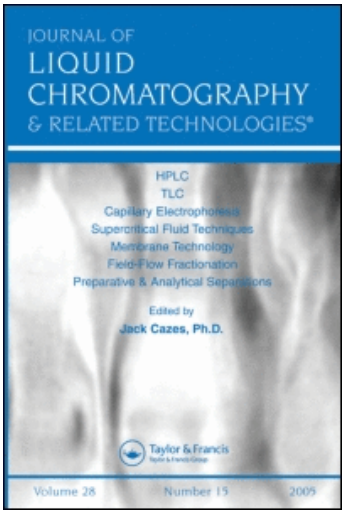

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Automatic Determination of Indomethacin in Human Plasma Using Liquid-Solid Extraction on Disposable Cartridges in Combination with HPLC

Ph. Hubert[a]; J. Crommen[a]
[a] Laboratory of Drug Analysis Institute of Pharmacy University of Liege rue Fusch, Liege, Belgium

To cite this Article Hubert, Ph. and Crommen, J.(1990) 'Automatic Determination of Indomethacin in Human Plasma Using Liquid-Solid Extraction on Disposable Cartridges in Combination with HPLC', Journal of Liquid Chromatography & Related Technologies, 13: 19, 3891 — 3907
To link to this Article: DOI: 10.1080/01483919008049576
URL: http://dx.doi.org/10.1080/01483919008049576

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(19), 3891-3907 (1990)

Original Article

AUTOMATIC DETERMINATION OF INDOMETHACIN IN HUMAN PLASMA USING LIQUID-SOLID EXTRACTION ON DISPOSABLE CARTRIDGES IN COMBINATION WITH HPLC

PH. HUBERT AND J. CROMMEN
Laboratory of Drug Analysis
Institute of Pharmacy
University of Liege
rue Fusch, 5
B-4000 Liege, Belgium

ABSTRACT

A sensitive and automatic method for the analysis of indomethacin in plasma has been developed using liquid-solid extraction (LSE) on disposable extraction cartridges (DECs) coupled to high-performance liquid chromatography (HPLC). The fully automated system handles the plasma samples by performing the same operations as in a manuel procedure by means of an autosampler equipped with a robotic arm at which is attached a needle dispensing the different liquids. The DEC is first conditioned with methanol and phosphate buffer pH 7.4. A 1.0-mL volume of plasma is then applied onto the DEC; the latter is washed with the same buffer before the elution with 0.25 mL of methanol. The eluting strength of the eluate is reduced by dispensing 0.30 mL of phosphate buffer pH 7.4 in the collection tube prior to the injection onto the HPLC column via a 0.1-ml loop. The chromatographic separation is performed on an octadecylsilica column with a mixture of methanol and phosphate buffer pH 7.4 as mobile phase (60:40, v/v) and indomethacin is monitored photometrically at 254 nm. The effect of the plasma dispensing flow rate on the drug recovery and the importance of the guard column for the stability of the

analytical column have been studied. The absolute recovery of the drug is 96.0 % and the limit of detection, 2 ng/mL. At the concentration of 100 ng/mL, relative standard deviations of 1.5 % (within-day) and 2.3 % (between-day) have been obtained.

INTRODUCTION

In order to eliminate proteins and other hydrophilic endogenous compounds which might interfere in the analysis, a preliminary clean-up step is usually needed in the determination of drugs in plasma by HPLC.

Moreover, the analyte is often present in plasma at lower concentrations than 100 ng/mL, as it is e.g. the case in the bioavailability study of indomethacin after external application to the skin as a spray solution. Under these conditions it is necessary to couple an enrichment step to the sample work-up in order to obtain a sufficient degree of sensitivity.

Indomethacin, widely used as anti-inflammatory drug, has, among other characteristics, a very low solubility in water at acidic pH and a strong tendency to be bound to plasma proteins (up to 99 %) (1).

Several papers can be found in the literature which describe the determination of indomethacin in plasma by HPLC. Most often, an off-line liquid-liquid extraction of the drug by organic solvents after acidification is used as sample purification step and the evaporation of the organic supernatant, as concentration step (2-9). The extraction is sometimes performed after precipitation of proteins by a chemical reagent (2,5,6,8,9). In some cases, the sample pretreatement only consists of such a protein precipitation (10,11,12).

The possibility of enhancing the selectivity and sensitivity of detection by measurement of fluorescence after alkaline hydrolysis of indomethacin has been exploited either in the pre-column mode (4) or in the post-column mode (3,10,13,14).

In recent years, liquid-solid extraction (LSE), which can easily be automated, has been used increasingly as an alternative to liquid-liquid

extraction for the clean-up of plasma samples (15-20). We have developed previously a fully automated HPLC method for the analysis of indomethacin in plasma involving a column switching system with elution in the fore-flush mode (19).

The purpose of this paper is to describe another automatic technique for the determination of indomethacin in plasma in which LSE is performed on disposable extraction columns (DECs) before on-line injection into the HPLC system. The plasma samples are treated in the same way as in a manual procedure by a robotic sampler with an XYZ-motion arm equipped with a needle dispensing the different liquids. The final extract, which has a lower eluting strength than the mobile phase, is then automaticaly introduced into the separation column with the same needle. This allows peak compression at the top of the analytical column (20). Indomethacin is quantified by UV detection at 254 nm.

The dependence of the drug recovery on the plasma dispensing flow rate and on the kind of sorbent used in the DECs have been studied. The possible effect of the air pressurizing volume applied to the plasma sample, as well as the influence of the volume of plasma extract injected and the guard column on the lifetime of the analytical column have also been investigated. The method developed has been validated and reproducibility results are presented.

MATERIALS AND METHODS

Apparatus

Liquid chromatography was performed on a Waters model 6000 A solvent delivery system coupled to a Waters model 440 single wavelength detector measuring at 254 nm (Waters Associates, Milford, MA, USA).

Liquid-solid extraction (LSE) was made on disposable extraction columns (DECs) and controlled by an ASPEC system (Automactic Sample Preparation with Extraction Columns) from Gilson (Villiers-le-Bel, France). The ASPEC system had three components : a set of racks (solvent rack, sample rack, LSE rack), a model 401 dilutor and an automatic sampling injector module which was equipped with an electrically actuated six port Rheodyne valve and with a robotic arm holding a needle through which the different liquids were dispensed. The sample loop had a volume of 0.1 mL. The LSE rack consisted of a DEC holder, a drain cuvette and a collection rack. The needle of the autosampler module could move the DEC holder in such a way that each DEC could be automaticaly placed above the drain cuvette during conditionning, sample loading and washing or above a collection tube during elution.

A LiChroCART analytical column (125 x 4 mm, i.d.) was used in combination with a LiChroCART guard column (4 x 4 mm, i.d.), both prepacked with the same support material (Merck, Darmstad, FRG). They were thermostatted at 35.0 $\pm$ 0.1°C in a model 02PT923 water-bath from Heto (Birkeröd, Denmark).

A model BD 9 two-channel recorder from Kipp and Zonen (Delft, The Netherlands) and a model 4270 integrator from Spectra-Physics (San-Jose, CA, USA) were used simultaneously for data collection.

Chemicals and Reagents

Indomethacin was obtained from Sigma (Saint-Louis, MO, USA). Potassium monohydrogen phosphate and sodium hydroxide were of p.a. quality from E. Merck (Darmstad, FRG). Methanol was of HPLC grade from Lab-Scan (Dublin, Ireland). Water was glass-distilled.

CHROMABOND DECs (capacity : 1 mL) packed with 100 mg of bonded silica material (particle size : 35-40 um) were used as supplied by Macherey-Nagel (Düren, FRG).

The LiChroCART columns were prepacked with LiChrospher 100 RP 18, 5 um (Merck).

Chromatographic Technique

The mobile phase consisted of methanol-phosphate buffer pH 7.4 (60:40, v/v). The flow rate was 0.7 mL/min. Before use, the mobile phase was degassed in a ultrasonic bath.

The phosphate buffer was prepared in a 1-L volumetric flask by mixing 250 mL 0.1 M potassium monohydrogen phosphate with 195.5 mL 0.1 M sodium hydroxide. Water was added to the volume. The buffer solution was then filtered through a paper filter. The same buffer was used in the second step of the DEC conditioning and as washing eluent for the clean-up and the dilution steps. The auto-sampler needle and the external tubing of the injection valve were also rinsed with phosphate buffer pH 7.4.

The stock methanolic solution of indomethacin at 1 mg/mL was further diluted with phosphate buffer before addition to the plasma samples used for the calibration curves and with methanol-phosphate buffer pH 7.4 (50-60, v/v) before direct injection in the recovery measurements. New diluted solutions were prepared every day.

Automatic Sample Preparation

The plasma sample was centrifuged at 3000 rpm during 10 minutes. A 2-mL volume of plasma was introduced into a vial placed on the appropriate rack of the auto-sampler.

After this manual operation, the automatic procedure was started. Before the beginning of the first cycle, the needle of the auto-sampler and the external tubing of the injection valve were washed with 1.0 mL of phosphate buffer pH 7.4. Between each step, the needle was rinsed with the same volume of buffer (flow rate : 12.0 mL/min) and a 10 mm air gap was generated inside the transfer tubing before pipetting the next liquid, in order to avoid cross-contamination.

The solvents and the plasma sample were dispensed under a positive pressure and they were then pushed through the packing of the DEC by air. For this reason, special caps were provided to seal the DECs during liquid and air delivery. The dispensing flow rates of liquids ranged from 0.18 to 96.0 mL/min. Air pressurizing volumes could also be varied.

The automatic sequence was performed in the following way (total cycle time : 17 min) :

- **DEC conditionning** (flow rate : 3.0 mL/min; air volume : 0.1 mL) :

At the beginning, the DEC holder is located above the drain cuvette (front position). The DEC (CHROMABOND C18) is first washed with 2.0 mL of methanol, then with 2.0 mL of phosphate buffer pH 7.4 in order to prepare the sorbent to receive the plasma sample.

- **Loading with plasma sample** (flow rate : 0.18 mL/min; air volume : 1.0 mL) :

The auto-sampler needle aspirates 1.0 mL of plasma from the corresponding vial and dispenses it on the DEC.

- **Washing** (flow rate : 1.5 mL/min; air volume : 1.0 mL) :

2.0 mL of phosphate buffer pH 7.4 are dispensed on the DEC. The packing is then flushed with 1.0 mL of air.

- **Elution** (flow rate : 1.5 mL/min; air volume : 1.0 mL) :

The DEC holder is pushed by the needle over the collection rack. A 0.25-mL volume of methanol is applied on the DEC. The eluate is collected in the tube positioned under the DEC.

- **Dilution** (flow rate : 1.5 mL/min; air volume : 1.0 mL) :

0.3 mL of phosphate buffer pH 7.4 are dispensed on the DEC. The DEC holder is then replaced in its front position.

- **Mixing** :

The diluted eluate is successively aspirated and dispensed in the collection tube by the needle. These operations are repeated three times.

- **Injection** :

The needle aspirates the whole volume of the final extract in the collection tube and dispenses it in the loop filler port. By switching of the injection

TABLE 1

Type of Stationary Phase Used in the Disposable Extraction Columns

Type of Sorbent	Absolute Recovery (%)
CN	3
C2	66
C8	89
C18	96

DECs : CHROMABOND (100 mg)
Dispensing flow rate of plasma : 0.18 mL/min; air pressurizing volume applied on plasma : 1.0 mL; washing eluent : phosphate buffer pH 7.4
Other chromatographic conditions as described in Materials and Methods
Sample : spiked plasma (indomethacin concentration : 100 ng/mL)

valve, 0.1 mL of the extract is injected into the HPLC column, the excess being directed to the waste.

The chromatographic separation of the prepared sample was performed during the LSE of the next sample (sequential mode).

RESULT AND DISCUSSION

Type of Sorbent Used for the Clean-up Step

The different kinds of DECs tested contained bonded silicas covering a relatively wide range of polarity (cfr Table 1). In all cases, phosphate

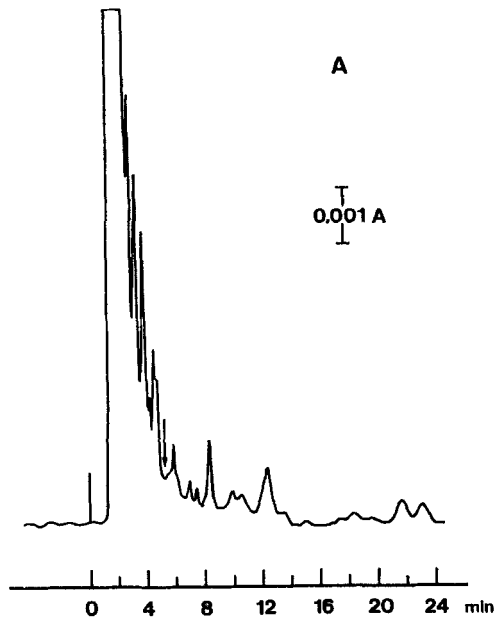

FIGURE 1

Typical chromatograms obtained by using LSE on disposable extraction cartridges in combination with HPLC.
DEC : CHROMABOND C18; dispensing flow rate of plasma : 0.18 mL/min.
Other chromatographic conditions as described in Materials and Methods.
Sample : A : blank plasma
B : spiked plasma (indomethacin concentration : 50 ng/mL).
Peak : 1 : Indomethacin (9.1 ng).

buffer pH 7.4 was selected as washing eluent in order to avoid the memory effects frequently observed with indomethacin in the acidic pH range (19).

As can be seen from Table 1, the fairly polar cyano phase gave a very low recovery under these conditions. Among the alkylbonded phases, the recoveries of indomethacin were found to increase, as expected, with the number of carbon atoms in the alkyl chains. High recoveries were obtained with DECs containing octyl and octadecylsilica (cfr Table 1). On the other hand, the

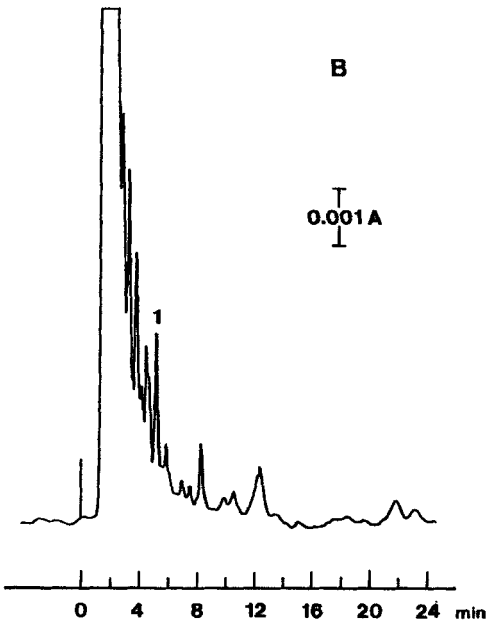

Figure 1 (continued)

two phases gave rise to very similar chromatograms, devoid of interferences from plasma components at the retention time of indomethacin (cfr Fig. 1). Finally, the C18 phase was given the preference owing to its somewhat higher affinity for the drug.

Dispensing Flow Rate of Plasma on the DEC

Fig. 2 shows that the flow rate at which the plasma is applied onto the DEC has a considerable influence on the absolute recovery of indomethacin. The latter approaches 100 % at the lowest flow rate when the DEC sorbent consists of octadecylsilica (see also Table 1) but decreases drastically with increasing flow rate. A fairly constant recovery of about 20 % is obtained at

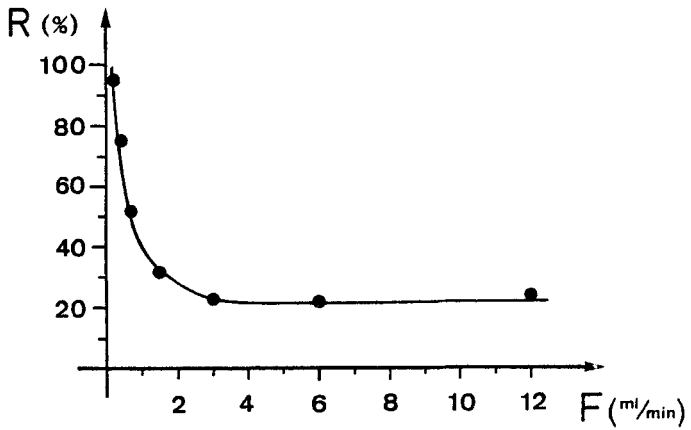

FIGURE 2

Influence of te dispensing flow rate of plasma on the recovery of indomethacin.
DEC : CHROMABOND C18 (100 mg).
Other conditions as described in Materials and Methods.
R : absolute recovery of indomethacin.
F : dispensing flow rate of the plasma sample.

dispensing flow rate higher than 2 mL/min. Under these conditions, the type of sorbent seems to have very little effect since DECs filled with the C2 phase give approximately the same recovery as those containing the C18 phase.

The further steps of the automatic sample preparation procedure (washing, elution, mixing) could be optimized by use of aqueous standard solutions of indomethacin instead of plasma samples (20). On the contrary, the strong effect of the dispensing flow rate illustrated in Fig. 2 was only observed when the DECs were loaded with plasma samples. This indicates that the effect can be correlated to the strong binding of indomethacin to plasma proteins (1). At high dispensing flow rates, the residence time of the plasma sample in the DEC is reduced to such an extent that only a part of the drug

TABLE 2

Recoveries obtained with different air pressurizing volumes dispensed on the DEC

Air pressurizing volume (mL)	Absolute recovery (%)
0.25	97.0
0.30	93.0
0.40	97.8
0.50	95.7
1.00	98.0
1.50	99.2
2.00	97.2
mean recovery	96.8
RSD (n= 7)	2.1

DECs : CHROMABOND C18 (100 mg)

Dispensing flow rate of plasma : 0.18 mL/min

Other conditions as described in Materials and Methods

Sample : spiked plasma (indomethacin concentration : 100 ng/mL)

is displaced from its binding to proteins and can be distributed to the DEC sorbent.

Air Pressurizing Volume Applied on the Plasma Sample

After delivery of liquids at the top of the packing, an air volume is aspirated outside the cartridge and then introduced into the DEC. This produces a positive pressure wich can force the liquids to flow through the sorbent. Consequently, the air volume applied on the DEC during the loading

step with plasma sample might also have an influence on the recovery of indomethacin.

However, the data given in Table 2 show that there are no significant differences in the recoveries obtained with air volumes ranging from 0.25 to 2.0 mL, the mean recovery being around 97 %. This is probably due to the fact that the plasma delivery is so slow in this case that there is virtually no residual volume of plasma at the top of the sorbent. Therefore, the air volume introduced into the DEC cannot influence significantly the passage of plasma through the cartridge and thus the drug recovery. With a dispensing flow rate higher than 0.18 mL/min, the residual volume of plasma increases and the influence of the air volume on the passage of plasma becomes significant, as it is the case with the other liquids which are delivered on the DEC at higher flow rates.

Effect of the Volume of Plasma injected and the Guard Column on the Stability of the Analytical Column

As can be seen from Fig. 3, the height of the indomethacin peak has a tendency to decrease with increasing total volume of plasma extract introduced into the chromatographic system, in which the analytical column is preceded by a short guard column packed with the same support material. The introduction of a total volume of 10 mL of plasma extract, corresponding to 100 injections in the method described, causes a reduction of about 30 % of the efficiency of the chromatographic system. This is accompanied by an increase in back pressure which also reaches about 30 % while the retention of indomethacin remains constant.

No improvements have been obtained by intensive washing of the columns in the back-flush mode. On the other hand, the replacement of the guard column practically restores the initial peak height (cfr dotted lines in Fig. 3) as well as the original pressure. Obviously the successive losses of efficiency observed in Fig. 3 are essentially due to the deterioration of the

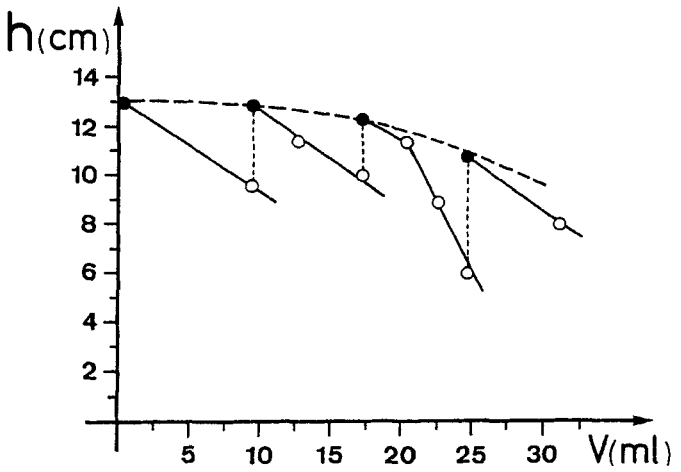

FIGURE 3

Influence of the volume of plasma extract injected and the guard column on the life-time of the analytical column.
Mobile phase : méthanol - phosphate buffer pH 7.4 (60-40).
Solid phase : LiChrospher 100 RP-18.
Injection loop : 0,1 mL.
Analytical column (125 x 4 mm i.d.) preceded by a guard column (4 x 4 mm i.d.).
● : new guard column
o : used guard column
h : height of the indomethacin peak
V : total volume of plasma extract injected.

guard column and it is therefore advisable to replace it regularly (every 50 injections in the present method) in order to maximize the lifetime of the analytical column.

Fixed-Wavelength UV Detector

As mentioned previously (19), a single wavelength detector, measuring at 254 nm, was selected for the determination of indomethacin at lower plasma

TABLE 3

Reproducibility of assay of indomethacin

Concentration (ng/mL)	Within-day RSD (%)	n	Between-day RSD (%)	n
500	1.8	6	-	-
100	1.5	6	2.3	9
20	3.8	6	-	-

concentrations than 100 ng/mL, even if the drug presents a slightly higher molar absorptivity at 260 nm. Among the instruments tested, the best signal-to-noise ratio was in fact obtained with the fixed wavelength detector, owing to its particularly low baseline noise.

Validation of the Method

The absolute recovery of indomethacin was found to be 96.0 $\pm$ 3.4 % (mean recovery $\pm$ S.D; n = 60) (cfr also Table 2). It was estimated by comparing the peak heights obtained by injection of spiked plasma samples into the ASPEC system with those found on direct injection of aqueous standard solutions of indomethacin onto the analytical column.

The mean capacity ratio of indomethacin is equal to 3.15 with a RSD of 0.6 % for successive injections of spiked plasma samples (n = 11).

Linear regression analysis made by plotting peak height (Y) in mm versus the indomethacin concentration of spiked plasma samples (X) in ng/mL (eight concentrations of indomethacin in duplicates, ranging from 10 to 200 ng/Ml)

gives the following equation with a coefficient of determination (r2) of 0.9986 : Y = 1.089 X + 0.857 (AUFS : 0.01 at 254 nm).

Calculated from the regression line (21), the limit of detection (L.O.D.) of indomethacin is equal to 2 ng/mL and the limit of quantification (L.O.Q.) to 6 ng/mL.

The precision of the method was estimated by calculating the RSD values at three different plasma concentrations for the within-day reproducibility and at 100 ng/mL for the between-day reproducibility (cfr Table 3).

At the retention time of indomethacin, the absence of interfering endogenous components is demonstrated in Fig. 1 which shows chromatograms obtained on injection of blank and spiked plasma samples.

The fully automated method has been applied successfully to bioavailability studies of indomethacin after percutaneous administration and has proved to be rugged.

ACKNOWLEDGMENTS

The loan of GILSON ASPEC system by Analis NV/SA (Ghent/Namur, Belgium) and the financial support of the pharmaceutical company SMB-Galephar (Brussels, Belgium) are gratefully acknowledged

REFERENCES

(1) Mason R.W. and McQueen E.G., Protein Binding of Indomethacin, Pharmacology, 12, 12, 1974.

(2) Terweij-Groen C.P., Heemstra S. and Kraak J.C., Rapid Determination of Indomethacin and Salicylic Acid in Serum by means of Reversed-Phase Liquid Chromatography, J. Chromatogr., 181, 385, 1980.

(3) Bayne W.F., East T. and Dye D., High-Pressure Liquid Chromatographic Method with Post-column, In-Line Hydrolysis and Fluorometric Detection for Indomethacin in Biological Fluids, J. Pharm. Sci., 70, 458, 1981.

(4) Bernstein M.S. and Evans M.A., High-Performance Liquid Chromatography Fluorescence Analysis for Indomethacin and Metabolites in Biological Fluids, J. Chromatogr., 229, 179, 1982.

(5) Astier A. and Renat B., Sensitive High-Performance Liquid Chromatographic Determination of Indomethacin in Human Plasma, J. Chromatogr., 223, 279, 1982.

(6) Cooper J.K., McKay G., Hawes E.M. and Midha K.K., HPLC Assay of Indomethacin and its Application in Pharmacokinetics in Healthy Volunteers, J. Chromatogr., 233, 289, 1982.

(7) Smith P.C. and Benet L.Z., HPLC Method for Determination of Indomethacin and its Two Primary Metabolites in Urine, J. Chromatogr., 306, 315, 1984.

(8) Sauvaire D. Cociglio M. and Alric R., Liquid Chromatographic Measurement for Plasma Indomethacin and its Prodrug Apyramide, J. Chromatogr., 375, 101, 1986.

(9) Clark C.R., McMillian C.L., Hoke J.F. Campagna K.D. and Ravis R., Liquid Chromatographic Determination of Sunlindac and Metabolites in Serum, J. Chromatogr. Sci., 25, 247, 1987.

(10) Stubs R.J., Swartz M.S., Chiou R., Entwistle L.A. and Bayne W.F., Improved Method for Determination of indomethacin in Plasma and Urine by Reversed-Phase High-Performance Liquid Chromatography, J. Chromatogr., 383, 432, 1986.

(11) Kaiko F., Nomura H. Makabe E. and Kato Y., Percutaneous Absorption of Indomethacin from Mixtures of Fatty Alcohol and Propylene Glycol through Rat Skin, Chem. Pharm. Bull., 35, 2928, 1987.

(12) Brown Y.L., Kandrotas R.J., Douglas J.B. and Gal P., High-Performance Liquid Chromatographic Determination of Indomethacin Serum Concentrations, J. Chromatogr., 459, 275, 1988.

(13) De Zeeuw D., Leinfelder J.L. and Brater D.C., Highly Sensitive Measurement of Indomethacin Using a HPLC Technique combined with Post-Column In-Line Hydrolysis, J. Chromatogr., 380, 157, 1986.

(14) Reginster J.Y. Crommen J., Renson M. and Franchimont P., Percutaneous Administration of Indomethacin in Rheumatoid Arthritis - Evaluation of Two Topical Preparations, Curr. Ther. Res., 47, 548, 1990.

(15) Frei R.W., New Sample Handling Strategies in HPLC, Swiss. Chem., 6, 55, 1984.

(16) McDowall R.D., Pearce J.C. and Murkitt G.S., Liquid-Solid Sample Preparation in Drug Analysis, J. Pharm. Biomed. Anal., 4, 3, 1986.

(17) Westerlund D., Direct injection of Plasma into Column Liquid Chromatographic Systems, Chromatographia, 24, 155, 1987.

(18) McDowall R.D., Pearce J.C. and Murkitt G.S., Sample Preparation Using Bonded Silica : Recent Experiences and New Instrumentation, TrAC, 8, 134, 1989.

(19) Hubert Ph., Renson M. and Crommen J., A Fully Automated Method for the Determination of Indomethacin in Plasma, J. Pharm. Biomed. Anal., 7, 1819, 1989.

(20) Hubert Ph. and Crommen J., Optimisation of Automated Liquid-Solid Extraction Using Disposable Cartridges in the HPLC Analysis of Drugs in Plasma, paper presented at the 2nd International Symposium on Pharmaceutical and Biomedical Analysis, York, U.K., April 4-7, 1990.

(21) Miller J.C. and Miller J.N., Statistics for Analytical Chemistry, Ellis Horwood, Chichester, 1984, p. 96.

Received: August 24, 1990
Accepted: August 29, 1990