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A rapid and sensitive high-performance liquid chromatographic method for the determination of diclofenac sodium in serum and its use in pharmacokinetic studies

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Abstract—A rapid, and sensitive high-performance liquid chromatographic method has been developed for the determination of diclofenac sodium in serum using flufenamic acid as the internal standard. Serum protein was precipitated with acetonitrile. The drugs were eluted from a 5 μm C-8 reversed-phase column at ambient temperature with a mobile phase consisting of acetonitrile-water (50:50% v/v) adjusted to pH 3·3 with glacial acetic acid, at a flow rate of 2 mL min^{-1} with UV detection at 280 nm. Each analysis required no longer than 10 min. Quantitation was achieved by the measurement of the peak-height ratio and the relative and absolute recoveries varied from 90 to 98%. Detection limits for diclofenac sodium in serum is 25 ng mL^{-1}. Intraday coefficients of variation (CV) ranged from 2·47 to 4·61% and interday CVs from 3·52 to 7% at three different concentrations. Preliminary stability tests showed that diclofenac sodium is stable for at least 2 weeks in serum after freezing. The method is applied for the determination of the pharmacokinetic parameters of diclofenac after administration of two formulations (enteric-coated tablet and slow-release tablet), to a healthy male volunteer.

Diclofenac sodium is a non-steroidal anti-inflammatory drug used in degenerative joint disease, rheumatoid arthritis, and allied conditions (Brodgen et al 1980). Diclofenac sodium particularly in comparison with other non-steroidal anti-inflammatory drugs such as acetylsalicylic acid, phenylbutazone, indomethacin, ibuprofen, and naproxen, appears to combine high therapeutic efficacy and improved tolerability (Zucker 1986).

A variety of analytical techniques are available for the quantitation of diclofenac sodium in biological fluids. These include, the use of radioactive isotope (Reiss et al 1978), thin layer chromatography (TLC) (Schumacher et al 1980) gas-liquid chromatography (GLC) (Geiger et al 1975; Ikeda et al 1980; Schneider & Degen 1981), and high-performance liquid chromatography (HPLC) (Said & Sharaf 1981; Chan et al 1982; Chan & Vyas 1985; Battista et al 1985).

The use of radioactive isotopes is generally unsuitable for pharmacokinetic and bioavailability studies because this technique requires the use of expensive equipments and the potential hazards of radioactivity. The reported TLC method, possesses adequate resolution for identifying diclofenac and its metabolites, but suffers from the inability to accurately and precisely quantitate diclofenac. It is a laborious and time-consuming technique with inadequate sensitivity for pharmacokinetic and bioavailability studies.

GLC analysis possesses adequate sensitivity, however, it requires a relatively large sample and a significant amount of time for sample preparation and derivatization.

Several HPLC techniques have been reported for determination of diclofenac sodium in plasma, urine and synovial fluid. Said & Sharaf (1981) developed an HPLC method for diclofenac in serum and urine with a sensitivity no lower than 1 μg mL^{-1}, about one fourth the peak plasma concentration arising from a 100-mg dose. As a consequence, plasma concentration could not be traced by this method for longer than about 4 h after a single dose. Three other reported HPLC techniques (Chan et al 1982, 1985; Battista et al 1985), although with improved sensitivity, are more complex, time-consuming, and involving tedious extraction steps.

In this report a simple, rapid, sensitive, accurate and reproducible HPLC assay for the determination of diclofenac in serum is described. The proposed method is also applied for the determination of the pharmacokinetic parameters of diclofenac after administration of two formulations (enteric-coated tablet and slow-release tablet), to a healthy male volunteer.

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Materials and methods

Apparatus. The HPLC equipment used comprised of a single piston pump (model 114 M; Beckman Instrument, Int., Geneva, Switzerland), a fixed wavelength detector (model 160; Beckman), and injector with a 100-μL loop size (model 210; Beckman) and a chart recorder (model JJ CR 452; JJ Lloyd Instrument Limited, Warsash, Southampton, England). Chromatographic separations were performed using spherisorb 5 μm RP-C8 column (4·6 × 250 mm, Phase Separations Inc., Norwalk, Connecticut, USA).

Reagents. All solvents used were of HPLC grade. All other chemicals and reagents were of spectroquality or analytical grade. Diclofenac sodium and flufenamic acid were obtained from Sigma Chemical Co. (St Louis Missouri, USA). The mobile phase comprised of acetonitrile: water (50:50% v/v) adjusted to pH 3·3 with glacial acetic acid. The mobile phase was degassed daily by passing it through a 0·45 μm membrane filter (Millipore, Bedford, MA, USA).

Standard solutions. Diclofenac sodium (10 mg) was dissolved in 10 mL methanol. This stock solution was diluted 100-fold in methanol to give the working standard solutions (10 μg mL^{-1}). Flufenamic acid (10 mg) was dissolved in 10 mL acetonitrile to yield stock solution (1 mg mL^{-1}).

Procedure. In a disposable 10 mL culture tube, diclofenac sodium working standard (10 μg mL^{-1}) was added in volumes of 0, 2·5, 5, 25, 100, and 200 μL to 0·5 mL of blank serum to provide calibration standards of 0 (no diclofenac sodium added), 50, 100, 300, 500, 1000, 2000, and 4000 ng mL^{-1}. A 50 μL aliquot of the internal standard (500 ng) was added to the mixture which was then shaken on a vortex mixer for 30 s. Precipitation of serum proteins was accomplished by addition of 2 mL acetonitrile. The mixture was shaken again on a vortex mixer for 1 min, and centrifuged for 10 min at 2500 rev min^{-1}. The supernatant was transferred to 10 mL centrifuge tube and evaporated to dryness at 45°C in a water bath under a stream of dry nitrogen. The residue is reconstituted in 200 μL of HPLC eluent, vortexed for 30 s and transferred to a disposable polypropylene microcentrifuge tube (1·5 mL, Eppendorf) and centrifuged for 2 min at 11500 rev min^{-1} in a microcentrifuge (Eppendorf) to ensure that no particulate matter would be injected into the column. An appropriate aliquot was then injected directly into the loop injector.

Serum samples were prepared in an identical manner except for the addition of diclofenac sodium.

The mobile phase was pumped isocratically at a flow rate of 2 mL min^{-1}, at ambient temperature. The chart speed was 2 mm min^{-1}, and the effluent was monitored at 280 nm, at 0·02 a.u.f.s.

Clinical study. Two formulations of Voltaren (2 × 50 mg enteric coated tablets, and 1 × 100-mg slow-release tablet) were administered to a healthy male volunteer separated by a one week washout period. The volunteer fasted overnight before the drug administration and continued fasting until 4 h post dose. The drug was administered at 0800 h, and the tablets were swallowed with 200 mL of water. Venous blood samples were drawn into plain tubes before drug administration and at 0·5, 1, 1·5, 2, 2·5, 3, 3·5, 4, 5, 6, 7, 8, 10, 12, 15, and 24 h after drug was given.

All blood samples were taken via an indwelling catheter. After clot retraction, the samples were centrifuged at 3000 rev min^{-1} for 10 min, the serum separated and frozen at −20°C until analysis.

Results and discussion

Selection of mobile phase. The composition and pH of the mobile phase were varied to achieve the optimum chromatographic conditions. A mobile phase consisting of acetonitrile-water (50:50% v/v) adjusted to pH 3·3 with glacial acetic acid gave optimum resolution of diclofenac sodium and flufenamic acid (I.S.), and no interference from other components in serum was observed.

The volume of acetonitrile in the mobile phase drastically affected the retention time of both diclofenac and the internal standard. For example, changing the acetonitrile ratio from 50 to 45% resulted in a retention time of 10 and 14 min for diclofenac and flufenamic acid, respectively. Increasing the percentage of acetonitrile decreased the retention times proportionately.

The effect of the pH of the mobile phase was also studied. At pH 7 the retention time of diclofenac peak moved from 6·5 min to the solvent front. However, at pH 3, the diclofenac peak was not resolved from the endogenous acidic components in serum. Using pH 3·3 resulted in sharpening the peak of diclofenac and no interference from other components in the serum was observed. The optimum flow-rate for the mobile phase, 2 mL min^{-1}, resulted in an analysis time of 10 min.

Fig. 1 shows chromatograms from a subject serum samples collected before and 3·5 h after administration of 100 mg oral dose of diclofenac sodium (Voltaren; enteric coated tablet).

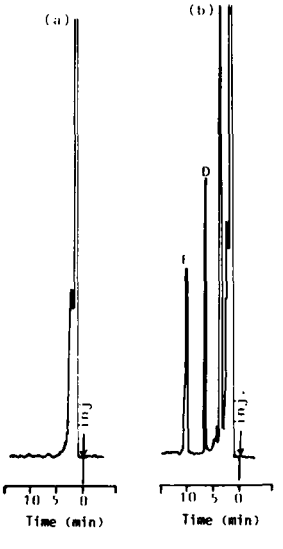

FIG. 1. Chromatograms from serum taken before (a) and 3·5 h after (b) a single 2 × 50 mg enteric-coated tablet. Both chromatograms were obtained at 0·02 a.u.f.s. D = diclofenac at concentration of 1250 ng mL^{-1} of serum; F = flufenamic acid (internal standard).

Quantitation. The quantitation of the chromatogram was performed using peak-height ratios of the drug to the internal standard. A representative standard curve of the diclofenac-flufenamic acid peak height ratio over the diclofenac serum concentration range 50-4000 ng mL^{-1} resulted in the following linear least-squares regression equation: $Y = 0{\cdot}0013 \times + 0{\cdot}0004$; $r = 0{\cdot}9999$. Standard curves of diclofenac sodium in serum were constructed on 10 different days to determine the variability of the slopes and intercepts. The results showed little day-to-day variability of slopes and intercepts as well as good linearity ($r > 0{\cdot}999$) over the serum concentration range studied. The coefficient of variation for the slope was 6·92%.

Sensitivity. The limit of quantitation for this method was attained with serum samples containing 25 ng mL^{-1} of diclofenac sodium.

Selectivity. Interference to the method was evaluated by injecting other drugs into the aforementioned HPLC system. Stock solutions of the following pure substances were prepared in the mobile phase and their retention times were determined: indomethacin, ibuprofen, mefenamic acid, ketoprofen, paracetamol, aspirin, phenobarbitone, and diflunisal. Table 1 lists the retention times for the drugs tested.

Table 1. Retention times of some tested drugs.

Drugs	Retention time (min)*
Paracetamol	1
Aspirin	2
Phenobarbitone	2·5
Diflunisal	2·5
Ketoprofen	4·5
Ibuprofen	6
Indomethacin	6·5
Diclofenac	6·5
Mefenamic acid	10
Flufenamic acid	10

*From time of injection into the column.

Precision. The intraday precision (random analytical variation) was evaluated by replicate analysis of pooled serum samples containing diclofenac sodium at three different concentrations (low, medium, and high). All specimens used to study precision and bias were interspersed with clinical specimens during analysis. The intraday precision showed a coefficient of variation of 2·47 to 4·61%. In addition, the assay was accurate even at plasma concentration as low as 200 ng mL^{-1} (bias = 2·33%).

The interday precision (total analytical variation) was similarly evaluated on several days up to 2 weeks. The interday CVs varied from 3·52 to 7·05%.

Recovery. The absolute and relative analytical recovery from serum for diclofenac sodium and flufenamic acid were measured

Table 2. Absolute* and relative† recovery of dicofenac sodium from serum.

Concn (ng mL^{-1})	Mean peak heights (mm)		Recovery %	Relative recovery % mean ± s.d.	Range
	Aqueous	Serum			
200	28·8	26·0	90·28	102·32 ± 4·72	94·35–108·95
800	56·7	51·8	91·36	101·81 ± 2·52	98·66–105·38
3000	101·3	99·7	98·42	99·42 ± 3·33	95·11–104·57
Flufenamic acid (I.S.)	93·2	90·7	97·32		

*Six replicate analyses for each concentration.
†Eight replicate analyses for each concentration.

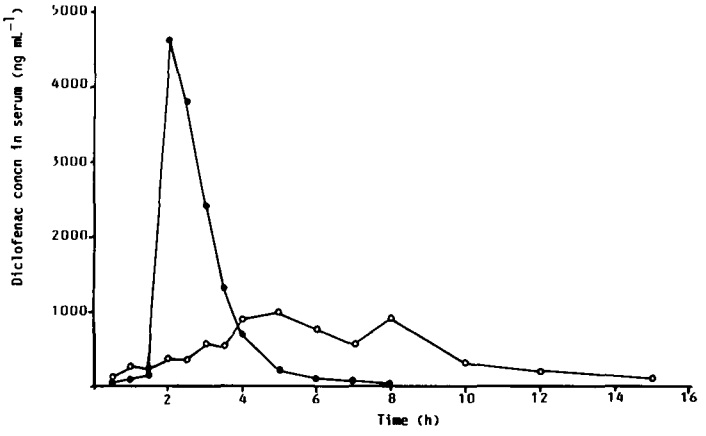

FIG. 2. Diclofenac serum concentrations in normal male volunteer after a single 2 × 50 mg oral dose of enteric-coated tablet (●), and a single 100 mg oral dose of sustained-release tablet (○).

in the following way. The drug and internal standard were added to drug-free serum to achieve the concentrations shown in Table 2. This serum was then analysed by the developed method. A carefully measured aliquot of the supernatants was then injected and the peak height measured. Absolute recovery was calculated by comparing these peak heights with the peak heights obtained by the direct injection of the pure aqueous drug standards. As shown in Table 2 absolute recoveries of the drugs ranged from 90 to 98%.

The relative recovery of diclofenac was calculated by comparing the concentrations obtained from the drug-supplemented serum with the actual added concentrations. The relative recovery ranged from 99 to 102% (Table 2).

Stability. Stability studies of serum spiked with diclofenac sodium (200, 800, 3000, ng mL^{-1}) were performed over a 15-day period (Table 3). Serum samples were stored in a freezer at −20°C until the time of analysis. The results demonstrated that diclofenac can be stored frozen in serum for at least two weeks without degradation.

Table 3. Effect of frozen storage on the diclofenac sodium stability in human serum.

Added concn (ng mL^{-1})	Measured concn/Added concn, % for days					
	0	1	2	3	8	10
200	99·4	99·5	105·15	107·45	107·0	103·15
800	99·1	101·73	102·24	98·66	95·1	104·13
3000	100·7	104·1	106·00	104·33	102·8	104·89

Clinical application. Fig. 2 shows a serum concentration-time profile of diclofenac sodium after administration of 2 × 50 mg enteric-coated tablets and slow-release tablet (1 × 100 mg) to a healthy male volunteer.

The peak concentration after the administration of the slow-release tablet (1 μg mL^{-1}) was considerably lower than the concentration found with standard tablets (4·6 μg mL^{-1}). The peak concentration was reached after ≃ 5 h with the slow-release tablet, while for the standard formulation was 2 h. The area under the serum concentration-time curves (AUC) were however well within the same range for both formulations 6·82 and 6·98 μg h mL^{-1}, respectively.

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