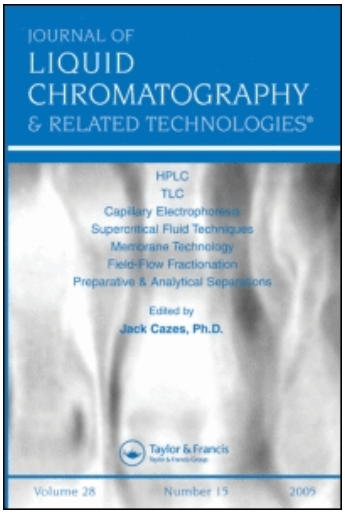


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Determination of Free Indomethacin in Human Plasma Using HPLC with UV Detection

Andrzej L. Dawidowicz[a]; Krzysztof Kondziola[a]; Mateusz Kobielski[a]
[a] Department of Chromatographic Methods, Faculty of Chemistry, Maria Curie-Sklodowska University, Lublin, Poland

To cite this Article Dawidowicz, Andrzej L. , Kondziola, Krzysztof and Kobielski, Mateusz(2009) 'Determination of Free Indomethacin in Human Plasma Using HPLC with UV Detection', Journal of Liquid Chromatography & Related Technologies, 32: 18, 2686 — 2698
To link to this Article: DOI: 10.1080/10826070903245748
URL: http://dx.doi.org/10.1080/10826070903245748

Journal of Liquid Chromatography & Related Technologies®, 32: 2686–2698, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070903245748

Determination of Free Indomethacin in Human Plasma Using HPLC with UV Detection

Andrzej L. Dawidowicz, Krzysztof Kondziola, and Mateusz Kobielski

Department of Chromatographic Methods, Faculty of Chemistry, Maria Curie-Sklodowska University, Lublin, Poland

Abstract: Determination of free indomethacin concentration in human plasma is an issue of great importance for the drug safety monitoring and the efficiency of the drug therapy, especially in the case of patent ductus arteriosus treatment (PDA) in neonates. The presented study describes a simple and sensitive method of free indomethacin analysis in plasma samples using high performance liquid chromatography (HPLC) combined with ultraviolet (UV) detection. In the developed method the unbound drug separation was performed by means of the ultrafiltration method on Amicon MPS units, utilizing the YM-10 membranes of 10 kDa molecular mass cutoff. The sample preparation step involved liquid–liquid extraction with ethyl acetate, providing very good absolute recovery of the analyte (ca. 97%, $n = 7$). Chromatographic separation was achieved on a C_{18} column using 63% acetonitrile and 37% water ($pH = 2.0$, adjusted with 0.2% orthophosphoric acid) as the mobile phase. The external standard calibration curves showed good linearity ($R^2 > 0.9995$) in the concentration range from 10 to 5000 ng/mL (total drug determination) and from 10 to 200 ng/mL (free drug determination). The intra-day and inter-day precision and accuracy were satisfactory, with relative standard deviations not exceeding 10%. The limit of detection (LOD) and the limit of quantitation (LOQ) were 3 ng/mL and 10 ng/mL, respectively. The presented analytical approach constitutes substantial improvement over previously reported methods for indomethacin analysis and thus seems to be the method of choice for determination of free indomethacin in plasma.

Correspondence: Andrzej L. Dawidowicz, Department of Chromatographic Methods, Faculty of Chemistry, Maria Curie-Sklodowska University, 20-031 Lublin, pl. Maria Curie-Sklodowska 3, Poland. E-mail: dawid@poczta.umcs.lublin.pl

Keywords: Free drug, HPLC, Indomethacin, Liquid–liquid extraction, Plasma, UV detection

INTRODUCTION

Indomethacin, 1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid is an indole derivative belonging to the group of nonsteroidal anti-inflammatory drugs (NSAIDs), which is used in the treatment of rheumatoid arthritis for antipyretic and analgesic effects. Indomethacin is also regarded as the drug of choice for the closure of patent ducts arteriosus in newborns.[1]

According to the literature, the indomethacin pharmacokinetics is characterized by high inter-individual variability.[2–4] Therefore, it would be desirable to adjust individual indomethacin dosages according to the plasma indomethacin concentration during the drug administration. Inability to maintain indomethacin at a therapeutic plasma concentration causes failure in the treatment of premature neonates.[1]

It should be emphasized that the pharmacologic effect of a drug is in fact exerted by its free fraction i.e., drug molecules not bound to plasma proteins.[5]

The degree of drug protein binding may be influenced by several factors such as disease states,[6–8] age,[9] and total drug concentration.[10] In the case of highly protein bound drugs (above 70%) even slight changes of drug binding may significantly affect their therapeutic efficacy.[5] Indomethacin is known to bind strongly to human serum albumin (about 97%).[8,11] Such a high degree of protein binding results in high interpatient variation in the fraction of the unbound NSAID.[12] For these reasons, reliable determination of free indomethacin concentration in human plasma is particularly important for the drug safety monitoring and the effectiveness of the drug therapy. It is worth mentioning that the issue of free indomethacin determination in plasma is still not fully recognized. Estimation of a free drug concentration requires the development of a efficient technique of unbound drug isolation as well as elaboration of a highly sensitive analytical method. Presently, the membrane separation techniques (i.e., ultrafiltration and equilibrium dialysis) are recognized as the most adequate ones for free drug isolation from a complex biological matrix.[13]

Several HPLC methods have been reported for the analysis of total indomethacin concentration in biological fluids, especially in plasma.[14–19] Recently, HPLC techniques coupled with protein precipitation were developed.[12,20] These methods, though relatively fast, simple, and of the lowest LOD value (5 ng/mL), seem not to be applicable for free drug analysis in plasma ultrafiltrate, due to sample dilution during the preparation procedure. Very low free indomethacin concentration in the ultrafiltrate demands application of an analytical procedure allowing for analyte

preconcentration. Therefore, the extraction technique seems to be the method of choice for the free drug analysis in plasma samples.

There are few analytical approaches described in literature involving liquid–liquid extraction for indomethacin analysis.[21–23] These methods suffer either from low recovery (not exceeding 81%)[21,22] or poor sensitivity (LOD of 250 ng/mL).[21]

The presented paper describes the analytical procedure of HPLC indomethacin determination in human plasma involving liquid–liquid extraction of the analyte and UV detection. The method is characterized by high yield (recovery about 97%) and detection limit 3 ng/mL, which is so far one of the lowest values reported for HPLC method. Due to this fact, the proposed analytical approach is the method of choice for determination of free (unbound) indomethacin concentration in plasma.

EXPERIMENTAL

Materials

Indomethacin (purity min. 99% TLC) was purchased from Sigma Aldrich, Inc. (St. Louis, USA). Acetonitrile and ethyl acetate (both HPLC grade) were purchased from POCH (Gliwice, Poland) and Merck (Germany), respectively. Water for chromatography was obtained by means of a Milli-Q (Millipore, USA) water purification system. All other chemicals were reagent grade. Pooled blank plasma was obtained from 20 healthy volunteer donors.

Chromatographic Conditions

A Gilson liquid chromatograph (Gilson, USA, controlled by UniPoint 2.10 System) consisting of a dual high pressure pump (Model 322), integrated with a manometric module and a dynamic mixer was employed for HPLC analysis. Indomethacin in plasma or in plasma ultrafiltrate was detected with an UV/VIS 155 detector (Gilson, France) working at $\lambda = 270$ nm. Chromatographic separations were carried out by using a 150 mm x 4.6 mm i.d. C_{18} silica gel column (Prodigy RP C_{18}, 5 μm, Phenomenex, USA) equipped with 0.5 μm prefilter (Upchurch Scientific, USA) and a guard column ODS C_{18} (Phenomenex, USA). All analyses were performed at room temperature (20°C ± 2°C). The samples (50 μL) were injected into the column by a Model 7125 injection valve from Rheodyne (Cotati, CA, USA). The mobile phase consisting of acetonitrile (63%) and water (37%) containing 0.2% orthophosphoric acid (Merck, Germany) was applied (pH = 2), with flow rate 0.8 mL/min and

backpressure 65 atmospheres. The assay of free indomethacin concentration can be treated as trace analysis. For this reason, peak height was assumed as an appropriate measure for indomethacin quantification.

Standard Calibration Curve for Total Indomethacin Determination

A primary stock solution of indomethacin (10 mg/mL) was prepared by dissolving 1000 mg of indomethacin in 100 mL of ethanol (96% v/v). Further working solutions were obtained by serial dilutions of the stock solution with ethanol/water mixtures to give 1, 50, 200, and 1000 μg/mL indomethacin concentration. A nine-point external standard calibration curve was prepared by adding these solutions to centrifuged blank plasma to obtain the following concentrations: 10, 25, 50, 100, 200, 500, 1000, 2000, and 5000 ng/mL. The ethanol concentration in the prepared standards did not exceed 1% (v/v). The indomethacin stock solution was stored at – 20°C and its stability period (6 months) was established experimentally.

Standard Calibration Curve for Free Indomethacin Determination

During this step of the experimental procedure, only one of previously mentioned working solutions of indomethacin (1 μg/mL) was applied. A six-point external standard calibration curve was prepared by adding this solution to Ringer's solution so as to obtain the following drug concentrations: 10, 15, 25, 50, 100, 200, ng/mL. The Ringer's solution was used as the imitation of plasma ultrafiltrate, which contains no plasma proteins but abounds with inorganic ions.

Sample Preparation Procedures

Sample Preparation for the Analysis of Total Indomethacin Concentration

The plasma samples were stored in the freezer at – 20°C and allowed to thaw at room temperature prior to the extraction procedure. To each plasma sample (400 μL), phosphate buffer (100 μL of 0.5 M $NaH_2PO_4 + H_3PO_4$, pH 3.5) and ethyl acetate (2 mL) were added. The mixtures were vigorously shaken for 10 minutes at 200 rpm. The organic layer (1.5 mL) was then transferred to a clean vial and the solvent was evaporated to dryness in a gentle stream of nitrogen at room temperature. The remaining residue was dissolved in 200 μL mobile phase and an aliquot of 50 μL was injected into the HPLC system.

Sample Preparation for the Analysis of Free Indomethacin Concentration

This part of the experiment required the unbound indomethacin isolation from the plasma sample. For this purpose, we applied the ultrafiltration technique, performed 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 cut-off. The ultrafiltration proceeded in a thermostated centrifuge (MPW-350-RH, MPW Med. Instruments, Poland) at the temperature of the human body (37°C). Of each solution, 1 mL was put into a sample compartment of the ultrafiltration unit. After the attachment of an ultrafiltrate collection container, the unit was centrifuged at $800 \times g$ till 400 μL of ultrafiltrate was obtained (usually for about 20 minutes).

In order to avoid analytical errors resulting from possible drug adsorption on the ultrafiltration membrane,[24,25] preliminary membrane saturation with the analyzed drug was performed. The saturation involved ultrafiltration of a few portions of analyzed sample, i.e., plasma spiked with indomethacin, until the free drug concentration in the ultrafiltrate was established at a constant level.

The extraction procedure of plasma ultrafiltrate was performed analogously, as in the case of the total drug concentration analysis. The slightly larger aliquot of organic layer (1.7 mL) transferred after the extraction process was the only difference.

RESULTS AND DISCUSSION

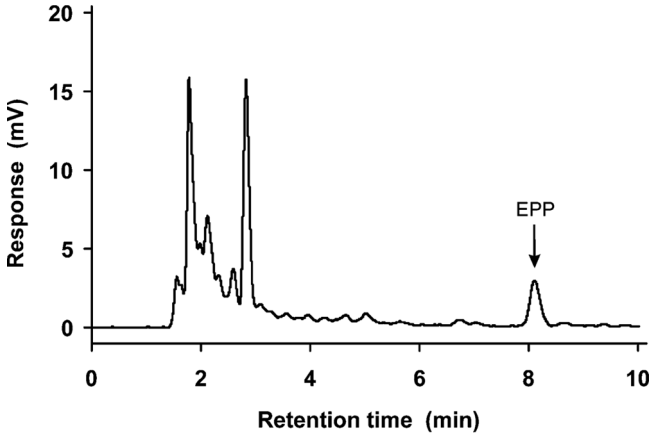
Determination of Total Indomethacin Concentration in Plasma

Figure 1 presents chromatograms of blank plasma (Figure 1a) and plasma spiked with indomethacin corresponding to a concentration of 100 ng/mL (Figure 1b). The retention time of the analyzed drug was 6.27 min with the total run time about 10 min. According to Figure 1, the applied chromatographic conditions allow for sufficient resolution of indomethacin from endogenous plasma peaks.

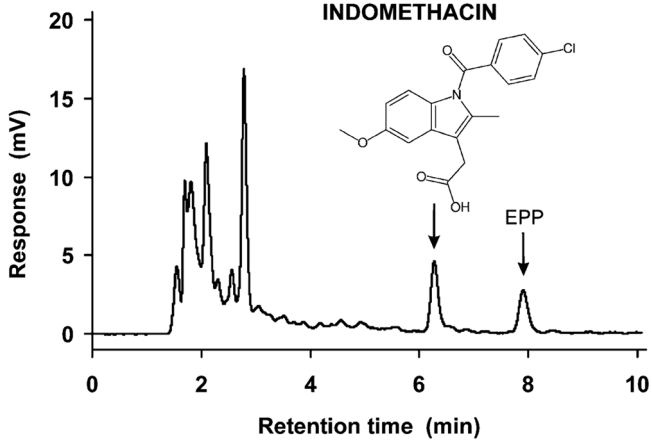
Figure 2 illustrates the calibration plot for indomethacin, performed in the concentration range $10 - 5000$ ng/mL with a regression coefficient $R^2 = 0.9999$, indicating very good linearity.

The imprecision and inaccuracy of the assay are exhibited in Table 1, which contains both the results of the intra- and inter-day precision and accuracy studies.

LOD (3 ng/mL) and LOQ (10 ng/mL) of the method were defined as the analyte concentration producing signal to noise ratio equal to 3:1 and 10:1, respectively.



(a)



(b)

Figure 1. HPLC chromatograms from a) blank plasma and b) plasma spiked with indomethacin (100 ng/mL); (EPP) – an endogenous plasma peak.

The mean extraction efficiency of indomethacin from plasma at concentrations 10, 200, and 5000 ng/mL were 96.59, 96.92, and 98.11%, respectively. Each sample was analyzed in seven replicates and the results were compared with those obtained by analyzing standard samples containing the same concentrations of the analyte dissolved in the mobile phase.

As it was mentioned in the Introduction, several methods of indomethacin determination in plasma have been reported in literature. In the most recently described procedures applying protein precipitation, the LOD values were 10[12] or 5 ng/mL.[20] The latter value seems to be

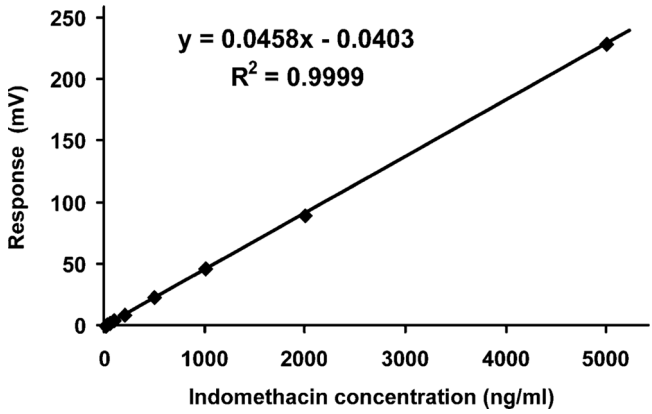


Figure 2. Linearity of the external standard calibration curve for determination of total indomethacin concentration in plasma samples.

questionable due to the fact that, according to the authors, the limit of detection is equated to the analyte amount of 350 ng introduced on the column in the form of a 100 μL sample. Moreover, the cited LOD was obtained at 8-fold dilution of the plasma sample. It should be additionally stressed, that the LOD value of 10 ng/mL reported by Boon et al.[12] was gained by using a similar sample preparation method considering 2-fold sample dilution.

The method described in the presented paper is characterized by LOD of 3 ng/mL. This value is comparable with the one obtained by the precipitation method developed by Al Za'abi et al.[20] and considerably better than the one reported by Boon et al.[12] Such high sensitivity of our approach was achieved by injecting twice as low a sample volume (50 μL), which was possible thanks to the analyte preconcentration during

Table 1. Statistic parameters and absolute analyte recovery calculated for the assay of total indomethacin in human plasma (n = 7)

Nominal concentration (ng/mL)	Calculated concentration (ng/mL, mean ± SD)	Inaccuracy (%)[a]	Intra-day imprecision (RSD %)[b]	Inter-day imprecision (RSD %)[b]	Absolute recovery (%) (mean ± SD)
10	10.92 ± 1.10	+9.24	5.51	5.76	96.59 ± 4.22
200	205.03 ± 7.82	+2.51	3.77	3.91	96.92 ± 2.97
5000	5011.36 ± 53.7	+0.23	1.11	1.88	98.11 ± 3.98

[a]Inaccuracy is defined as the error % estimated as [(calculated concentration – nominal concentration)/nominal concentration] × 100%.
[b]RSD % = (SD/mean) × 100%.

the sample preparation. In consequence, the method seems to be applicable for the determination of free indomethacin concentration in plasma.

Determination of Free Indomethacin Concentration in Plasma

Chromatograms of the blank plasma ultrafiltrate and the ultrafiltrate of plasma spiked with indomethacin (3000 ng/mL) are presented in

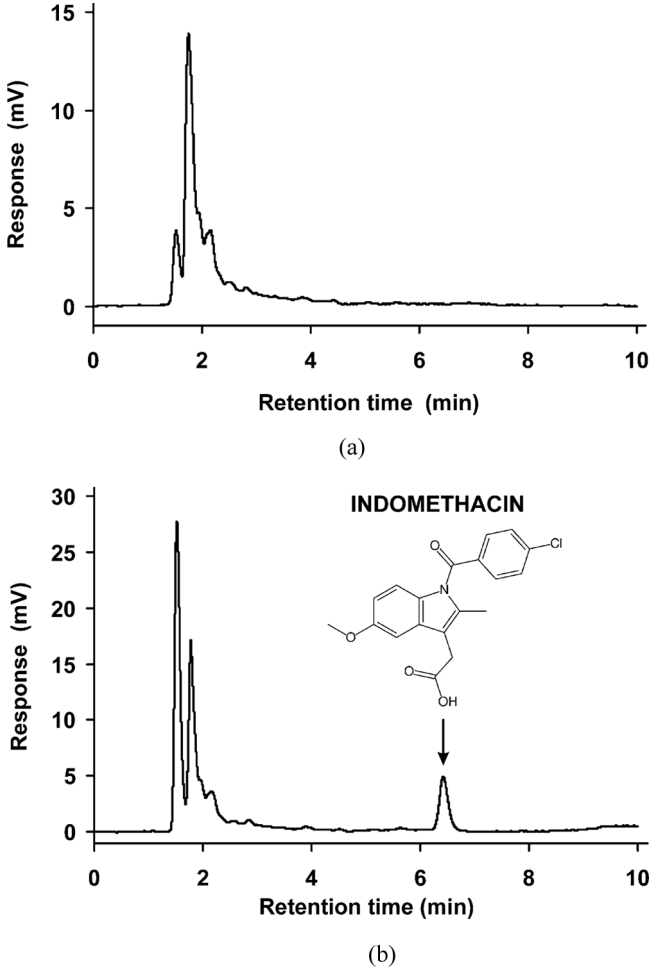


Figure 3. HPLC chromatograms from a) blank plasma ultrafiltrate and b) ultrafiltrate of plasma spiked with indomethacin (the total drug concentration in the spiked plasma was 3000 ng/mL). The obtained peak corresponds with free indomethacin concentration 96 ng/mL (about 3% of total drug concentration in plasma).

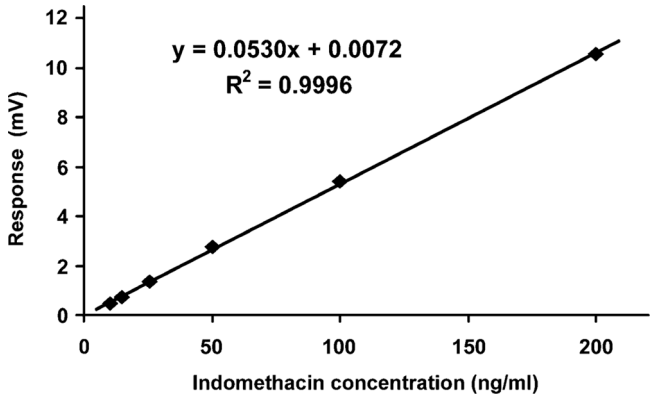


Figure 4. Linearity of the external standard calibration curve for determination of free indomethacin concentration in plasma.

Figures 3a and Figure 3b, respectively. The chromatographic assay of free drug concentration was identical with the one applied for total drug determination. Hence, the retention time of the analyte and the total run time correspond with those presented in Figures 1a and b. As it can be seen in the Figure 3, both chromatograms display a lower number of interfering plasma peaks thanks to the ultrafiltration process applied for sample preparation.

The calibration curve for indomethacin performed in the concentration range of $10-200\,ng/mL$ is presented in Figure 4. The plot is characterized with a very good linearity with a regression coefficient (R^2) of 0.9996.

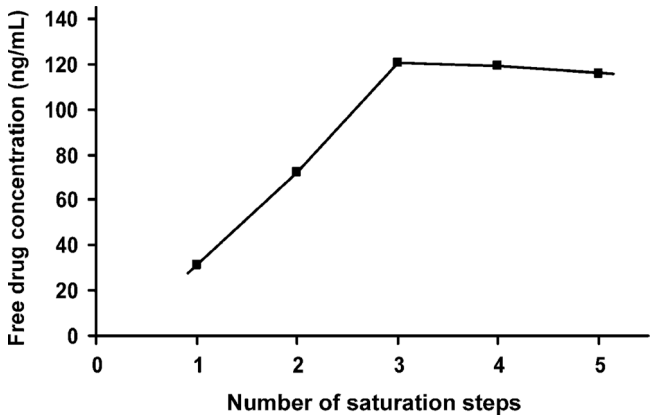


Figure 5. The effect of the number of ultrafiltrate membrane saturation steps on indomethacin concentration in the obtained ultrafiltrates.

Table 2. Statistic parameters and absolute analyte recovery calculated for the assay of free indomethacin in plasma (n = 7)

Nominal concentration (ng/mL)	Calculated concentration (ng/mL, mean ± SD)	Inaccuracy (%)[a]	Intra-day imprecision (RSD %)[b]	Inter-day imprecision (RSD %)[b]	Absolute recovery (%) (mean ± SD)
10	9.02 ± 1.22	−9.79	3.91	5.00	99.60 ± 2.62
50	51.75 ± 4.18	+3.50	3.83	5.38	96.72 ± 3.48
200	198.92 ± 8.76	−0.54	1.91	2.09	96.48 ± 4.28

[a]Inaccuracy is defined as the error % estimated as [(calculated concentration – nominal concentration)/nominal concentration] × 100%.
[b]RSD % = (SD/mean) × 100%.

The analytical procedure of free drug concentration in plasma requires separation of the free analyte from drug protein complex. As it was described in Experimental, an ultrafiltration technique was applied for this purpose in the developed approach. This type of separation method may produce analytical errors due to uncontrolled drug adsorption on the ultrafiltration membrane.[24,25] Primary saturation of the applied membrane is the simplest and the most efficient way of avoiding drug loss in an ultrafiltration process. Figure 5 shows the number of saturation steps required for the complete membrane saturation with indomethacin. As the results from the picture show, only two saturation steps are necessary for the saturation of the used membrane. After that, the free drug concentration in the obtained ultrafiltrate is settled at the constant level. The percentage of free indomethacin calculated by means of ultrafiltration is about 3%, which is consistent with the data reported in literature.[8]

Table 2 shows the data concerning intra- and inter-day imprecision and inaccuracy of the assay, as well as the analyte recovery of the overall sample preparation procedure. Imprecision expressed as RSD% was in the range of 1.91–5.38 and inaccuracy expressed as error % did not exceed 10%. These data indicate the highly satisfactory parameters characterizing the quality of the analytical approach.

CONCLUSION

The problem of free indomethacin determination in plasma is an important, yet not extensively studied issue in the field of clinical analysis. This fact results from difficulties in development of an analytical method sensitive enough to determine the free indomethacin concentration, which is known to constitute only about 3% of total drug concentration.[8]

The analytical approaches recently described in literature, involving plasma protein precipitation, are successful in the determination of total

indomethacin concentration. However, they seem not to be applicable for the free drug estimation as they do not provide enrichment of the analyte (sample undergoes dilution during the preparation procedure).

Following the above, the extraction technique seems to be the method of choice for the free drug analysis in plasma samples. So far, the analytical approaches involving liquid–liquid extraction have not provided indomethacin recovery and detection limit sufficient for the determination of free drug concentration. The analytical method described in this paper is characterized by very high indomethacin recovery (ca. 97%) and very low LOD value (3 ng/mL), comparable with the lowest value reported in literature obtained by the precipitation method.[20] Such high sensitivity justifies the application of the presented method for the analysis of free indomethacin.

It should be emphasized that the research on drug pharmacokinetics frequently requires not only the evaluation of free drug concentration but also its protein binding degree. For this purpose, the reliable method of total drug concentration analysis is necessary.

As results from the paper prove, the presented method can also be successfully applied for the determination of total indomethacin concentration in plasma. Liquid–liquid extraction procedure is more selective and allows for better sample cleanup in comparison to the one involving protein precipitation. Moreover, that way of sample preparation reduces the risk of analytical error, which may possibly occur due to drug coprecipitation with plasma proteins.[26] Inaccurate estimation of total drug concentration results in erroneous calculation of unbound drug fraction. Therefore, the extraction technique seems to be preferred to the precipitation procedure, especially in the cases when total drug concentration is going to be applied for evaluation of drug protein binding degree.

The paper presents a sensitive, accurate, and reproducible HPLC-UV detection method for the determination of free indomethacin concentration in human plasma. High recovery and selectivity of the plasma sample preparation procedure, as well as the very low limit of detection recommend the described approach for its application for the pharmacokinetic studies of indomethacin.

REFERENCES

1. Lin, S.-J.; Chen, Y.-R.; Su, Y.-H.; Tseng, H.-I; Chen, S.-H. Determination of indomethacin in plasma by micellar electrokinetic chromatography with UV detection for premature infants with patent ducts arteriosus. J. Chromatogr. B. **2006**, *830*, 306–313.
2. Brash, A.R.; Hickey, D.E.; Graham, T.P.; Stahlman, M.T.; Oates, J.A.; Cotton, R.B. Pharmacokinetics of indomethacin in the neonate. Relation

of plasma indomethacin levels to response of the ductus arteriosus. N. Engl. J. Med. **1981**, *305*, 67–72.
3. Mrongovius, R.; Imbeck, H.; Wille, L.; Muller, H.; Seyberth, H.W. Variability of serum indomethacin concentrations after oral and intravenous administration to preterm infants. Eur. J. Pediatr. **1982**, *138*, 151–153.
4. Yaffe, S.J.; Friedman, W.F.; Rogers, D.; Lang, P.; Ragni, M.; Saccar, C. The disposition of indomethacin in preterm babies. J. Pediatr. **1980**, *97*, 1001–1006.
5. Mehta, A.C. Therapeutic monitoring of free (unbound) drug levels: Analytical aspects. Trends Anal. Chem. **1989**, *8*, 107–112.
6. Svensson, C.K.; Woodruff, M.N.; Baxter, J.G.; Lalka, D. Free drug concentration monitoring in clinical practice. Rationale and current status. Clin. Pharm. **1986**, *11*, 450–469.
7. Olsen, G.D.; Bennett, W.M.; Porter, G.A. Morphine and phenytoin binding to plasma proteins in renal and hepatic failure. Clin. Pharmacol. Ther. **1975**, *17*, 677–684.
8. Raveendran, R.; Heybroek, W.M.; Caulfield, M.; Abrams, S.M.; Wrigley, P.F.; Slevin, M.; Turner, P. Protein binding of indomethacin, methotrexate and morphine in patients with cancer. Int. J. Clin. Pharmacol. Res. **1992**, *12*, 117–122.
9. Jacob, R.; Krishanan, B.S.; Venkatesan, T. Pharmacokinetics and pharmacodynamics of anaesthetic drugs in paediatrics. Indian J. Anaesth. **2004**, *48*, 340–346.
10. Dawidowicz, A.L.; Kalitynski, R.; Kobielski, M.; Pieniadz, J. Influence of propofol concentration in human plasma on free fraction of the drug. Chem. Biol. Int. **2006**, *159*, 149–155.
11. Lin, J.H.; Cocchetto, D.M.; Duggan, D.E. Protein binding as a primary determinant of the clinical pharmacokinetic properties of non-steroidal anti-inflammatory drugs. Clin. Pharmacokinet. **1987**, *12*, 402–432.
12. Boon, V.; Glass, B.; Nimmo, A. High-performance liquid chromatographic assay of indomethacin in porcine plasma with applicability to human levels. J. Chromatogr. Sci. **2006**, *44*, 41–44.
13. Wan, H.; Rehngren, M. High-throughput screening of protein binding by equilibrium dialysis combined with liquid chromatography and mass spectrometry. J. Chromatogr. A. **2006**, *1102*, 125–134.
14. Brown, Y.L.; Kandrotas, R.J.; Douglas, J.B.; Gal, P. High-performance liquid chromatographic determination of indomethacin serum concentrations. J. Chromatogr. **1988**, *459*, 275–279.
15. Liu, S.; Kamijo, M.; Takayasu, T.; Takayama, S. Direct analysis of indomethacin in rat plasma using a column-switching high-performance liquid chromatographic system. J. Chromatogr. B. **2002**, *767*, 53–60.
16. Ou, C.-N.; Frawley, V.L. Liquid-chromatographic determination of indomethacin in blood from newborns with patent ductus arteriosus. Clin. Chem. **1984**, *30*, 898–901.
17. Roberts, I.; Smith, I.M. A high performance liquid chromatography method for the analysis of total and free indomethacin in serum. Ann. Clin. Biochem. **1987**, *24*, 167–171.

18. Sato, J.; Amizuka, T.; Niida, Y.; Umetsu, M.; Ito, K. Simple, rapid and sensitive method for the determination of indomethacin in plasma by high-performance liquid chromatography with ultraviolet detection. J. Chromatogr. B **1997**, *692*, 241–244.
19. Bernstein, M.S.; Evans, M.A. High-performance liquid chromatography-fluorescence analysis for indomethacin and metabolites in biological fluids. J. Chromatogr. B. **1982**, *229*, 179–187.
20. Al Za'abi, M.A.; Dehghanzadeh, G.H.; Norris, R.L.G.; Charles, B.G. A rapid and sensitive microscale HPLC method for the determination of indomethacin in plasma of premature neonates with patent ductus arteriousus. J. Chromatogr. B. **2006**, *830*, 364–367.
21. Grippa, E.; Santini, L.; Castellano, G.; Gatto, M.T.; Leone, M.G.; Saso, L. Simultaneous determination of hydrocortisone, dexamethasone, indomethacin, phenylbutazone and oxyphenbutazone in equine serum by high-performance liquid chromatography. J. Chromatogr. B **2000**, *738*, 17–25.
22. Tomuta, I.; Vlase, L.; Leucuta, S.E. HPLC determination of indomethacin from human plasma. Ovidius University Annals of Medical Science-Pharmacy **2003**, *1*, 182–186.
23. Johnson, A.G.; Ray, J.E. Improved high-performance liquid chromatographic method for the determination of indomethacin in plasma. Ther. Drug Monit. **1992**, *14*, 61–65.
24. Chan, G.L.-Y.; Axelson, J.E.; Price, J.D.E.; McErlane, K.M.; Kerr, C.R. In vitro protein binding of propafenone in normal and uraemic human sera. Eur. J. Clin. Pharmacol. **1989**, *36*, 495–499.
25. Lohman, J.J.; Merkus, F.W.; Costongs, G.M.; Hegtermans, E.P.; Hooymans, P.M. Pitfalls in the determination of unbound carbamazepine concentrations in plasma. Pharm. Weekl. Sci. **1984**, *6*, 91–95.
26. Dawidowicz, A.L.; Fornal, E.; Fijalkowska, A. Problems in the analysis of propofol in blood when protein precipitation is used in sample preparation. Chromatographia **1998**, *47*, 523–528.

Received May 3, 2009
Accepted June 9, 2009
Manuscript 6546