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A fully automated high-performance liquid chromatographic method for the determination of indomethacin in plasma*

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Abstract: A fully automated method is described, which enables the determination of indomethacin in plasma by reversed-phase HPLC following on-line sample enrichment and clean-up on a short pre-column.

The plasma sample is introduced directly into the column switching system. The pre-column, filled with a pellicular bonded phase, is first washed with phosphate buffer, pH 7.4. The compounds retained on the pre-column are then eluted in the fore-flush mode and separated on an octadecylsilica column with a methanolic phosphate buffer (pH 7.4) mobile phase. Indomethacin is determined spectrophotometrically at 254 or 260 nm.

The effect of changes in the pH and flow rate of the washing eluent are studied. Under the conditions selected, memory effects can be avoided, the absolute recovery of the drug is 70% and the limit of detection 10 ng ml^{-1} for a 100 µl injection of plasma. At a concentration of 100 ng ml^{-1}, the relative standard deviations (RSD) are 1.7% (within-day) and 3.5% (between-day), respectively.

Keywords: *Indomethacin; reversed-phase HPLC; determination of drugs in biological fluids; automated on-line sample clean-up; column switching; direct injection of plasma.*

Introduction

It is usually not possible to analyse plasma samples by direct injection onto a HPLC column due to the incompatibility of plasma proteins with microparticulate stationary phases and with the organic solvents which are often used as mobile phase modifiers. It is necessary, therefore, to perform a preliminary clean-up step that will eliminate the proteins and other endogenous components which might interfere in the HPLC determination.

Indomethacin is a well known anti-inflammatory drug which has the following properties: it is a weak acid, practically insoluble in water in its unionized form and strongly bound to plasma proteins (up to 99%) [1–4]. So far, most methods described for the LC analysis of indomethacin in plasma involve an off-line liquid–liquid extraction of the drug by organic solvents, after acidification, as a sample purification step [5–11]. This extraction is sometimes applied after precipitation of the proteins by a suitable chemical

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reagent [5, 8, 9, 11]. In some cases, the sample handling is limited to such a protein precipitation [12, 13].

As far as detection is concerned, indomethacin is monitored spectrophotometrically or spectrofluorometrically after alkaline hydrolysis. The possibility of enhancing the selectivity and sensitivity of detection by measurement of fluorescence has been exploited by several authors, the hydrolysis of the drug being effected either in the pre-column [7] or in the post-column mode [6, 13, 14].

In the present paper, a fully automated LC method for the determination of indomethacin in plasma is described. The sample is injected directly into a column switching system where the enrichment/clean-up step is performed on-line by liquid–solid extraction, using a short pre-column. After elution from the pre-column in the fore-flush mode and passage through the separation column, indomethacin is determined by UV absorption measurements. The dependence of the drug recovery on the pH and flow rate of the washing eluent has been studied. In the selection of the most suitable pH for the clean-up step, other factors also have been taken into account, such as the washing efficiency and the necessity of avoiding memory effects. The method developed is validated and applied to bioavailability studies of indomethacin.

Experimental

Apparatus

The column switching was controlled by an automatic sampler-injector Model 232–401 from Gilson (Villiers-le-Bel, France). This auto-sampler was equipped with two electrically actuated six port Rheodyne valves, one for sample injection and the other for column switching. The sample loop had a volume of 100 μl.

The pre-column used for sample enrichment and clean-up consisted of a Macherey–Nagel (Düren, FRG) empty cartridge (15 × 4 mm, i.d.), supplied with filters and filled manually with pellicular material. The washing eluent was delivered by means of a Gilson Model 302 pump equipped with a Model 802 manometric module.

The analytical column was a LiChroCART prepacked cartridge (125 × 4 mm, i.d.) from E. Merck (Darmstadt, FRG), preceded by a short LiChroCART guard cartridge (4 × 4 mm, i.d.) packed with the same support material. The column was thermostatted at 35.0 ± 0.1°C in a Model 02 PT 923 water bath from Heto (Birkerød, Denmark). The mobile phase was pumped through the column by a Model 6000 A solvent delivery system from Waters Associates (Milford, MA, USA).

The UV monitors were either a Waters Model 440 single wavelength absorbance detector, measuring at 254 nm, or a Model 4020 variable wavelength detector from Pye–Unicam (Cambridge, UK), set at 260 nm. The detector signal was recorded simultaneously by a Model BD 9 two-channel recorder from Kipp and Zonen (Delft, The Netherlands) and by a Model 4270 integrator from Spectra–Physics (San Jose, CA, USA).

Chemicals and reagents

Indomethacin was obtained from Sigma (Saint Louis, MO, USA). Potassium monohydrogenphosphate and sodium hydroxide were of p.a. quality from Merck. Methanol was of LiChrosolv quality from Merck. The water used was glass-distilled.

Superficially porous Vydac 201 RP reversed-phase packing material (bead diameter: 30–40 μm), supplied by Macherey–Nagel, was used to fill the pre-column cartridge. The

LiChroCART cartridges were prepacked with LiChrospher 100 RP–18 (5 μm) from Merck.

Chromatographic procedure

The plasma samples were centrifuged at 3000 rpm for 10 min before they were introduced into the auto-sampler.

The phosphate buffer (pH 7.4) used as the washing eluent for the clean-up step was prepared as follows: 250 ml of 0.1 M potassium monohydrogenphosphate were mixed with 195.5 ml of 0.1 M sodium hydroxide in a 1 l volumetric flask and water was added to the volume. The flow rate of the washing eluent was 0.25 ml min^{-1}. The needle of the auto-sampler and the external tubing of the injection valve were rinsed with the same buffer.

The mobile phase used for the elution from the pre-column and the chromatographic separation of the retained compounds consisted of a mixture of phosphate buffer (pH 7.4) and methanol (40:60, v/v). The flow rate of the mobile phase was 0.7 ml min^{-1}.

The spiked plasma samples used for the calibration curves were prepared by adding small volumes (20–100 μl) of standard indomethacin solutions to 2 ml samples of pooled plasma. New standard aqueous solutions of indomethacin were prepared every day by dilution with phosphate buffer (pH 7.4) of a stock methanolic solution of the drug at 1 mg ml^{-1}. The concentration range of the spiked plasma samples was 100–2500 ng ml^{-1} when indomethacin was analysed after oral administration and 25–250 ng ml^{-1} when it was determined after external application.

Sequence of column switching events

At $t = 0$ min, the two valves are in the INJECT position. The needle of the diluter and the external tubing of the injector are rinsed with 2 ml of phosphate buffer (pH 7.4) (first cycle only). The washing eluent is pumped through the pre-column.

At $t = 1$ min, a 250 μl volume of plasma sample is introduced into the loop, the excess being directed to the waste (switching of the injection valve to the LOAD position).

At $t = 2$ min, the sample is injected into the column switching system (switching of the injection valve to the INJECT position) and the enrichment/clean-up step is started. Meanwhile, the needle and the external tubing of the injection valve are rinsed with 2 ml of phosphate buffer (pH 7.4).

At $t = 6$ min, the mobile phase is directed to the pre-column (rotation of the switching valve to the LOAD position) and elutes the compounds retained on the pre-column in the fore-flush mode to the analytical column on which they are separated.

At $t = 10$ min, the switching valve returns to the INJECT position. The pre-column is reconditioned with the washing eluent and a new cycle is started ($t = 11$ min equivalent to $t = 1$ min).

Results and Discussion

pH and flow rate of the washing eluent

As can be seen from Fig. 1, the absolute recovery of indomethacin increases with decreasing pH. This is due to the fact that the distribution of the acidic drug to the hydrophobic stationary phase of the pre-column is enhanced at low pH (≤ 3), the carboxy group of the drug being unionized under these conditions.

However, at acidic pH, the solubility of indomethacin in aqueous solutions is very low,

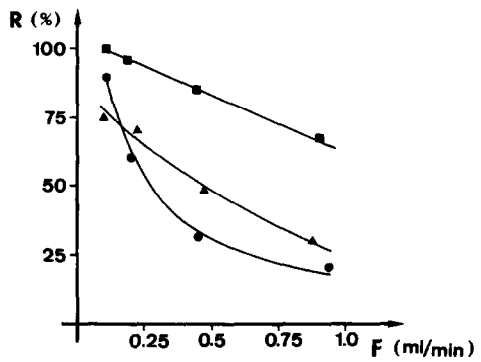

Figure 1
Influence of the pH and flow rate of the washing eluent on the indomethacin recovery in plasma. Pre-column solid phase: Vydac 201 RP. R: absolute recovery of indomethacin. F: flow rate of the washing eluent. Washing eluent: ■ phosphate buffer (pH 3); ▲ phosphate buffer (pH 7.4); ● water.

Table 1
Influence of the composition and volume of the eluents on indomethacin memory effects

Rinsing liquid (volume in ml)	Washing eluent	Mobile phase (elution time on pre-column in min)	RSD (%)	*n*	Residual peak
Water (1)	B pH 5	Methanol–B pH 3 75:25 (1)	9.8	6	+
B* pH 5 (1)	B pH 5	Methanol–B pH 5 75:25 (1)	9.9	4	+
B pH 7.4 (1)	B pH 7.4	Methanol–B pH 7.4 60:40 (1)	2.6	5	+
B pH 7.4 (2)	B pH 7.4	Methanol–B pH 7.4 60:40 (1)	2.0	5	+
B pH 7.4 (2)	B pH 7.4	Methanol–B pH 7.4 60:40 (4)	1.8	6	–

*B = phosphate buffer.
Stationary phase: pre-column — Vydac 201 RP; analytical column — LiChrospher 100 RP-18.
UV detection at 254 or 260 nm.
Sample: aqueous standard solution of indomethacin (concentration: 1 $\mu g\ ml^{-1}$).

which gives rise to memory effects. These effects are responsible to a large extent for the poor reproducibility obtained on injection of standard aqueous solutions when an acidic buffer (pH 5) was used as washing eluent (cf. Table 1). The washing of the pre-column, the diluter needle and the external tubing of the injector with phosphate buffer (pH 7.4) could eliminate these adsorption effects. Consequently, the RSD for successive assays of standard aqueous solutions was considerably reduced, as shown in Table 1, although the drug recovery was lower in this case (cf. Fig. 1).

By decreasing the flow rate of the washing eluent to 0.25 ml min^{-1}, the indomethacin recovery could, however, be maintained at a level of 70% (cf. Fig. 1). This favourable effect of flow rate reduction on the recovery is probably related to the strong binding of indomethacin to plasma proteins. A low flow rate increases the residence time of the plasma sample in the pre-column and therefore compensates to some extent for the small size of the latter as well as for the use of a pellicular stationary phase with a limited adsorption capacity.

Also, Table 1 shows that an increase in the volume of buffer used for rinsing the needle and external tubing of the auto-injector causes a further reduction of the RSD of the method.

The selection of a buffer (pH 7.4) as washing eluent also has the advantage of improving the clean-up efficiency. Figure 2 shows a comparison of chromatograms obtained when the clean-up step was performed with phosphate buffer (pH 5 and 7.4, respectively). It should be emphasized that in both cases the mobile phase used for the elution of the compounds retained on the pre-column and their separation on the analytical column was a mixture of methanol and the phosphate buffer selected for the clean-up. Indeed it has been observed that differences in pH between the washing eluent and the mobile phase could give rise to disturbing system peaks (Ph. Hubert, M. Renson and J. Crommen, to be published). When the pH was increased to pH 7.4, the volumetric fraction of methanol in the mobile phase had to be reduced to 60% in order to give indomethacin a sufficient retention.

As can be seen from Fig. 2A, endogenous plasma components interfere at pH 5 with the assay of indomethacin by UV detection, whilst the chromatogram of Fig. 2B, obtained by use of buffer (pH 7.4), is devoid of such interferences (cf. also Figs 3 and 4). In the latter case, UV detection is adequate for the determination of the drug at low plasma concentrations.

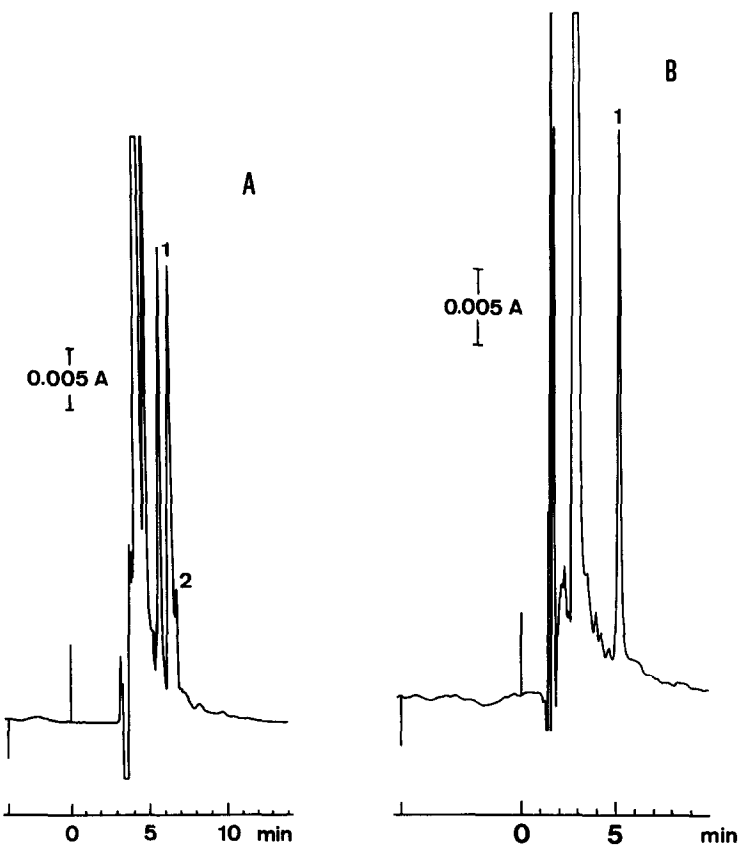

Figure 2
Reversed-phase HPLC of spiked plasma samples after on-line clean-up with phosphate buffer. Buffer pH: A. 5; B. 7.4. UV detection at 254 nm. Other chromatographic conditions as described in the Experimental. Peaks: 1. Indomethacin (100 ng); 2. Endogenous plasma component.

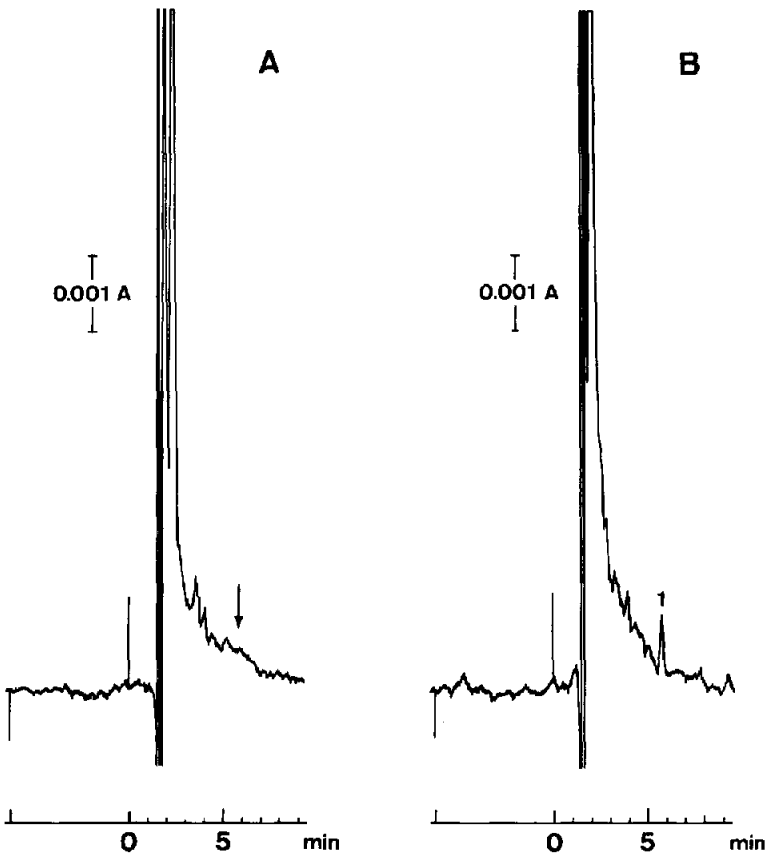

Figure 3
Typical chromatograms obtained by use of a variable wavelength UV detector. Detection wavelength: 260 nm. Other chromatographic conditions as described in the Experimental. Sample: A. Blank plasma; B. Spiked plasma (indomethacin concentration: 50 ng ml^{-1}). Peak: 1. Indomethacin (5 ng).

When acidic buffers (pH 5) were chosen as washing eluents, a more selective detection mode was needed to eliminate the interfering peaks, i.e. the fluorimetric detection of indomethacin after post-column hydrolysis with sodium hydroxide (M. Renson and J. Crommen, paper presented at 9th ISCLC, Edinburgh, July 1985). However, the introduction of a post-column reactor in the HPLC system makes the method more complicated and requires the use of an additional pump.

Transfer of indomethacin from the pre-column to the analytical column

When the elution of the compounds retained on the pre-column was effected by passage of the mobile phase for only 1 min, the total quantity of indomethacin adsorbed did not reach the analytical column, in spite of the fairly high percentage (60%) of methanol in the eluent. This was demonstrated by injecting successively an aqueous standard indomethacin solution and phosphate buffer (pH 7.4). On the second chromatogram, a small residual peak was observed at the retention time of the drug (cf. Table 1).

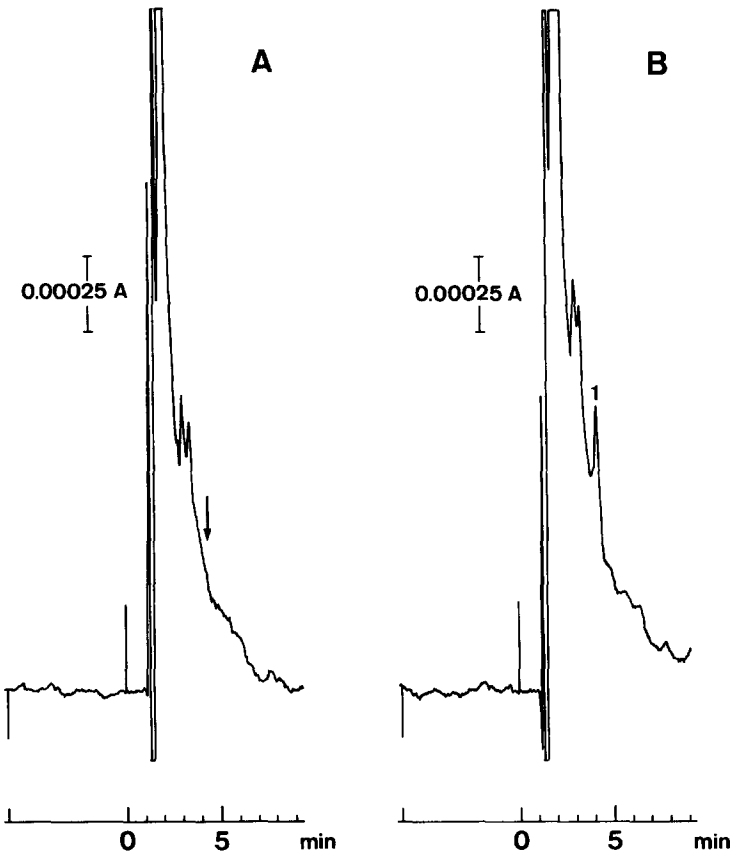

Figure 4
Typical chromatograms obtained by use of a fixed wavelength UV detector. Detection wavelength: 254 nm. Other chromatographic conditions as described in the Experimental. Sample: A. Blank plasma; B. Spiked plasma (indomethacin concentration: 50 ng ml^{-1}). Peak: 1. Indomethacin (5 ng).

This problem could be solved by increasing the time during which the mobile phase passes through the pre-column from 1 to 4 min. Under these conditions, the duration of a complete switching cycle was still less than 10 min (cf. Experimental).

The transfer of the compounds retained on the pre-column to the separation column was performed in the fore-flush mode, i.e. the mobile phase was pumped through the pre-column in the same direction as the washing eluent. A good stability of the pre-column was obtained in this case: a total volume of up to 12 ml of plasma, corresponding to 120 injections in the method described, could be applied on the pre-column before an increase of the back-pressure in the system and/or a loss of separation efficiency was observed. This volume is significantly larger than the one reached with the same kind of pre-column when the transfer was made in the back-flush mode (M. Renson and J. Crommen, paper presented at 9th ISCLC, Edinburgh, July 1985).

In order to minimize the band broadening which can occur in the fore-flush mode, since the compounds migrate through the entire bed of the pre-column, the latter consisted of a very short cartridge (15 mm long) filled with superficially porous packing material.

Fixed and variable wavelength UV detectors

With a variable wavelength detector, 260 nm was found to be the most suitable wavelength for the determination of indomethacin, as the absorbance spectrum of this compound displays a maximum at that wavelength. However, the molar absorptivity of indomethacin at 260 nm is only slightly higher than that at 254 nm. Therefore, a fixed wavelength detector also was tested, as it is well-known that this kind of apparatus usually gives a lower baseline noise. With the detectors used in this work, short-term noises equivalent to 2×10^{-5} and 1×10^{-4} au were obtained for the fixed and variable wavelength monitors, respectively.

When indomethacin has to be analysed in the low plasma concentration range (<100 ng ml^{-1}), such as that found in pharmacokinetic studies made on preparations for external use, it seems preferable to choose the fixed wavelength detector since it gives a somewhat better signal-to-noise ratio than the variable wavelength equipment at 260 nm. A comparison of chromatograms obtained for spiked plasma samples with the two detectors at the same drug concentration of 50 ng ml^{-1} is shown in Figs 3B and 4B.

Validation of the method

The mean adjusted retention time, measured from the void volume peak, was 4.0 min, with a RSD of 1.1% for successive injections of spiked plasma samples ($n = 5$).

The absolute recovery of the drug was 70%. It was estimated by comparing the peak areas obtained by injection of spiked plasma samples into the column switching system with those found on direct injection of aqueous standard solutions onto the analytical column.

The minimum detectable concentration of indomethacin in plasma, considered to correspond to three times the baseline noise, was equal to 10 ng ml^{-1} with the fixed wavelength detector (254 nm) and to 23 ng ml^{-1} at 260 nm, using the variable wavelength monitor. The sample volume injected was 100 μl in all cases.

Good linearity was obtained for calibration curves. By plotting the peak height (in mm) vs the indomethacin concentration (in ng ml^{-1}), the following regression equations were found:

$Y = 0.153\,X + 0.748$ (260 nm; AUFS: 0.01; concentration range: 100–2500 ng ml^{-1}; $r = 0.99945$; $n = 5$);

$Y = 0.269\,X + 0.055$ (254 nm; AUFS: 0.0025; concentration range: 25–250 ng ml^{-1}; $r = 0.99936$; $n = 4$).

The precision of the method was estimated by calculating the RSD values for the results obtained at two different plasma concentrations. At 1000 ng ml^{-1}, the within-day reproducibility was 1.3% ($n = 6$), while at 100 ng ml^{-1}, the within-day and the between-day reproducibilities were 1.7% ($n = 8$) and 3.5% ($n = 5$), respectively.

The absence of interfering endogenous components is demonstrated in Figs 3 and 4, which show chromatograms obtained on injection of blank and spiked plasma samples at 260 nm (variable wavelength) and 254 nm (fixed wavelength), respectively.

The method described above has been applied to the bioavailability studies of indomethacin, not only after oral administration but also after external application to the skin as a spray solution.

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