Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Pharmaceutical and Biomedical Analysis 41 (2006) 973–978
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Application of adsorption method to the chromatographic analysis of free drug concentration

Andrzej L. Dawidowicz *, Mateusz Kobielski, Rafal Kalitynski

Faculty of Chemistry, Maria Curie-Sklodowska University, Pl. M. Curie-Sklodowskiej 3, PL 20-031 Lublin, Poland

Received 8 October 2005; received in revised form 30 January 2006; accepted 1 February 2006
Available online 20 March 2006

Abstract

The development of analytical techniques for estimation of free drug concentration is crucial for the needs of modern pharmacology. Up to now, many different methods of free drug assay have been used. The methods involving isolation of free drug from a sample are most frequently applied because they can be easily adopted for the processing of real samples. Among the methods that do not require free drug isolation only the adsorption method could be promising in application to samples of this kind.

The successful use of the adsorption method is possible only if two requirements are fulfilled: (I) the chosen adsorbent does not bind proteins from the sample and (II) the processes of adsorption of the assayed drug on the adsorbent used and on the protein should be independent.

This paper discusses the possibilities of application of the adsorption method for determination of free drug concentration using propofol as the model drug. The presented results show that the fulfillment of the above conditions may be very difficult if not impossible not only for propofol, but for other drugs as well.

Keywords: Propofol; HSA; Unbound drug; Plasma; Adsorption; PVP; Charcoal

1. Introduction

Blood is the fundamental medium for drug transport in the organism. Drugs exist in blood in two forms: free and bound with plasma proteins and blood cells. It is generally assumed that only the unbound drug can reach the site of action by diffusing across the membranes and exert pharmacological effect by interacting with receptors [1]. The bound form serves as a kind of drug storage from which the free drug is released following the decrease of its blood concentration. That is the reason for correlating the therapeutic effect of a drug with the unbound rather than with the total drug concentration [1]. Hence the development of analytical techniques for estimation of free drug concentration is crucial for the needs of modern pharmacology.

Up to now, many different methods of free drug assay have been developed. In general, two groups can be distinguished. The methods involving the separation of a free drug from a bound drug can be included in the first group. In these methods free drug isolation is accomplished by ultrafiltration [2,3], dialysis [1,2,4,5], ultracentrifugation [1] or in a chromatographic process [3]. The first three methods are most frequently used in the processing of real samples as they allow to isolate free drug and discard the remaining part of the sample which simplifies the final analysis. Most widely employed are membrane methods: equilibrium dialysis because of real equilibrium conditions and ultrafiltration in cases when smaller sample volumes are available and the shorter separation time is preferable. The main problem of these methods (handled in different ways) is analyte adsorption on the separation membrane. Ultracentrifugation (centrifugation of the sample at up to $350{,}000 \times g$ for several hours) does not suffer from this drawback, but it is time-consuming and requires costly equipment [1]. In contrast to the described methods of free drug isolation, the application of chromatographic separation is limited mainly to artificial drug–protein systems.

The second group of free drug assay methods includes methods that do not require unbound drug isolation. In such methods as the absorption spectrum changes [6,7], fluorescence quenching [7] and circular dichroism [8] free drug concentration is estimated by measurement of optical protein–drug system prop-

* Corresponding author. Tel.: +48 81 537 55 45; fax: +48 81 533 33 48.
E-mail address: dawid@hermes.umcs.lublin.pl (A.L. Dawidowicz).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.02.002

erties which depend on the bound drug concentration. Proton titration [4] and potentiometric ion [9] or fluorescence [5] probe methods use titration-like approach to determine drug–protein binding parameters from which free drug concentration can be estimated. All these methods are suitable only for artificial drug–protein systems.

According to the literature [10], there is another free drug assay method that can be included in the second group—the adsorption method. Here free drug concentration is calculated basing on the change of total drug concentration in the sample after addition of an appropriate adsorbent and after completing the reequilibration. This method assumes independence of the drug adsorption on the protein and the introduced adsorbent. The estimated adsorbed amount of the drug is related to a calibration curve prepared for the protein-free drug-sorbent system, defining the drug binding properties of the adsorbent used. This method seems to be especially interesting and promising for free form assay of highly bound drugs because it is easy to perform and does not require instrumentation for trace analysis (with sensitive detection system), as it has to be employed when the methods from the first group (with free drug separation) are applied. Moreover, the whole experimental setup is very simple and the analytical procedure is inexpensive. In addition, the method has been applied for assay of unbound propofol [10] and the authors have obtained free propofol concentration in agreement with literature data [11–14].

On the one hand, the advantages of the adsorption method, pointed out above, encourage to its wide application. On the other hand, it should be stressed that the method requires the use of an adsorbent that does not adsorb protein and, in addition, assumes the independence of drug adsorption on the adsorbent used and on the protein. As both the mentioned requirement and the assumption are quite restrictive from the physicochemical point of view, the practical application of the adsorption method raises certain doubts, because there is only one publication reporting its employment [10]. Therefore the main aim of the presented paper is a more detailed reconsideration of this method's application for the determination of free drug concentration. The model drug chosen for the method evaluation was propofol, which is a favorable choice since the same drug was used in [10]. Besides, its chromatographic analysis is very well validated in our laboratory.

2. Experimental

The adsorption method requires two types of independent experiments. The first is performed with protein-free drug solutions and adsorbent. The second one is performed with the same adsorbent as before and with the real sample assayed for free drug concentration. In the first experiment, the dependence between the amount of the drug adsorbed (per mass unit of the adsorbent) and drug concentration in the protein-free solution is determined and plotted as "calibration curve" (as it was done for the purposes of this paper in Figs. 1 and 2a–c). In the second experiment, the difference between two total drug concentration measurements before and after introduction of the adsorbent into the sample (i.e. drug loss resulting from drug adsorption

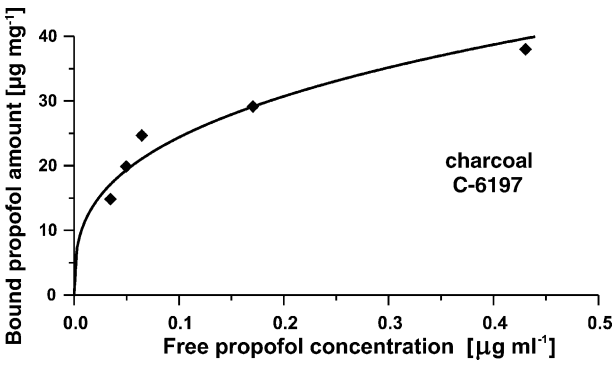

Fig. 1. Calibration curve for the determination of free propofol concentration by adsorption method using charcoal C-6197.

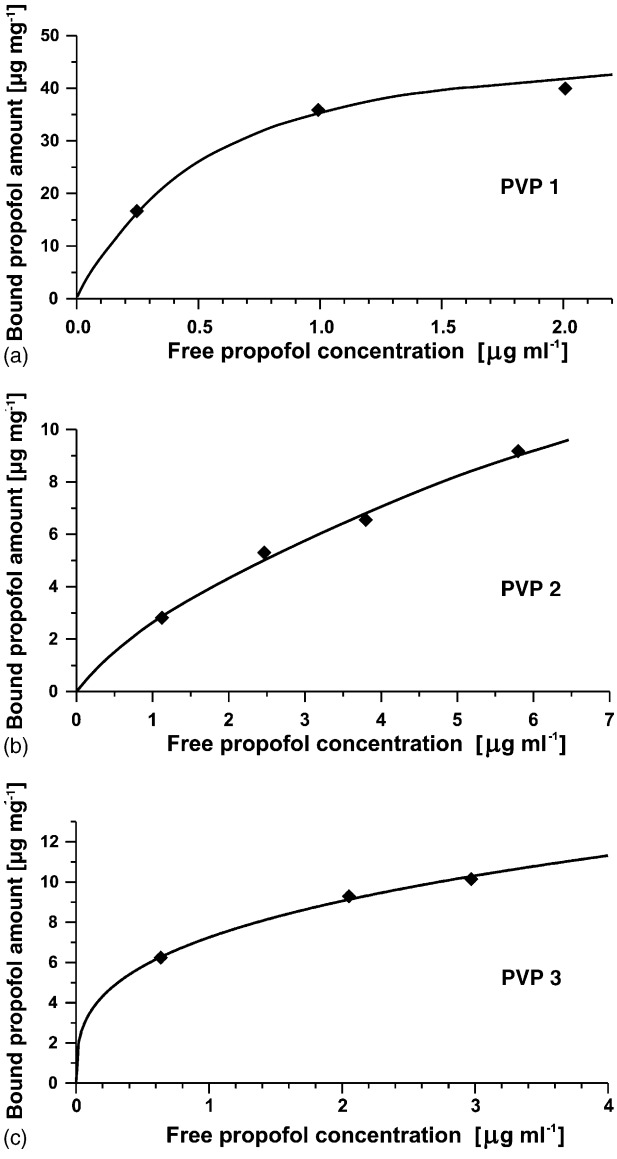

Fig. 2. (a–c) Calibration curves for the determination of free propofol concentration by adsorption method using VP-DVB adsorbents batch 1–3.

on the adsorbent used) is calculated. Free drug concentration is estimated relating the drug loss to the calibration curve constructed in the first experiment. Hence, the applied adsorbent is a crucial element of the free drug assay by adsorption method. Two types of adsorbents were used in the experiments. One type is the commercially available dextran coated charcoal (C-6197, Sigma–Aldrich, St. Louis, Montana, USA)—the same as in [10]. The other type is vinylpyrrolidone–divinylbenzene synthesized in our laboratory.

2.1. Synthesis of the VP-DVB

VP-DVB sorbents were prepared by polymerization of vinylpyrrolidone (VP) crosslinked with divinylbenzene (DVB). The sorbents marked as VP-DVB, batch 1 and 2 were obtained by suspension–emulsion polymerization and batch 3 was synthetized by suspension polymerization similarly to the procedure described elsewhere [15]. The monomers (VP and DVB) were solved in a mixture of porogenic solvents (sol, nonsol) and added to an aqueous phase containing a suspension stabilizer or surfactant. Crosslinked copolymers were obtained by free-radical polymerization initiated by AIBN (azo-diisobutylnitrile).

2.2. Determination of free drug concentration

2.2.1. By adsorption method

Calibration curve construction constitutes the first part of the assay. For this task, 1 mg portions of the adsorbent were introduced to a series of propofol solution aliquots (5 ml) of different drug concentration. Propofol solutions were obtained from the solution of the drug in 40% ethanol (50 $\mu g\,ml^{-1}$) by dilution with Ringer solution to the required concentrations. After the equilibration the suspensions were centrifuged to remove the adsorbent and the supernatant was analyzed to determine propofol concentration by HPLC as described further (Section 2.3). The calibration curve was constructed with the use of the drug amounts calculated from the difference between drug concentrations before and after adsorption.

Determination of the drug loss after introduction of the adsorbent to the investigated sample is the second part of the free drug assay. It was decided to perform the experiments using an easily reproducible artificial test sample containing human serum albumin (HSA) and the drug concentrations similar to those typical for the clinical conditions. Thus all determinations were performed with the same test sample containing HSA in Ringer solution (40 $mg\,ml^{-1}$) and propofol (total concentration 4 $\mu g\,ml^{-1}$). One milligram of a given adsorbent was introduced into 1.5 ml of the test sample. After the equilibration the suspension was centrifuged to remove the adsorbent and the supernatant was analyzed to determine total propofol concentration by HPLC as described further (Section 2.3). The drug loss was calculated as the difference between total drug concentrations before and after adsorption. Free drug concentration was estimated relating the drug loss per 1 mg of the adsorbent to the calibration curve.

2.2.2. By ultrafiltration

Unbound propofol was isolated from the sample by ultrafiltration on Amicon MPS (Millipore, Bedford, MA, USA) units, utilizing 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 millilitres of each sample was put into the sample compartment of the ultrafiltration unit. After the attachment of the ultrafiltrate collection container, the unit was centrifuged at 2500 rpm till 400 μl of ultrafiltrate was obtained. The ultrafiltrate was subsequently analyzed as described in the next section.

2.3. Chromatographic analysis

For propofol assay HPLC was used. Three types of samples were examined: two types for adsorption method (the sample before and after addition of the adsorbent) and one for ultrafiltration method. The way of sample preparation was described in Section 2.2. Each sample was analyzed according to the same following procedure. To the sample (1 ml in case of adsorption method and 400 μl in case of ultrafiltration) thymol, dihydrogen sodium phosphate (1 ml of 0.1 M NaH_2PO_4), and cyclohexane (3 ml for ultrafiltrate and 5 ml for other solutions) 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 (2 ml and 4 ml, respectively) was transferred to a clean tube with TMAH solution (10 μl and 20 μl, respectively). The solvent was evaporated to dryness in a stream of nitrogen. The residue was re-dissolved in the mobile phase and injected into the chromatographic column. The lower limit of propofol detection in sample ultrafiltrate was 1.1 $ng\,ml^{-1}$ with the 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 $ng\,ml^{-1}$. The lower limit of propofol detection in plasma was 43 $ng\,ml^{-1}$ with the 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 $ng\,ml^{-1}$.

Other details of sample preparation and analytical procedure can be found in [13,14,16,17].

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 two high-pressure pumps (Model 306), with a manometric module (Model 805) and a dynamic mixer (Model 811C), was employed for HPLC analysis. Total concentrations of propofol in the samples (high levels) were detected with an UV/VIS DAD detector working at 270 nm (Model 170), whereas unbound propofol concentration in ultrafiltrates (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 prefilter (Supelco, Bellefonte, PA, USA) and a guard column ODS

Table 1
Free propofol concentration estimated by adsorption and ultrafiltration methods for the test sample composed of HSA in Ringer solution (40 mg ml^{-1}) and propofol (total concentration 4 μg ml^{-1})

Measurement method	Free propofol concentration (ng/ml)	Free propofol (%)
Adsorption method (charcoal C-6197)	8.7 ± 0.1	0.22 ± 0.00
Ultrafiltration	110.5 ± 10.0	2.83 ± 0.26

Shown mean values ± S.D. ($n = 3$).

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. Protein determination

The protein concentration in the tested samples was determined by spectrophotometry from the measurements of sample solutions absorbance at λ = 280 nm. The apparatus used was the spectrophotometer model UV-160A (Shimadzu, Tokyo, Japan).

3. Results and discussion

The comparison of the value of free drug concentration determined by means of the examined method and another well validated one is the simplest way of the method verification. In [10] the discussed adsorption method was applied for free propofol assay in plasma using dextran coated charcoal C-6197 as an adsorbent. Taking the above into consideration, it was decided to compare the result of free propofol determination in HSA solution obtained by adsorption method using the same adsorbent, with the result obtained by ultrafiltration (taken as a standard method). Such approach was chosen in order to eliminate the potential discrepancies resulting from different properties of adsorbent and adsorbate.

The free propofol concentration determined in the test sample, obtained by adsorption method (using the calibration curve presented in Fig. 1) and ultrafiltration method, are presented in Table 1. As it can be seen, the concentration of free propofol determined by adsorption method is about 14 times lower than the one obtained by ultrafiltration. According to the literature [11–14], the free propofol concentration in plasma and in the HSA solutions of similar protein and propofol concentrations is in the range of 1–3%. The above indicates that ultrafiltration is a good method of choice. At this point a question appears about the reason for such a low free propofol concentration assayed by the adsorption method.

As it was mentioned above, the authors of [10] used the dextran coated charcoal claiming that the absence of protein binding to this adsorbent had been experimentally verified. The charcoal adsorbents are known to have high hydrophobic properties and exhibit strong protein adsorption. On the highly hydrophobic surface the protein adsorbs, frequently unfolding its structure, due to hydrophobic interactions between the adsorbent and the adsorbate [18]. Dextran coating of the adsorbent surface is made in order to increase compatibility of the adsorbent with an aqueous protein solution and to lower the amount of adsorbed proteins. As results from Fig. 1, the dextran-coated charcoal shows strong adsorption properties towards propofol in Ringer solution. Mere 1 mg of this adsorbent adsorbs 98.8% of the drug from the solution (5 ml) containing 4 μg/ml of propofol. These data suggest that propofol should be also strongly absorbed on the charcoal in the presence of protein. Yet, the very low free fraction of propofol estimated by means of adsorption method using charcoal (see Table 1) indicates a very small adsorption of the drug on this adsorbent in the presence of protein. The decrease in propofol adsorption on the charcoal from protein solution suggests the changes of adsorption properties of the charcoal used. The most probable explanation of this fact is the adsorption of protein on the adsorbent used, resulting in a blockage and deactivation of the adsorbent's surface. Moreover, adsorbed proteins may undergo structural changes, which leads to the release of previously bound propofol, decreasing the difference in the total drug concentration before and after adsorption (the drug loss used for the determination of free drug concentration). The explanation considering the protein adsorption is quite reasonable since a protein molecule has many different kinds of functional groups and adsorption centers of different character, so it is very difficult to find a material that does not adsorb proteins at all.

The experimental verification of the literature data [10,19] concerning protein adsorption on the dextran coated charcoal (C-6197) is the natural consequence of the obtained results and the entire above reasoning. In the first approach it was decided to

Table 2
Protein adsorption from the HSA in Ringer solution (40 mg ml^{-1}) on the adsorbents used for free drug determination

Adsorbent	Adsorbent weight (mg)	Albumin loss in (%)	Mass of HSA calculated for 1 mg of adsorbent[a] (mg)
Charcoal C-6197	1	0.0	0.00
	100	52.5	0.32
VP-DVB, batch 1	200	0.0	0.00
VP-DVB, batch 2	200	3.1	0.0093
VP-DVB, batch 3	200	0.0	0.00

[a] Estimated for the adsorbent weight used for free drug determination (1 mg, see Table 3) assuming linear dependence between the adsorbent weight and the amount of adsorbed drug.

measure the decrease of HSA concentration resulting from the introduction of this adsorbent into HSA solution in the amount used for free propofol assay by adsorption method (i.e. 1 mg). No measurable decrease of protein concentration in the test sample was registered (see Table 2). However, the experiment with a larger amount of the adsorbent (100 mg) revealed significant adsorption of HSA on this adsorbent. Thus, too low free propofol concentration determined by the adsorption method can be actually explained by protein adsorption on the charcoal. The information about the absence of protein adsorption on charcoal C-6197 [10,19], encouraging the use of this material in the adsorption method, probably resulted from the lack of measurable protein adsorption on a small amount of this adsorbent used by the cited authors.

If protein adsorption is responsible for wrong results of free propofol determination by the adsorption method, it is logical to use the adsorbent which intrinsically adsorbs protein to far less extent. Poly(vinylpyrrolidone) adsorbents belong to this group [20]. Although the interior of their particles is more hydrophobic, the pores of particles are small enough to prevent HSA molecules from entering inside. The external surface of the particles is hydrophilic. The presence of pyrrolidone moieties on the external surface of particles makes them biocompatible with blood proteins preventing the proteins from denaturation. Due to this feature, vinylpyrrolidone polymers have been used for a long time as blood replacement fluids [21].

The adsorption properties of VP-DVB adsorbents towards HSA are presented in Table 2. The most useful results are collected in the last column of Table 2, showing the amount of adsorbed protein per mass of the adsorbent used for free propofol assay.

It should be stressed that these data are only approximate because they were calculated with the assumption of linear dependence between the adsorbent weight and the amount of adsorbed protein. Nevertheless, VP-DVB adsorbents actually adsorb significantly lower (sometimes even impossible to measure) HSA amount than dextran-coated charcoal does (see Table 2).

Table 3 lists free propofol percentages determined by adsorption method using the polymeric adsorbents. The calibration curves corresponding to the appropriate adsorbents are shown in Fig. 2a–c. All the free propofol percentages are considerably higher than the value obtained by ultrafiltration—2.83%. This difference makes the results obtained with polymeric adsorbents unacceptable.

Table 3
Free propofol concentration estimated for the test sample containing HSA (40 mg ml^{-1}) and propofol (total concentration 4 μg ml^{-1}) in Ringer solution, using adsorption method and the adsorbents from Table 2

Adsorbent	Free propofol concentration (ng ml^{-1})	Free propofol (%)
Charcoal C-6197	8.7 ± 0.1	0.22 ± 0.00
VP-DVB, batch 1	237.0 ± 0.7	6.1 ± 0.0
VP-DVB, batch 2	342.0 ± 2.8	8.7 ± 0.1
VP-DVB, batch 3	1670 ± 2.1	42.7 ± 0.1

Shown mean values ± S.D. (n = 3).

Although the polymeric adsorbents do adsorb negligible quantities of HSA, the free propofol percentage assayed using these materials is overestimated. Therefore, the application of materials not adsorbing proteins does not lead to correct results either.

As it was mentioned in Section 1, there is still another requirement for the method: the independence of analyte binding to the protein and to the adsorbent. The incorrect results obtained in the conditions which fulfill the first requirement (no protein adsorption), suggest that the second requirement stated in Section 1 (independence of drug adsorption on the sorbent and on the HSA) is not satisfied. Thus processes of the drug binding to the protein and the adsorbent are dependent on each other. As it is known, vinylpyrrolidone adsorbents show high affinity towards phenols (adsorption of phenols is one of their typical applications in analytical chemistry [22]). It cannot be excluded that strong adsorption of propofol by VP-DVB adsorbents disturbs the equilibrium of propofol binding to HSA, which results in the determination of false free propofol percentage.

The presented paper shows the difficulties in employing the adsorption method for free drug assay. Practical application of the adsorption method does not seem to be highly probable because of slight possibility of fulfilling the two initial assumptions of this method. The results presented in the paper stem from testing the method with the use of merely two types of adsorbents (dextran coated charcoal and three kinds of VP-DVB of different physicochemical characteristics). In fact, during verification of the method a far higher number of adsorbent types (different types of carbonaceous adsorbents, silica gels, polyamides, adsorbents with chemically bonded phases and materials with the surface adhesively coated by stationary phases) were tested. The results of their employment, being even worse, were not included to avoid excessive extension of the text. It is doubtful whether the choice of a proper adsorbent meeting both initial requirements (no protein adsorption and binding independence) is possible in practice at all, even in the case of drugs other than propofol.

References

[1] A.C. Mehta, Trends Anal. Chem. 8 (1989) 107–112.
[2] W.F. Bowers, S. Fulton, J. Thompson, Clin. Pharmacokinet. 9 (1984) 49–60.
[3] B. Sebille, R. Zini, C. Madjar, J. Tillment, J. Chromatogr. 531 (1990) 51–77.
[4] F. Brée, S. Urien, P. Nguyen, J.P. Tillement, A. Steiner, C. Vallat-Molliet, B. Testa, J. Visy, M. Simonyi, J. Pharm. Pharmacol. 45 (1993) 1050–1053.
[5] M.H. Rahman, T. Maruyama, T. Okada, K. Yamasaki, M. Otagiri, Biochem. Pharmacol. 46 (1993) 1721–1731.
[6] J. Gonzàlez-Jimènez, Chem. Biol. Interact. 91 (1994) 65–74.
[7] J. Bartošová, I. Kalousek, Z. Hrkal, Int. J. Biochem. 26 (1994) 631–637.
[8] V. Russeva, R. Rakovska, N. Stavreva, D. Mihailova, N. Berova, Pharmazie 49 (1994) 519–522.
[9] A.T. Angelakou, E.E. Sideris, G.N. Valsami, M.A. Koupparis, P.E. Macheras, J. Pharm. Sci. 83 (1994) 1150–1154.
[10] J.X. Mazoit, K. Samii, Br. J. Clin. Pharmacol. 47 (1999) 35–42.
[11] J. Vuyk, F.H.M. Engbers, H.J. Lemmens, A.G. Burm, A.A. Vletter, M.P. Gladines, J.G. Bovill, Anesthesiology 77 (1992) 3–9.
[12] T. Gin, Anesthesiology 78 (1993) 604–605.

[13] A.L. Dawidowicz, R. Kalitynski, Biomed. Chromatogr. 17 (2003) 447–452.
[14] A.L. Dawidowicz, R. Kalitynski, A. Fijalkowska, Clin. Neuropharmacol. 27 (2004) 129–132.
[15] M. Maciejewska, B. Gawdzik, J. Appl. Polym. Sci. 95 (2005) 863–870.
[16] A.L. Dawidowicz, A. Fijalkowska, J. Liq. Chromatogr. 19 (1996) 1423–1435.
[17] A.L. Dawidowicz, E. Fornal, M. Mardarowicz, A. Fijalkowska, Anesthesiology 93 (2000) 992–997.
[18] K. Nakanishi, T. Sakiyama, K. Imamura, J. Biosci. Bioeng. 91 (2001) 233–244.
[19] J. Yuan, D.C. Yang, J. Birkmeier, J. Stolzenbach, J. Pharmacokinet. Biopharm. 23 (1995) 41–55.
[20] S. Sun, Y. Yue, X. Huang, D. Meng, J. Membr. Sci. 222 (2003) 3–18.
[21] E.A. Moffitt, Can. Anaesth. Soc. J. 22 (1975) 12–19.
[22] J. Hradil, M.J. Beneš, Z. Plichta, React. Funct. Polym. 44 (2000) 259–272.