Journal of Chromatography, 620 (1993) 158–163
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7046

Short Communication

High-performance liquid chromatographic method for the routine determination of diclofenac and its hydroxy and methoxy metabolites from *in vitro* systems

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(First received June 9th, 1993; revised manuscript received July 8th, 1993)

ABSTRACT

A rapid and sensitive high-performance liquid chromatographic method is presented for the determination of diclofenac and its hydroxylated and methoxylated metabolites. The procedure describes extraction of diclofenac and its metabolites from acidified incubation medium into *tert.*-butylmethyl ether. Separation is achieved with a C_{18} reversed-phase column and quantification by UV detection at 280 nm. The method employs an internal standard resulting in good accuracy and precision. The limit of detection is 5 ng/ml for diclofenac and 10 ng/ml for its metabolites. One analysis requires no more than twelve minutes so that the assay is very suitable for the determination of a large number of samples.

INTRODUCTION

Diclofenac, 2-(2,6-dichloroanilino)phenylacetate, is a nonsteroidal anti-inflammatory drug successfully used for several years in the treatment of rheumatic diseases. Several methods have been described for determination of diclofenac and its hydroxy metabolites, including high-performance liquid chromatography (HPLC) [1–5] and gas chromatography (GC) [6–9]. However, most of these assays were developed for determination of diclofenac itself [3–5] or they allowed the detection of only one or two main metabolites, *i.e.* 4′-hydroxydiclofenac and 5-hydroxydiclofenac [1,2,6]. Working with a complex instrumentation and using a special derivatization method as used in GC with electron-capture detection only allowed the determination of diclofenac and four of its known metabolites [7,9].

Recently a highly sensitive HPLC assay with electrochemical detection for the determination of diclofenac and its metabolites has been published [10]. The method lacks in a good separation of the metabolites and is not applicable for higher concentrations of metabolites (> 100 ng/ml); furthermore 5-hydroxydiclofenac and 3′-hydroxy-4′-methoxydiclofenac coeluted in a single peak and running time for one analysis was more than 24 min. Lansdorp *et al.* [11] presented the first HPLC assay with UV detection for diclofenac and its four hydroxy metabolites. However, this method did not employ an internal standard

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0378-4347/93/$06.00

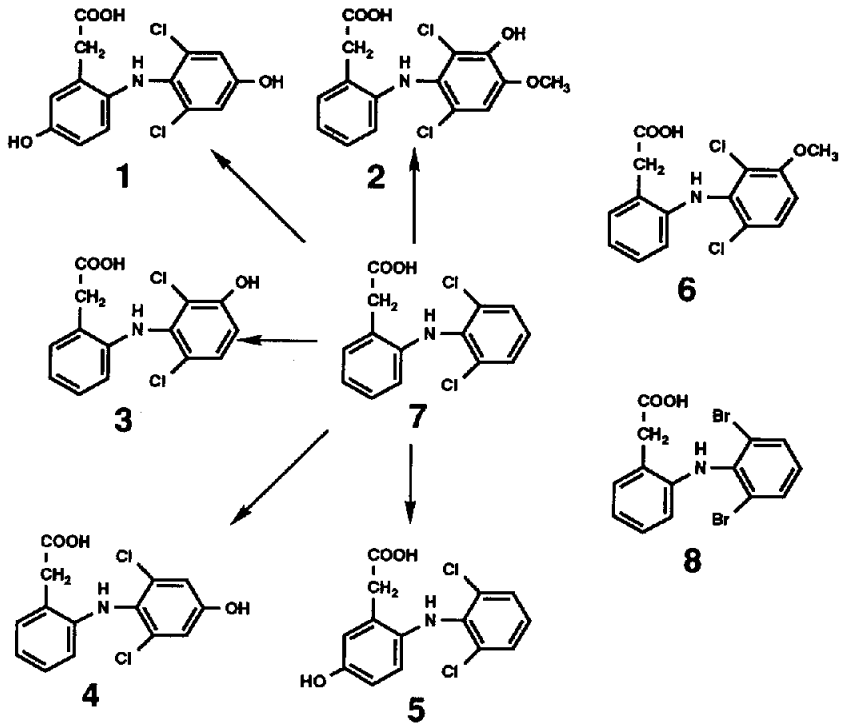

Fig. 1. Structures of diclofenac, its metabolites and the internal standard. (1) = 4′,5-dihydroxydiclofenac, (2) = 3′-hydroxy-4′-methoxydiclofenac, (3) = 3′-hydroxydiclofenac, (4) = 4′-hydroxydiclofenac, (5) = 5-hydroxydiclofenac, (6) 3′-methoxydiclofenac, (7) = diclofenac, (8) = dibromfenac (= internal standard).

(I.S.) and was not able to separate all hydroxylated metabolites completely. Methoxylated metabolites were not included. Furthermore, 45 min were needed for running the assay, and the limit of detection was found to be 20 ng/ml for diclofenac.

We therefore developed a much more rapid and sensitive method with a high separating performance for determination of diclofenac and all its known hydroxylated and methoxylated metabolites (Fig. 1).

EXPERIMENTAL

Chemicals

Diclofenac and *tert.*-butylmethyl ether were obtained from Sigma (Deisenhofen, Germany). The metabolites 3′-hydroxydiclofenac, 4′-hydroxydiclofenac, 5-hydroxydiclofenac, 4′-5-dihydroxydiclofenac, 3′-methoxydiclofenac and 3′-hydroxy-4′-methoxydiclofenac were a gift from Ciba-Geigy (Basel, Switzerland). Dibromfenac was synthesized and kindly provided by Heumann Pharma (Nürnberg, Germany). All other organic solvents and chemicals were of HPLC or reagent grade. The stock solutions of diclofenac and its metabolites were prepared in methanol (0.5 mg/ml) and stored at −20°C. A 50-μl aliquot of dibromfenac standard solution (10 μg/ml in phosphate buffer, pH 7.0) was added as internal standard to each sample before extracting.

Chromatographic system

HPLC analysis was performed at 20°C on a gradient instrument system using a Gynkotek High Precision Pump Model 480 (Gynkotek, Germering, Germany) attached to a Gilson Model 231 diluter-autosampler (Gilson/Abimed, Langenfeld, Germany). Eluents were analyzed by either an HP 1040 A diode-array detector (Hew-

lett-Packard GmbH, Böblingen, Germany) to confirm the peak identities or an Uvikon 430 double-channel UV-Vis detector (Kontron, Neufahrn, Germany) set at 280 nm, both connected to a Hewlett-Packard HP 79994 A analytical workstation.

Separation was achieved with a pre-column (5 mm × 4 mm I.D.) and an analytical column (250 mm × 4 mm I.D.) both packed with Eurospher 100 C18 5 μm (Knauer, Berlin, Germany). The mobile phase, composed of solvent A (acetonitrile–tetrahydrofuran 7:3, v/v) and solvent B (phosphate buffer, 45 m*M*, pH 7.0), which were both on-line degassed by placing them under helium atmosphere, was freshly prepared and passed through an 0.2 μm membrane filter (Schleicher und Schuell, Dassel, Germany). The gradient started linearly from 72% to 62% solvent B for a 0–6 min period followed by an isocratic period of 55% solvent B over 2 min and then back to 72% of solvent B. The flow-rate was at 1.1 ml/min and UV detection was performed at 280 nm. The runtime between two injections was 12 min.

Incubations

Isolated hepatocytes were prepared from male Wistar-rats (190–230 g) by collagenase perfusion as described by Berry and Friend [12] and modified by Seglen [13] and Bauer *et al.* [14]. The cells were suspended in Williams Medium E (Sigma, Deisenhofen, Germany) with a density of 1×10^6 cells/ml (cytochrome P-450 content: ~200 pmol per ml; protein content: ~1.87 mg protein per ml). One ml of the cell suspensions was incubated with 100 μ*M* diclofenac for 60 min at 37°C. The details are described elsewhere [15]. Rat liver microsomes were prepared according to previously described procedures [16] and stored in 0.1 *M* potassium phosphate buffer (pH 7.4). Diclofenac (100 μ*M*) was incubated in a volume of 1 ml of 0.1 *M* potassium phosphate buffer pH 7.4 for 60 min at 37°C with rat liver microsomes (estimated at 200 pmol cytochrome P-450) and an NADPH-generating system consisting of 13.7 m*M* glucose 6-phosphate, 0.66 m*M* $NADP^+$ and 2.8 units of glucose 6-phosphate dehydrogenase. The cytochrome P-450 content of hepatocytes and microsomes was measured according to the method of Omura and Sato [17]. Protein was determined according to Bradford [18] with bovine serum albumin as standard.

Analytical procedure

For the quantification of diclofenac and its metabolites 1.0 ml of the cell suspensions or samples from microsomal incubations were treated in a 10-ml glass tube with 100 μl of phosphoric acid (43%) to stop the reaction by inactivation of the enzymes and then sonicated for 10 sec. Before extracting in 3 ml of *tert.*-butylmethyl ether cellular particles were removed by centrifugation (13 000 g; 1 min) and 50 μl of the I.S. solution were added (= 0.5 μg/ml). The organic layer was evaporated to dryness under nitrogen. The dry residue was redissolved in 200 μl of 45 m*M* phosphate buffer of pH 7.0. A 50-μl aliquot of the sample was injected onto the HPLC column. Standard curves were obtained by injecting extracts from blank samples spiked with various concentrations of diclofenac or its metabolites respectively. For the quantification of the samples peak-area ratios relative to the internal standard were used.

Recovery values

Recovery values were calculated by comparing extracted spiked-samples with the unextracted solutions in the mobile phase in the range 0.005 μg/ml–3.5 μg/ml.

RESULTS AND DISCUSSION

The assay described allows simple sample preparation and was applied for the analysis of incubation media from drug-metabolism studies with isolated hepatocytes or liver microsomes. The analytical procedure consists of a single extraction with organic solvent, employing an I.S., followed by the separation with an analytical reversed phase HPLC column and detection at 280 nm.

Analysis of a reference mixture (Fig. 2) shows separation of diclofenac, its metabolites and di-

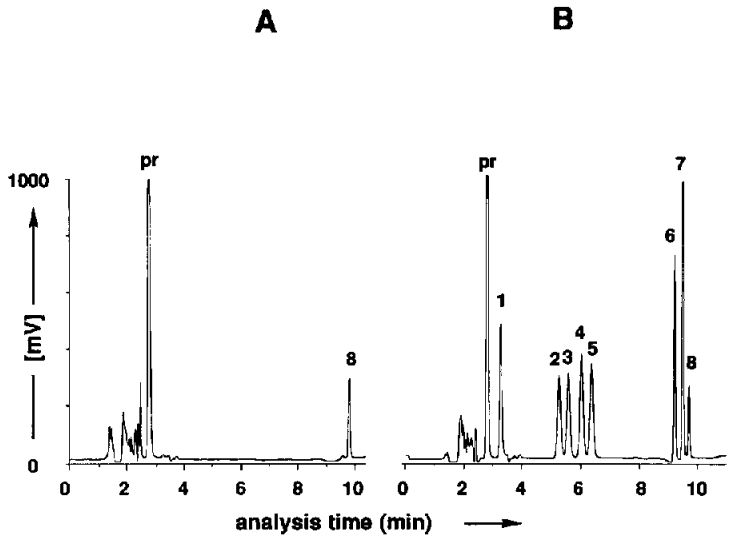

Fig. 2. Analysis of diclofenac its metabolites and internal standard. Representative sample chromatograms from: (A) blank control of hepatocyte incubation mixture with I.S. (0.5 μg/ml) and (B) reference mixture of diclofenac (1 μg/ml), the metabolites (1 μg/ml) and internal standard (0.5 μg/ml). Peak pr (2.8 min) = phenol red, peak 1 (3.1 min) = 4′,5-dihydroxydiclofenac, peak 2 (5.2 min) = 3′-hydroxy-4′-methoxydiclofenac, peak 3 (5.5 min) = 3′-hydroxydiclofenac, peak 4 (5.9 min) = 4′-hydroxydiclofenac, peak 5 (6.3 min) = 5-hydroxydiclofenac, peak 6 (9.1 min) = 3′-methoxydiclofenac, peak 7 (9.5 min) = diclofenac, peak 8 (9.9 min) = dibromfenac (IS).

bromfenac to be complete. Blank samples from cell cultures as well as microsomal incubations yielded straight baselines with no interfering peaks (Fig. 2). Analysis of cell culture medium (Fig. 3) or microsomal incubation mixtures (Fig. 3) yielded sharp and fully separated peaks of diclofenac, 4′-hydroxydiclofenac, 5-hydroxydiclofenac and dibromfenac. Additionally, the extrac-

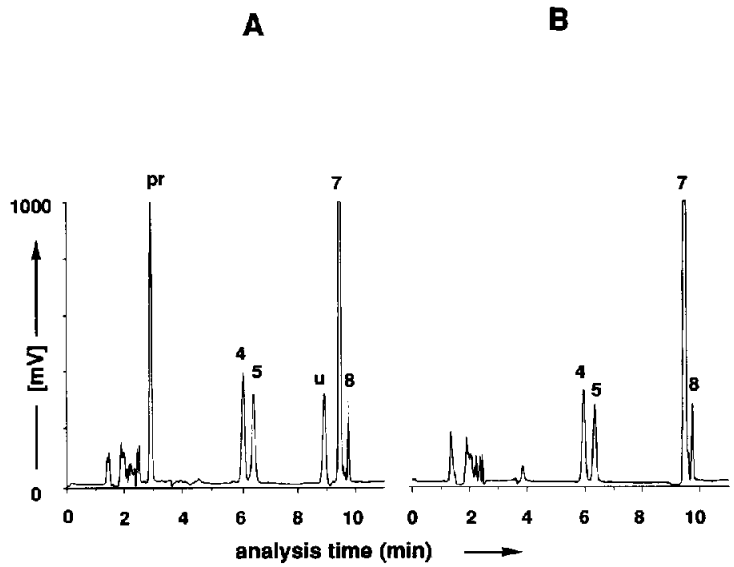

Fig. 3. Analysis of *in vitro* metabolism samples. Representative sample chromatograms from: (A) hepatocyte incubation, and (B) microsomal incubation mixture with: peak pr (2.8 min) = phenol red, peak 4 (5.9 min) = 4′-hydroxydiclofenac, peak 5 (6.3 min) = 5-hydroxydiclofenac, peak u (8.9 min) = unidentified, peak 7 (9.5 min) = diclofenac, peak 8 (9.9 min) = dibromfenac (IS).

tion from the hepatocyte incubation mixtures produces a peak at 2.8 min which could be detected as phenol red (pH indicator in Williams medium E) and furthermore an unidentified peak at 8.9 min. As this peak does not occur after extracting microsomal incubation mixtures it is assumed that this peak represents a phase-II metabolite, formed by active conjugating enzymes of the intact cells. It can be ruled out that the unidentified peak represents the 3'-methoxy metabolite of diclofenac because the synthetic reference compound moves more slowly (Fig. 2) and differs in its UV-spectrum.

The extraction recovery (mean ± S.D., $n = 4$) varies between 85 ± 6.9% and 101 ± 3.7% for concentrations of 0.005–3.5 μg/ml and is not significantly different from diclofenac and its hydroxylated and methoxylated metabolites.

For diclofenac, 3'-hydroxydiclofenac, 4'-hydroxydiclofenac, 5-hydroxydiclofenac, 4',5-dihydroxydiclofenac, 3'-methoxydiclofenac, 3'-hydroxy-4'-methoxydiclofenac and dibromfenac, calibration curves were constructed using spiked blank samples for analysis. Linear correlations ($r = 0.995 \rightarrow 0.999$) were found for concentrations ranging from 0.01 μg/ml to 3.5 μg/ml (0.005 μg/ml)–0.7 μg/ml for diclofenac and 3'-methoxydiclofenac).

The inter-assay precision for diclofenac and its metabolites (4 consecutive assays) was better than 7% (coefficient of variation, C.V.). The intra-assay precision (4 samples of each standard in the range of 0.005–3.5 μg/ml were determined) was better than 6% (C.V.). In the analytical system described the limit of detection (signal-to-noise ratio = 3) was 5 ng/ml for diclofenac and 10 ng/ml for its metabolites.

There are several methods described for separating metabolites of diclofenac from the parent compound. The GC methods [6–9] based on derivatization and those involving electron-capture detection are extremely sensitive. However, they need an extensive sample clean-up before derivatization and require more than 30 min for the analysis of one sample. Godbillon *et al.* [1] have published a more simple and quicker HPLC method for the determination of unchanged diclofenac and its monohydroxylated metabolites, but 3'- and 4'-hydroxydiclofenac were not separated, and 4',5-dihydroxydiclofenac could not be measured. Recently, a highly-sensitive HPLC method with electrochemical detection for the determination of diclofenac and four metabolites (including one methoxylated) has been published [10]. Separation of the metabolites was not complete and useful for low concentrations (< 100 ng/ml) only. Furthermore, 5-hydroxydiclofenac and 3'-hydroxy-4'-methoxydiclofenac coeluted in a single peak and running time for one analysis was more than 24 min. Lansdorp *et al.* [11] published an HPLC method with UV detection for determination of diclofenac and its total hydroxylated metabolites, but the assay described did not allow a complete separation of the 3'- and 4'-hydroxydiclofenac, lacked an internal standard and needed more than 40 min to run one analysis which is unexceptable for a routine determination of a large number of samples.

The HPLC assay presented here is more sensitive (5 ng/ml for diclofenac *vs.* 20 ng/ml as described by Lansdorp *et al.* [11]) and allows a complete separation of all existing metabolites, including the 3'-hydroxy-4'-methoxydiclofenac recently isolated and identified by Faige *et al.* [19], and additionally the 3'-methoxydiclofenac, a synthetic reference compound.

By using dibromfenac as internal standard we could demonstrate that our method works with good accuracy, precision and reproducibility within the whole concentration range. Furthermore, the analytical procedure is easy to handle and because of the short time between two injections it is very suitable for the routine determination of a large number of samples.

ACKNOWLEDGEMENT

This work was supported by the Wilhelm Sander Stiftung (Grant No. 89.103.2). We wish to thank CIBA Geigy (Basel, Switzerland) and Heumann Pharma (Nürnberg, Germany) for generous gifts of the reference substances and internal standard.

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