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Determination of diclofenac in plasma using a fully automated analytical system combining liquid–solid extraction with liquid chromatography

A. SIOUFI*, J. RICHARD, P. MANGONI and J. GODBILLON

Laboratoires Ciba-Geigy, Biopharmaceutical Research Centre, B.P. 308, 92506 Rueil-Malmaison Cedex (France)

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ABSTRACT

A fully automated analytical system based on liquid–solid extraction combined with column liquid chromatography is described for the determination of diclofenac in plasma. After addition of pH 5 buffer and the internal standard solution to the plasma sample, both sample preparation via a C_{18} disposable extraction column and injection were performed by a Gilson ASPEC system. Diclofenac and the internal standard were separated on a reversed-phase column, using methanol–pH 7.2 phosphate buffer (56:44, v/v) as mobile phase at a flow-rate of 0.4 ml/min. The reproducibility and accuracy of the method were acceptable over the concentration range 31–3140 nmol/l in plasma.

INTRODUCTION

Numerous methods for the determination of diclofenac in plasma have been reported, using either gas chromatography–mass spectrometry (GC–MS), gas chromatography (GC) or high-performance liquid chromatography (HPLC). Most of them permit the determination of diclofenac in plasma with good sensitivity, 0.2 ng/ml for GC–MS [1], 5 ng/ml for GC [2–4] and 10 ng/ml for HPLC [5,6], but all of them are time-consuming, *e.g.*, involving shaking for liquid–liquid extraction, centrifugation, evaporation of the organic phase to dryness and derivatization for the GC methods. Recently, fully automated systems based on liquid–solid extraction (LSE) via disposable extraction columns (DECs) combined with HPLC have been introduced, permitting rapid preparation and analysis of samples.

This paper describes an automated procedure for the determination of diclofenac in plasma samples using the Gilson Automatic Sample Preparation with Extraction Column (ASPEC) system.

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EXPERIMENTAL

Chemicals and reagents

Diclofenac sodium ($C_{14}H_{10}NO_2Cl_2Na$; MW 318.13) and CGP 4287 (internal standard) ($C_{15}H_{13}NO_3Cl_2$; MW 326.18) were supplied by Ciba-Geigy (Lyon, France) and Ciba-Geigy (Basle, Switzerland), respectively.

All chemicals were of analytical reagent grade except methanol, for spectrophotometry (Carlo Erba, Milan, Italy). Water was deionized on a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Phosphate buffer pH 7.2 was prepared by dissolving 0.71 g of anhydrous disodium hydrogenphosphate (Merck, Darmstadt, Germany) in 2000 ml of distilled water. Citrate buffer (pH 5) was supplied by Merck (Titrisol).

Extraction columns

Bakerbond SPE C_{18} columns (100 mg) with a capacity of 1 ml (Baker, Deventer, The Netherlands) were used as DECs.

Chromatographic equipment and conditions

The HPLC system was composed of a Model 302 solvent-delivery pump (Gilson, Villiers-le-Bel, France), an ASPEC system (Gilson) and a Model 783 variable-wavelength UV detector (Kratos, Ramsey, NJ, U.S.A.). A Model 3388A computing integrator (Hewlett Packard, Palo Alto, CA, U.S.A.) was used for data acquisition.

The ASPEC system, already described [7], combined an automatic sampling injector module with a stroke vertical arm and a Model 7010 Rheodyne injection valve with a 100-μl sample loop, and a Model 401 diluter. All were controlled by a sample controller keypad. A set of rack and accessories was used for handling DECs and solvents.

The operating cycle of the ASPEC system has already been described by Rouan *et al.* [7]. The analytical column was a stainless-steel tube (15 cm × 3.9 mm I.D.) packed with Novapak C_{18} (4 μm) (Waters Assoc., Milford, MA, U.S.A.).

Chromatography was carried out at room temperature. Methanol–pH 7.2 phosphate buffer (56:44, v/v) used as the mobile phase was prepared freshly each day. The flow-rate of the mobile phase was 0.4 ml/min. The detection wavelength was set at 282 nm.

Stock and working standard solutions

Stock standard solutions were prepared by dissolving diclofenac sodium in methanol. Working standard solutions were obtained by dilution. The internal standard was dissolved in methanol.

Calibration graphs

Aliquots of the working standard solutions and a constant amount of internal standard (0.383 nmol) were introduced into a tube and evaporated to dryness at 45°C under a stream of nitrogen. Then 0.5 ml of plasma was added to produce calibration samples in the concentration range 31.4–3140 nmol/l (10–1000 ng/ml).

Extraction procedure

A 1300-μl volume pH 5 buffer solution was added manually to the plasma sample containing diclofenac and the internal standard. The tube was shaken on a vortex mixer for 10 s and placed on the rack of the ASPEC system.

All the following steps were performed automatically by the ASPEC system. At every step, the elution through the DEC was performed by pressurizing air dispensed above the packing, to induce the desired flow-rate through the DEC. The automatic sequences were as follows:

1. DEC conditioning. A 1-ml volume of methanol and 1 ml of water were successively dispensed on the DEC (flow-rate, 25 μl/s; air volume, 70 μl).

2. Sample transfer. A 1500-μl volume of the diluted plasma sample was transferred to the DEC (flow-rate, 6 μl/s; air volume, 150 μl).

3. Column washing. A 2-ml volume of water and 2 ml of methanol–water (25:75, v/v) were successively dispensed on the DEC (flow-rate, 50 μl/s; air volume, 700 μl).

4. Elution. A 1-ml volume of the mobile phase was dispensed on the DEC (flow-rate, 25 μl/s; air volume, 750 μl). Then 200 μl of water were added to the eluate and the mixture was bubbled twice with 2000 μl of air, to obtain a homogeneous mixture.

5. Injection. Before injection onto the analytical column, 420 μl of the diluted eluate were dispensed through the 100-μl injection loop.

In all instances, the needle was rinsed with 1 ml of water before pipetting the liquid to be transferred. The preparation of a sample started immediately after the injection of the previous one.

RESULTS

Automatic procedure

The modularity of the ASPEC system allowed suitable automation of the solid-phase extraction procedure. The system hardware configuration was designed in order to offer the choice of five possible solvents and elution through the DEC by pressurizing air. The software system permitted the parameters to be chosen for the different steps of the preparation (number of conditionings, washings, eluting solvents, their volume and flow-rate and the pressurizing air volume).

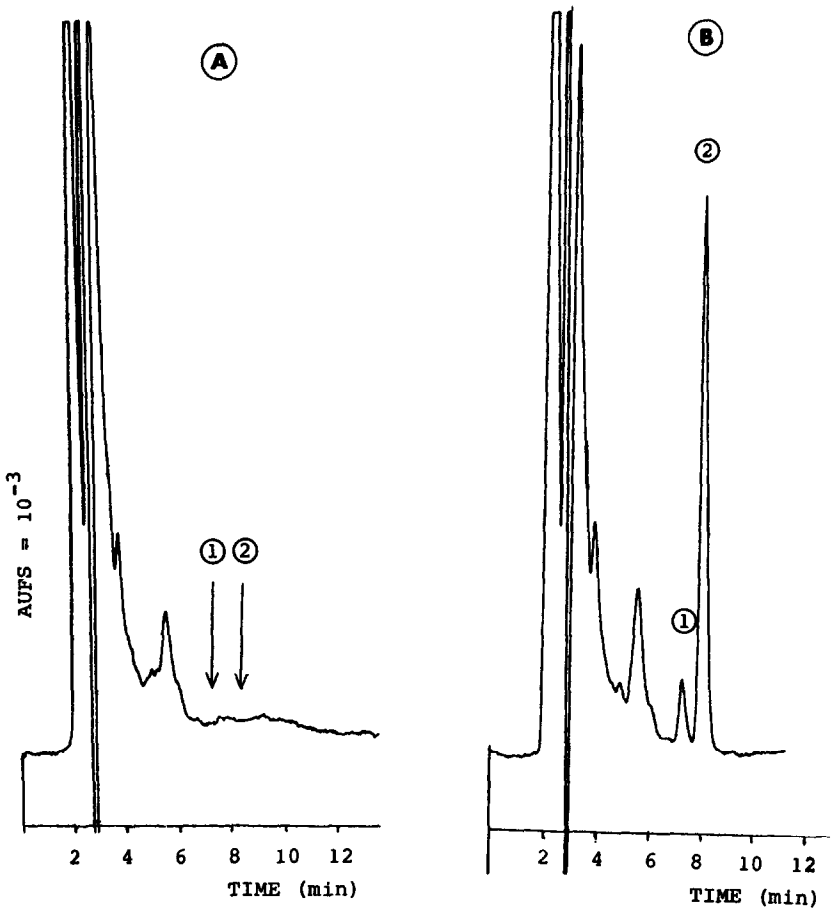

Fig. 1. Examples of chromatograms. (A) Extract of 0.5 ml of drug-free human plasma. (B) Extract of 0.5 ml of human plasma spiked with (1) 78.5 nmol/l diclofenac sodium and (2) 766 nmol/l internal standard.

Separation from plasma components

An example of a chromatogram of extracts from blank plasma and plasma spiked with 80 nmol/l diclofenac sodium is shown in Fig. 1. No interfering peaks derived from endogenous components were observed.

Calibration graph

The ratio of the peak heights of diclofenac and the internal standard was plotted against the diclofenac concentration in plasma. The equation of the graph was calculated by the least-squares method using weighted linear regression with a weighting factor of $1/(\text{concentration})^2$ [8]. It corresponds to the regression equation $y = 0.0015358x + 0.0072404$, where y = peak-height ratio and x = concentration, with a correlation coefficient higher than 0.997.

TABLE I

DAY-TO-DAY PRECISION AND ACCURACY OF THE DETERMINATION OF DICLOFENAC

Taken (nmol/l)	Mean recovery[a] (%)	R.S.D. (%)
31.4	95.9	13.2
126.0	98.5	5.4
629.0	102.0	4.0
1890.0	103.0	6.0
2830.0	106.0	5.0

[a] (Found × 100)/taken. n = 10. Overall recovery = 101% (R.S.D. 8%)

Within-day accuracy and precision

Plasma samples spiked with different amounts of diclofenac sodium were analysed six times on the same day. The overall accuracy was characterized by a mean recovery (found × 100/taken) ranging from 95 to 101%. The precision of the method [relative standard derivation (R.S.D.) of the mean recoveries] was 1.0–9.3%.

Day-to-day accuracy and precision

Plasma samples spiked with different amounts of diclofenac sodium were analysed in duplicate on five days. The overall accuracy was 101% (Table I) with an R.S.D. of 8%. These results demonstrate the good accuracy and precision of the method within the concentration range 31.4–2830 nmol/l. The limit of determina-

TABLE II

STABILITY OF DICLOFENAC SODIUM IN PLASMA SAMPLES DILUTED WITH pH 5 BUFFER LEFT AT ROOM TEMPERATURE

Time passed before sample preparation (h)	Concentration (nmol/l)		Recovery (%)
	126 nmol/l	1260 nmol/l	
0	124		98.4
2		1192	94.6
4	133		105
6		1284	102
8	127		101
10		1229	97.5
12	139		110
13		1333	106

tion established on the basis of the day-to-day reproducibility by taking the lowest concentration determined with an R.S.D. of *ca.* 10% was 31.4 nmol/l (10 ng/ml).

Stability of diclofenac sodium in plasma samples diluted with pH 5 buffer

Diluted plasma samples were left for up to 13 h on the ASPEC system before preparation. They were analysed at the time indicated in Table II. There was no decrease in the concentration of diclofenac up to 13 h at room temperature.

Application

The method described was applied to the determination of diclofenac in plasma samples collected after oral administration to healthy volunteers of 100 mg of diclofenac sodium as two 50-mg Voltaren enteric-coated tablets or as one 100-mg Voltaren suppository.

DISCUSSION

Plasma samples from one subject given one 100-mg Voltaren suppository, already analysed by the traditional HPLC method [6], were reanalysed by single determination using the automated ASPEC method. The results obtained with the two methods were in good agreement (Fig. 2), proving the reliability of the

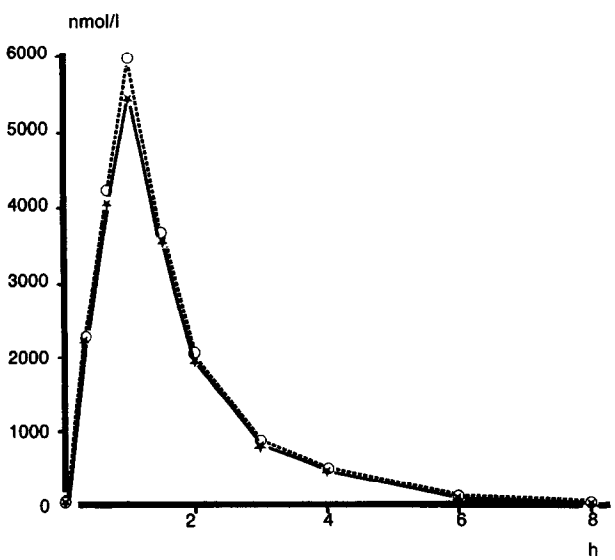

Fig. 2. Comparison between (★) the traditional HPLC method [6] and (○) the automated ASPEC method: diclofenac plasma concentrations for one subject given 100 mg of diclofenac sodium as one Voltaren suppository.

automated procedure. The limits of determination and the reproducibilities of the two methods were similar.

With the ASPEC system, the time required to analyse the first plasma sample is *ca.* 40 min. For subsequent samples, this time is reduced to *ca.* 15 min, which corresponds to the time needed for the actual HPLC. Hence it is clear that the time required for a sample to be analysed using the traditional HPLC method is longer than that with the ASPEC method owing to the need for liquid transfer, extraction by horizontal shaking, centrifugation and evaporation. Liquid–solid extraction involves less preparation than liquid–liquid extraction.

The ASPEC system can automate the entire process from the initial sampling to the final measurement by HPLC and detection. The automated determination of drugs in biological fluids increases the sample throughput and simplifies the transfer of an assay from one laboratory to another.

CONCLUSION

The automated method using the Gilson ASPEC system permits the determination of diclofenac in human plasma with acceptable precision and accuracy.

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