentration typical for totally anaesthetised patients.

Table 1 illustrates the free propofol concentrations in plasma isolated from blood samples taken using different

Table 1
Influence of anticoagulant type used during blood sampling on unbound propofol concentration in plasma (mean values ± S.D., $n = 3$)

Anticoagulant type	Free propofol concentration (ng ml^{-1}) in plasma from patient no.		
	1	2	3
Citrate	29.2 ± 1.2	14.5 ± 0.8	44.4 ± 2.9
Heparin	25.1 ± 2.3	15.7 ± 1.1	44.5 ± 4.8
EDTA	–	15.4 ± 0.6	45.8 ± 2.3
Oxalate	29.3 ± 1.9	15.3 ± 0.9	50.3 ± 4.1

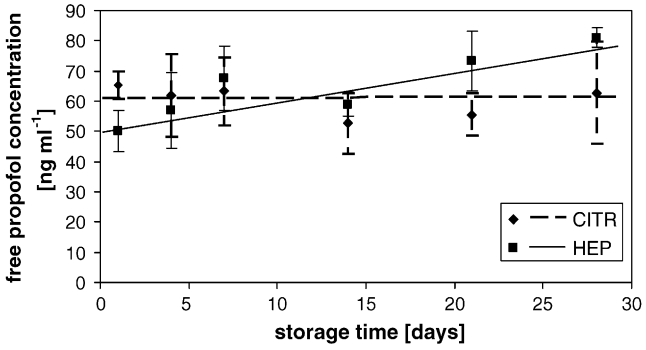

Fig. 2. Changes of free propofol concentration in plasma vs. sample storage time (HEP—plasma isolated from blood with heparin, CITR—plasma isolated from blood with citrate). Shown are mean values ± S.D., $n = 3$.

types of anticoagulants. Each column presents data for one patient. The influence of plasma storage time at 4 °C on free propofol level is presented graphically in Fig. 2. The curves correspond to blood samples taken on citrate and heparin. The investigations were performed within 28 days of plasma storage.

Table 2 contains the concentrations of free propofol assayed before and after plasma freezing. The investigations were carried out using blood samples with citrate and heparin as anticoagulants.

4. Discussion

4.1. Influence of infusion fluid type on unbound propofol concentration

The application of blood replacement and nourishing fluids belongs to typical procedures employed during surgery. As stated in Section 1, it can change free drug concentration in blood. Due to the variety of blood replacement fluids and drugs administered to the patient during surgery this part of the research was conducted using artificial plasma, i.e. solutions of human serum albumin in each infusion fluid. This simplified the analysis of the examined influence. The following conclusions can be drawn from the data in Fig. 1:

1. Free drug percentage is virtually independent of its total concentration in the range of clinically relevant concentrations.
2. The type of electrolyte solution used for diluting albumin (Ringer or PWE) does not influence free propofol level.
3. Carbohydrate fluids (glucose, HAES) decrease the unbound propofol fraction. Lower free drug fraction was observed in samples containing HAES.

Table 2
Influence of plasma freezing on free propofol concentration (mean values ± S.D., $n = 3$)

Anticoagulant type	Patient no.	Free propofol concentration (ng ml^{-1})	
		Before freezing	After thawing
Citrate	1	49.9 ± 1.4	59.2 ± 2.3
	2	60.7 ± 2.7	77.0 ± 4.8
	3	140.6 ± 5.5	145.6 ± 6.4
Heparin	3	142.9 ± 8.9	156.8 ± 15.2

The third conclusion deserves further comment. Increased propofol binding in carbohydrate infusion fluids can result from glycoproteins and glycoprotein-like macromolecules formation. It cannot be excluded that the formed glycoproteins exhibit higher affinity towards propofol than protein molecules unmodified with carbohydrates. The obtained results prove that some infusion fluids can change free drug fraction in plasma, which is clinically important. If so, the influence of infusion fluid on the free drug level must be investigated, especially when potentially dangerous drugs are used.

4.2. Influence of anticoagulant type on unbound propofol concentration

As seen in Table 1, anticoagulant type has no evident influence on free propofol level. This refers to both types of anticoagulants used: anti-thrombin activating (heparin) and Ca^{2+} blockers (citrate, EDTA and oxalate) at all examined free propofol levels.

4.3. Influence of storage time on unbound propofol concentration

Sample storage in relatively mild conditions (4 °C) is frequently necessitated by laboratory circumstances, especially when high sample throughput is required. Fig. 2 presents the relationship between free propofol concentration in plasma (corresponding to citrated or heparinised blood) and sample storage time. In the case of citrate, free propofol concentration in plasma is almost constant. A slight rise in its concentration is observed for heparin. The increase of free propofol form in plasma stored for a long time can be explained by the well known phenomenon of protein structure modification occurring in heparinised plasma [9].

Hence, from the analytical point of view, it is advisable to use citrate as blood anticoagulant for free propofol (and possibly for other drugs) assay.

4.4. Influence of plasma freezing on unbound propofol concentration

Freezing is the most popular way of protecting samples against the changes occurring during their storage in higher temperatures. As appears from the data listed in Table 2, the freezing of plasma samples leads to the increase of free drug concentration. This phenomenon can be explained by the properties of propofol and by the changes occurring in

plasma during its freezing [10]. Due to its chemical character, propofol exhibits high affinity to lipids. According to Albani et al. [11], plasma freezing results in the destruction of some lipoproteins and the formation of higher density lipid aglomerates. Consequently, a part of protein-complexed propofol passes into the aqueous phase, richer in lipids, thus increasing the free drug concentration determined by ultrafiltration. The observed increase of apparent free drug fraction as a result of free fatty acids concentration increase (lipoproteins' lysis) was also observed for valproic acid [11].

Our results demonstrate that although the effect of the discussed factors on free propofol concentration in plasma can be registered, it is relatively small. The biggest changes are induced by infusion fluids and plasma freezing. Our conclusions are important for clinical and pharmacological research and useful to analytical chemists. Yet it should be remembered that the same factors may significantly influence free form concentration for other drugs and thus deserve further study.

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