Journal of Chromatography, 577 (1992) 185–189
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6298

Short Communication

Extractionless determination of diclofenac sodium in serum using reversed-phase high-performance liquid chromatography with fluorimetric detection

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(First received September 9th, 1991; revised manuscript received January 28th, 1992)

ABSTRACT

The author describes a method of using reversed-phase high-performance liquid chromatography with fluorimetric detection for the assay of diclofenac sodium in serum. The method is sensitive down to 20 ng/ml (250-μl loop). Elution is at pH 6.2 with methanol in 0.05 *M* phosphate buffer (43:57, v/v) on a 25-cm Spherisorb S5 ODS2 column. Detection is at an excitation wavelength of 282 nm and an emission wavelength of 365 nm. Serum sample size is 100 μl. Sample protein, to which diclofenac is highly bound, is first denatured by heat and then with methanol to release the diclofenac prior to centrifugation and injection of 100 μl (or 250 μl) of the clear supernatant. Harmol, with similar fluorescence and polarity characteristics to diclofenac, is a satisfactory internal standard. At the 1 μg/ml level intra-sample reproducibility is better than 2%, whilst inter-sample reproducibility is 4.6%. Detector response is linear from 40 ng/ml to 20 μg/ml (100-μl loop).

INTRODUCTION

Diclofenac sodium, sodium [*o*-(2,6-dichloroanilino)phenyl]acetate (Fig. 1), is a potent non-steroidal anti-inflammatory drug used in the treatment of rheumatoid arthritis and degenerative bone disease [1,2]. Several methods have been developed for the determination of diclofenac itself or along with its metabolites in body fluids [3–20]. Various analytical technical techniques have been used for the assay, such as radioisotope assay [2], gas chromatography (GC) [3,7,17–20], thin-layer chromatography (TLC) [12] and high-performance liquid chromatography (HPLC) [9–11,17–20]. The use of radioactive drugs is counter-indicated for on-going pharmacokinetic and bioavailability studies because of the radiation hazard and TLC is difficult to quantify with sufficient accuracy. The GC methods are highly sensitive and specific, especially the capillary methods, and gas chromatography–mass spectrometry (GC–MS) adds further to this specificity; however, all require extensive sample preparation by extraction and derivatization prior to GC separation.

HPLC has been performed using 5-μm ODS reversed-phase (RP) columns with UV detection [9,11,17,19,20] or electrochemical detection [10,18]. Since diclofenac sodium is approximately 99% bound to serum protein, particularly albumin [21], the normal methods of displacing bound materials by acid denaturation and pre-

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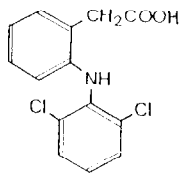
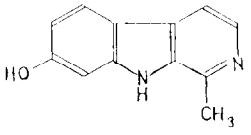


DICLOFENAC



HARMOL

Fig. 1. Structures of diclofenac and harmol.

cipitation of the proteins is only partially effective and very irreproducible. Because of this protein binding, all the separation methods published so far have employed at least one extraction step with or without derivatization prior to chromatographic separation and detection, resulting in a loss in reproducibility. The high potency of diclofenac sodium means that therapeutic levels of the drug lie in the upper ng/ml serum level. Pharmacokinetic studies require that the assay method must be sufficiently sensitive to accurately assay the drug for at least four half-lives after peak time (T_{max}), *i.e.* about one twentieth of the 2 μg/ml C_{max} level found after a single 50-mg dose (one fortieth of the 4 μg/ml level found after a single 100-mg enteric-coated dose) [17].

Whilst a few authors achieve a limit of detection in the 10 ng/ml region using HPLC with UV detection [9–11,17,19], the present author was unable to reproduce this sensitivity because of high background absorbance form residual components in the cleaned-up samples. The chemical structure of diclofenac sodium suggested that fluorescence detection, with its greater selectivity than UV detection, may reduce the background and give greater sensitivity.

In this paper the author presents a simple method of effectively displacing diclofenac sodium from serum protein by denaturation and its HPLC assay using only a 100-μl serum sample and fluorimetric detection.

EXPERIMENTAL

Reagents

All solvents used were spectroscopic grade from Burdick and Jackson (Muskegon, MI, USA) and all water was purified by a Milli-Q system (Millipore, Milford, MA, USA). The diclofenac sodium standard was donated by Ciba Geigy (Isando, RSA) and harmol, the internal standard, was supplied by Sigma (St. Louis, MO, USA). All other reagents were analytical-reagent grade. The elution buffer was 0.05 *M* sodium phosphate, pH 6.2, containing 500 μl/l triethylamine and was prepared by mixing equal volumes of 0.05 *M* sodium dihydrogenphosphate and 0.05 *M* disodium hydrogenphosphate, adding the triethylamine and adjusting to pH 6.2 with 0.1 *M* phosphoric acid.

Internal standard

Harmol was found to have similar fluorescence and polarity characteristics to diclofenac sodium and proved to be a satisfactory internal standard for monitoring volume changes due to evaporation during heating, in the serum clean-up, elution and detection conditions used. A 10 μg/ml solution of harmol in elution buffer was satisfactory as the internal standard for the concentration range investigated. For very low levels of < 80 ng/ml, a 4 μg/ml harmol concentration was necessary when using the chart recorder.

Sample collection

Human blood samples were collected in plain Vacu-test tubes from the antecubital vein after ingestion of 50 mg diclofenac sodium with 250 ml water by drug-free staff volunteers. After 15 min when clotting was complete, each sample was centrifuged and the serum transferred to a clean glass vial. All samples were stored at -18°C until assayed, within two weeks after collection.

Sample preparation

Serum (100 μl) was added to 50 μl elution buffer in a centrifuge tube and heated to 85°C for 10 min. Methanol (200 μl) was immediately added to the hot serum mixture and allowed to stand for 2 min. Internal standard solution (50 μl) was then added, the mixture vortex-mixed for 10 s and centrifuged at 2000 *g* for 3 min. The clear supernatant was injected into the chromatograph through a 100-μl loop injector valve.

Preparation of standards

Standard solutions in elution buffer were prepared in the range 80 ng/ml to 40 μg/ml to give final serum concentrations of 40 ng/ml to 20 μg/ml. Blank undosed serum (100 μl) was added to 50 μl standard solution in a centrifuge tube and gently shaken. After standing for 5 min at ambient temperature, the mixture was heated to 85°C for 10 min. Methanol (200 μl) was added immediately to the hot mixture which was then allowed to cool for 2 min. Internal standard solution (50 μl) was then added, the contents vortex-mixed for 10 s, then centrifuged at 2000 *g* for 3 min and the supernatant injected.

Chromatography

HPLC was performed on a Spectra Physics 8100 liquid chromatograph with a Valco autoinjector valve with a 100-μl loop. Separation was achieved on a 250 mm × 4.6 mm I.D. Spherisorb S5 ODS2 reversed-phase column preceded by a 10 mm × 4.6 mm I.D. home-made guard column packed with Shandon Hyperspheres 5-μm ODS. Isocratic elution with methanol–pH 6.2 sodium phosphate elution buffer (43:57, v/v) at 2 ml/min was performed at a column temperature of 40°C. The retention times for diclofenac and harmol were 5.92 and 4.70 min respectively.

Detection

A Perkin-Elmer 650-10 dual-monochromator fluorescence detector was used. The best response to diclofenac sodium in mobile phase was at $\lambda_{excitation}$ = 282 nm and $\lambda_{emission}$ = 365 nm. For method development the output was recorded simultaneously on a Parkin-Elmer 56 strip-chart recorder and a Spectra Physics SP4200 integrator. The concentrations of diclofenac in serum were estimated on the basis of peak-height ratio from the standards calibration curve for the same detector.

RESULTS AND DISCUSSION

Using the above method, good separation and detectability of diclofenac sodium in serum was obtained with minimal interference from serum components (Fig. 2). The UV–VIS absorption spectrum for diclofenac in mobile phase showed an absorption maximum at 282 nm. Fluorescence with a 282-nm excitation wavelength gave maximum emission at 365 nm, so these were selected as the detection wavelengths. Detection was linear from 50 ng/ml to 20 μg/ml on the basis of peak-height ratio. Many interfering serum component peaks that were present when using UV detection were suppressed by fluorescence detection. When using a strip-chart recorder it was found necessary to use a weaker internal standard solution when very low levels (<80 ng/ml) of diclofenac were being assayed. Due to the high protein binding of diclofenac, direct cold precipitation of the protein with perchloric acid or trichloroacetic acid was incapable of displacing the diclofenac from the protein. Trials with the published extraction methods [3,9–11,14,18,20] showed that, at best, less than 80% of the spiked diclofenac was being recovered by these tedious extraction processes, and one method [14] showed only about 20% recovery of the analyte as diclofenac. A different approach using physical rather than exclusively chemical denaturation of the protein was attempted by the author. Serum samples containing diclofenac sodium were heated to 85°C for 10 min, partially denaturing the protein and releasing the diclofenac. Immediately on removal from the oven, methanol was added, further denaturing the protein and preventing reabsorption of the released diclofenac. Methanol was used for the final denaturation as it was found that both perchloric acid and trichloroacetic acid, the most commonly used protein precipitants, attack diclofenac (Fig.

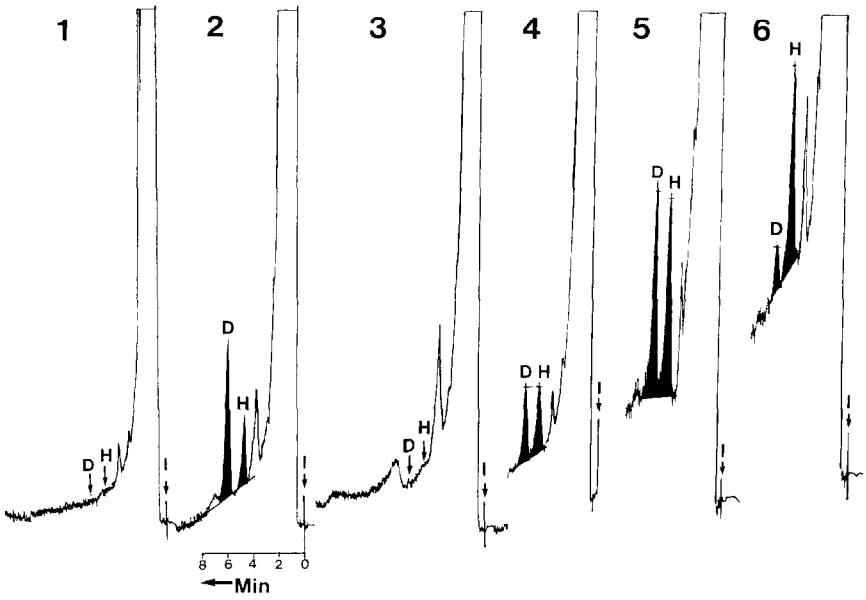


Fig. 2. Chromatograms of serum and standard samples. (1) Blank serum; (2) serum 1 h after ingestion of 50 mg diclofenac sodium; (3) serum 25 h after ingestion of 50 mg diclofenac sodium; (4) 1 μg/ml standard in serum with internal standard harmol (10 μg/ml); (5) 400 ng/ml standard in serum with internal standard (4 μg/ml); (6) 80 ng/ml standard in serum with internal standard (4 μg/ml). Peaks: D = diclofenac; H = harmol; I = Inject.

3). Precipitation with acetonitrile as used by El-Sayed *et al.* [17] also reduced the recovery of diclofenac. Contrary to the claim that diclofenac sodium solutions are stable for one month at 5°C [11], the author found them stable for less than one week at 4°C and two days at 20°C resulted in the production of three breakdown products (Fig. 4). Thus all samples were stored at −18°C

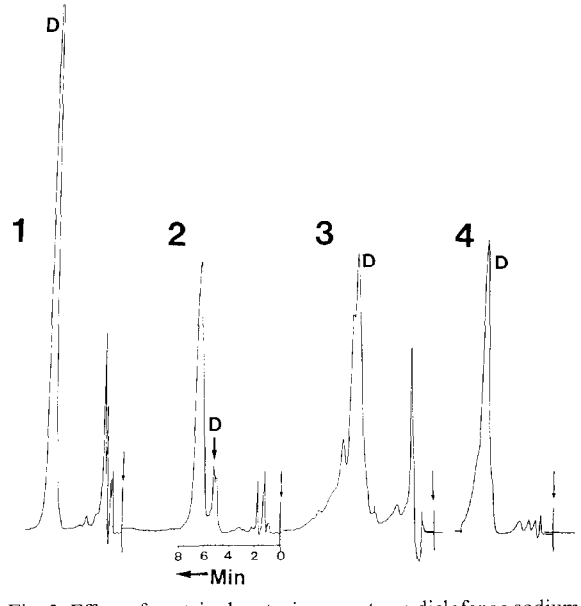


Fig. 3. Effect of protein denaturing agents on diclofenac sodium. (1) Fresh diclofenac sodium standard solution; (2) fresh diclofenac sodium in 4% perchloric acid; (3) diclofenac sodium in 5% trichloroacetic acid; (4) diclofenac sodium solution in methanol (1:1). Peak D = diclofenac sodium.

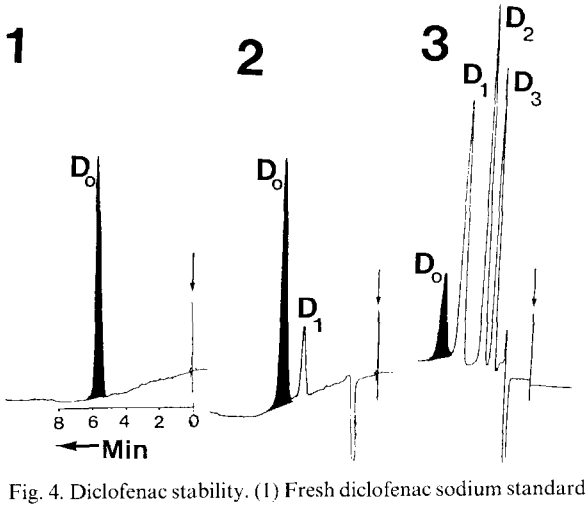


Fig. 4. Diclofenac stability. (1) Fresh diclofenac sodium standard solution; (2) same solution after 24 h at room temperature; (3) diclofenac sodium solution after eight days at room temperature. Peaks: D_0 = diclofenac; D_1 = first breakdown product; D_2 and D_3 = subsequent breakdown products.

and for less than two weeks prior to assay. Standards were stored for up to five days at −18°C.

Both harmol and harmane were suitable as the internal standard. Harmol was chosen because it eluted earlier than harmane. Initially the internal standard was added prior to heating, but better reproducibility was attained by adding it after the addition of methanol. The intra-sample standard deviation was <2% (0.993 ± 0.019) over ten assays at the mid-range concentration of 1 μg/ml and the inter-sample standard deviation was 4.6% (1.016 ± 0.046) over eight assays at the same concentration. Using the criterion of minimum detectability as three times the system noise, the detection limit for diclofenac sodium in serum was 40 ng/ml using a 100-μl loop. With a 250-μl loop the detection limit was extended to 20 ng/ml. The absolute recovery of diclofenac sodium from blank serum spiked with 1 μg/ml was >90% and the relative recovery was 98.2–102%.

The results obtained indicate that this simple and rapid method for the assay of diclofenac sodium in serum is sufficiently sensitive to follow therapeutic blood levels for several hours after dosing. Whilst the sensitivity is less than that found with GC–MS [8,15,16] or electrochemical detection [10,18], the method is sufficiently sensitive to follow the pharmacokinetics of all dosing routes for diclofenac other than topical application.

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