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Efficient high performance liquid chromatograph/ultraviolet method for determination of diclofenac and 4′-hydroxydiclofenac in rat serum

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Abstract

A rapid and sensitive high-performance liquid chromatographic method was developed for determination of diclofenac and its major metabolite, 4′-hydroxydiclofenac, in serum from rats treated with diclofenac. The method is simple with a one-step extraction procedure, isocratic HPLC separation, and UV detection at 280 nm. Use of *N*-phenylanthranilic acid as the internal standard provided good accuracy without interference by endogenous compounds or 5-hydroxydiclofenac, another metabolite of interest. Limits of detection for diclofenac and 4′-hydroxydiclofenac were 0.0225 and 0.0112 μg/ml, respectively. Average extraction efficiencies of diclofenac, 4′-hydroxydiclofenac, and the internal standard were ≥76%. The method was applied to serum collected at 3 h after rats were treated with an experimentally useful dosage range of 3, 10 and 50 mg/kg diclofenac. Recovery (as a percentage of dose) for the 4′-hydroxy metabolite in serum was found to consistently average from 0.10 to 0.12% following each dosage, whereas recovery of diclofenac in serum declined from 0.45 to 0.37%. Thus, the method is suitable for measurement of a major diclofenac metabolite in experimental studies.

Keywords: Diclofenac; 4-Hydroxydiclofenac; Metabolism; Cytochrome P450 3A4

1. Introduction

Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) that is widely prescribed for the treatment of rheumatoid arthritis, osteoarthritis, musculoskeletal injuries, and post surgery analgesia in human and veterinary medicine. Patients are frequently given special formulations of diclofenac or a co-treatment agent as a therapeutic strategy to attenuate the gastrointestinal tract complications that limit the use of diclofenac and other NSAIDs [1–3]. Many patients prescribed diclofenac for arthritis also take additional drugs for other chronic health problems, such as hypertension [4,5]. The literature contains multiple reports about alterations in the pharmacological efficacy, uptake, metabolism or toxicity of diclofenac by special formulations of this drug or by its interactions with other compounds [6–9]. Insight into the mechanistic basis for such alterations has been obtained from experimental investigations with rats that assessed levels of diclofenac or its metabolites in blood and other compartments [10–12].

Information about metabolites should provide mechanistic insight since the clearance of diclofenac in rats and humans is driven by its biotransformation to hydroxylated and/or conjugated metabolites via multiple competing or sequential reactions [13–15]. The relative amounts of specific metabolites formed have toxicological relevance since several of its metabolites, notably 5-hydroxydiclofenac and the acyl glucuronides of diclofenac and 4′-hydroxydiclofenac, are reactive entities capable of forming potentially injurious adducts with proteins [15–17]. Since the enzymes responsible for the formation of these metabolites exhibit basic kinetic differences (e.g., K_m and V_{max}) for diclofenac [15], dosage should influence the extent of biotransformation to specific metabolites. However, we could not find any information about dose-dependent changes

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in the extent of diclofenac metabolism in vivo since studies of diclofenac metabolism in humans or experimental animals have typically only looked at one dose [15,18].

Limitations of described techniques for measuring levels of diclofenac and its metabolites in serum or plasma by HPLC also present a problem for researchers. Specifically, each of the described relevant HPLC techniques for the analysis of both diclofenac and its metabolites in serum or plasma [19–22] has one or more limitations or complexities, namely, low extraction efficiency (recovery) for one or more compounds [19,21], long retention times [19,21], use of an electrochemical detector [20], and use of a mobile phase gradient with a multi-step flow rate change [22].

The purpose of this study was to develop a simple, reproducible method for the analysis of diclofenac and a representative hydroxylated metabolite in serum. We selected 4′-hydroxydiclofenac as the representative metabolite since this major metabolite in rats and humans [13,14] is commercially available. Utility of our method was assessed in serum collected from rats treated with 3, 10 or 50 mg/kg diclofenac which is a meaningful dosage range for experimental studies because the low doses have reported pharmacological efficacy for analgesia and inflammation [23] while the highest dose reproducibly produces substantial enteropathy [24].

2. Experimental

2.1. Chemicals, standards and solvents

Diclofenac, Tween-20, diethyl ether, sodium acetate, quinidine and NADPH were purchased from Sigma Chemical Company (St. Louis, MO, USA). 4′-Hydroxydiclofenac was obtained from BD Biosciences (Woburn, MA, USA). The internal standard, *N*-phenylanthranilic acid was obtained from Fluka Chemie (Buchs, Switzerland). Injectable solution of Nembutal® (sodium pentobarbital) was obtained from Abbott Laboratories (North Chicago, IL, USA). Acetic acid, phosphoric acid, HPLC grade acetonitrile and other chromatography solvents were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Unless specified, all other chemicals and reagents were from Sigma. Stock solutions of diclofenac and internal standard (1 mg/ml) were prepared in ethanol and stored at 4 °C wrapped in aluminum foil. The 4′-hydroxydiclofenac standard was dissolved in a solution of 40% acetonitrile and 1% glacial acetic acid and then stored at 4 °C wrapped in aluminum foil. Further dilutions of standards were made with double distilled water.

2.2. Animals

Male Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN, USA). The protocol for animal care and handling was approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston in accordance with NIH guidelines. Animals were housed on wire-floor cages above absorbent paper, maintained in a 12 h light/dark cycle, and given free access to Purina lab chow and water. After at least 7 days of acclimation to these conditions, groups of four to eight animals weighing ∼360 g were treated with 3, 10 or 50 mg diclofenac/kg body weight in a volume of 2 ml 0.1% Tween-20/kg by gavage. Control animals received only an equivalent amount of 0.1% Tween-20 (2 ml/kg) by gavage. At 3 h after drug treatment, animals were anesthetized by intraperitoneal injection of Nembutal® (∼80 mg/kg). Blood was aspirated from the inferior vena cava and transferred to a serum separation tube containing clot activator to facilitate separation of serum by centrifugation. Serum was frozen at −80 °C until analysis.

2.3. Chromatography system and operating conditions

We used a Waters 626 LC System (Waters, Milford, MA, USA) with a 2487 dual λ absorbance detector and a 600S controller that was equipped with 20 μl loop for sample injection. System operation was controlled by a Millenium 32 Chromatography Manager (Waters, Milford, MA, USA). Separation was achieved using a C-18 analytical reversed phase column (250 mm × 4.6 mm i.d., 5 μM particle size, Beckman, Palo Alto, CA, USA) and a mobile phase consisting of an acetonitrile:sodium acetate buffer (75 mM, pH adjusted to 5.0 with acetic acid) in a ratio of 2:1.5 (v/v). The mobile phase was filtered through a Millipore 0.22 μm filter and degassed prior to use. Flow rate was set at 0.5 ml/min. Effluent was monitored at 280 nm and output was recorded and printed. We found that both daily preparation of fresh mobile phase and daily cleaning of the column with water followed by methanol were critical for reproducible results.

2.4. Choice of internal standard

A number of other NSAIDs have been used as an internal standard for the analysis of diclofenac and its metabolites from the biological matrix [20,25,26]. However, we found very poor base line separations of ibuprofen and indomethacin from diclofenac under the experimental conditions used in the present study. Use of another reported internal standard, namely, *N*-phenylanthranilic acid [27], provided a clear base line separation from diclofenac and 4′-hydroxydiclofenac (Fig. 1).

2.5. Extraction and sample analysis

Initial assessment of solid phase extraction using a SPE-octadecyl C_{18} column according to Zecca et al. [20] was not promising since <50% of the diclofenac was recovered. Diethyl ether was found to give a better extraction efficiency for diclofenac and the internal standard from rat serum than other solvents described for the extraction of diclofenac, including cyclohexane–diethyl ether, hexane–isopropanol, and dichloromethane [28–30]. Rat serum (200 μl) was added to a glass-stopper tube and mixed with 100 μl of internal standard (1.56 μg/ml). The contents were acidified by addition of 100 μl of 1 M phosphoric acid to facilitate release of acidic compounds from proteins and then extracted twice with 3.0 ml HPLC grade diethyl ether. The organic phase was collected after centrifuging

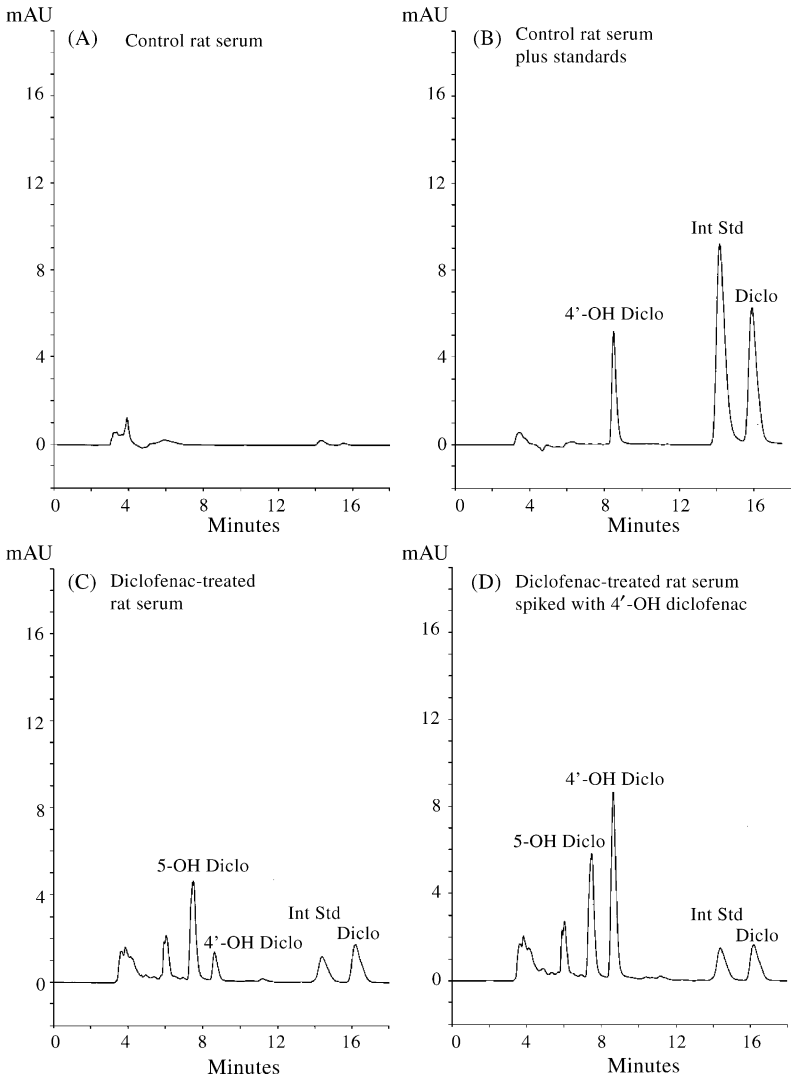

Fig. 1. Representative HPLC profiles of: (A) serum from a control rat; (B) serum from a control rat plus standards for diclofenac (diclo), 4′-OH-diclofenac (4′-OH-diclo) and the internal standard (Int Std); (C) serum from a rat at 3 h after treatment with 10 mg/kg diclofenac showing 5-OH-diclofenac (5-OH diclo); and (D) 4′-OH diclofenac (4′-OH diclo) spiked serum from a rat at 3 h after treatment with 10 mg/kg diclofenac.

the tubes at 950 × *g* for 20 min. The organic phase was aspirated, placed in screw-capped tubes, and dried under nitrogen at 37 °C. The dried residue was re-dissolved in 100 μl mobile phase.

Performance of the chromatographic system was verified daily by injecting 20 μl of a calibration standard containing a mixture of diclofenac (3.125 μg/ml), 4′-hydroxydiclofenac (3.125 μg/ml), and internal standard (1.56 μg/ml).

2.6. Precision and accuracy

The within-day precision and accuracy of the method were evaluated by spiking 200 μl of control rat serum with 0.39, 0.78, 3.13, or 6.25 μg/ml of either diclofenac or 4′-hydroxydiclofenac plus the internal standard. The samples were extracted with diethyl ether and analyzed by HPLC as described in Sections 2.3 and 2.5. Observed concentrations were calculated using standards curves as described in Section 2.8.

2.7. In vitro system for synthesis of 5-hydroxydiclofenac

Human cytochrome P450 3A4 expressed as His-tagged proteins in *Escherichia coli* TOPP3 and purified using Talon affinity columns [31] was kindly provided by Dr James R. Halpert. Recombinant NADPH-cytochrome P450 oxidoreductase and cytochrome b_5 (b_5) from rat liver were prepared as described by Harlow and Halpert [32]. The NADPH-supported enzyme system for quinidine-enhanced synthesis of 5-OH diclofenac was carried out essentially as described [33,34]. In brief, a substrate mixture containing (0.5 mM diclofenac, 0.2 mM quinidine, and 5 mM glutathione) was prepared in 80 μl of 50 mM Hepes, pH 7.4, 15 mM $MgCl_2$ with 2% as the final concentration of methanol. The substrate mixture was pre-incubated with reconstituted P450 system at 37 °C for 5 min. The reconstituted P450 3A4 system (10 μl) contained 0.1 M MOPS, pH 7.4, 0.04% CHAPS, 0.1 mg/100 μl DOPC, 80 pmol P450 oxidoreductase, 40 pmol b_5, and 20 pmol P450 3A4 in this

order. The reactions were initiated by the addition of 10 μl NADPH (1 mM final concentration). The total reaction volume of the assay was 100 μl. After 20 min of incubation, the reactions were stopped by adding 50 μl 20% TCA. Subsequently, the final acidified reaction mixture (150 μl) was extracted twice with diethyl ether, and analyzed by HPLC as described in Sections 2.3 and 2.5. Observed concentrations were calculated using standards curves as described in Section 2.5.

2.8. Calibration curves and data analysis

Amounts of diclofenac and 4′-hydroxydiclofenac in duplicate samples of 200 μl serum from drug-treated animals were calculated using calibration curves. These calibration curves were generated by diluting stock solutions (described in Section 2.1) to prepare at least five concentrations of diclofenac (0.097–100 μg/ml) and 4′-hydroxydiclofenac (0.047–100 μg/ml) using integrated peak area data obtained with a Millenium Chromatography Manager (Waters, Milford, MA, USA). Data presented for observed serum concentrations of diclofenac and 4′-hydroxydiclofenac were corrected for the percentage extraction efficiency of the internal standard. Sigma Stat for Windows version 2.3 (Jandel Scientific, San Rafael, CA, USA) was used for statistical analysis of data.

3. Results and discussion

3.1. Chromatography and selectivity

Clear base line separation of diclofenac and its major metabolite 4′-hydroxydiclofenac from the internal standard *N*-phenylanthranilic acid was achieved by HPLC with an isocratic mobile phase. Retention times for 4′-hydroxydiclofenac, internal standard and diclofenac were found to be approximately 8.6, 14.4 and 16.2 min, respectively, as shown in representative chromatographs of control serum plus standards and serum from a rat at 3 h after treatment with 10 mg diclofenac/kg body weight (Fig. 1B and C). Chromatograms from the serum of control rats showed no endogenous compounds at the retention time of diclofenac, 4′-hydroxydiclofenac or the internal standard (Fig. 1A). Preliminary studies indicated that use of naproxen as an internal standard could be problematic since the peak for naproxen eluted close to peak at 13.6 min for the injectable anesthetic Nembutal® (not shown) which is given to all our animals at the time of sacrifice. These preliminary studies about specificity of chromatographic analysis indicate the importance of making certain that other agents given to the experimental animal do not have a retention time similar to compounds of interest.

Several peaks with elution times close to that of 4′-hydroxydiclofenac were consistently detected in serum from diclofenac treated rats but not control rats (Fig. 1C). These peaks could represent other hydroxylated metabolites of diclofenac [22,35]. Of particular relevance for future application to toxicology investigations is potential interference by 5-hydroxydiclofenac since this reactive metabolite is capable of injurious adduction reactions [15,16] and since appreciable amounts of this metabolite have been detected in the urine of rats treated with diclofenac [14]. Therefore, we synthesized a small amount of 5-hydroxydiclofenac using a reaction system containing CYP3A4 which is reported to be specific for quinidine-enhanced conversion of diclofenac to 5-hydroxydiclofenac [34]. As shown in Fig. 2B, incubation of the CYP3A4 reaction system with the diclofenac and quinidine lead to a presumptive 5-hydroxydiclofenac peak which eluted at a time distinct from standards for 4′-hydroxydiclofenac and diclofenac (Fig. 2C). Further confirmation of distinct peaks for the 4′-OH and 5-OH metabolites was obtained by spiking experiments. As shown in Figs. 2C and 1D, spiking with 4′-hydroxydiclofenac increased the peak for the 4′-OH metabolite, but not for the presumptive 5-OH metabolite, in chromatograms from the CYP3A4 plus diclofenac reaction system or serum from diclofenac-treated animals.

3.2. Linearity and sensitivity

Calibration curves of peak areas versus the concentrations of diclofenac or 4′-hydroxydiclofenac were found to be linear in the range of 0.097–100 μg/ml and gave correlation coefficients (R) of >0.999). The mean linear regression equations of the standard curves are: $y = 4785x + 57.72$ for diclofenac and $y = 5665x + 15.01$ for 4′-hydroxydiclofenac. For each calibration curve, the intercept was not statistically different from zero. The lower limits of quantitation for diclofenac and 4′-hydroxydiclofenac were found to be 0.097 and 0.045 μg/ml, respectively. The limits of detection for diclofenac and 4′-hydroxydiclofenac were 0.0225 and 0.0112 μg/ml, respectively. The large range of linearity combined with the low limits of detection facilitates use of small serum samples. Thus, our method is suitable for measuring a wide range of concentrations of diclofenac and its major metabolite in the small volume of serum obtained from experimental animals.

3.3. Precision and accuracy

The intra- and between-days precision and accuracy for the determination of diclofenac and 4′-hydroxydiclofenac are presented in Table 1. For these parameters, we focused on the 16-fold range of 0.39–6.25 μg/ml based on preliminary observations of the serum levels detected in rats given the dosage range of interest, namely 3–50 mg/kg. The CV% of intra- and inter-day variabilities of extracted standards from spiked rat serum samples were less than 10%, which is within the limit of acceptability (±15%). Values for accuracy (percent bias), which were calculated from the spiked concentration and the mean value of the observed concentration, are also within the range of acceptability. This documentation of good precision for our method over a 16-fold range for each compound compares well with the precision reported by others for detection of hydroxylated metabolites of diclofenac in urine over a narrower concentration range of 3-fold [25].

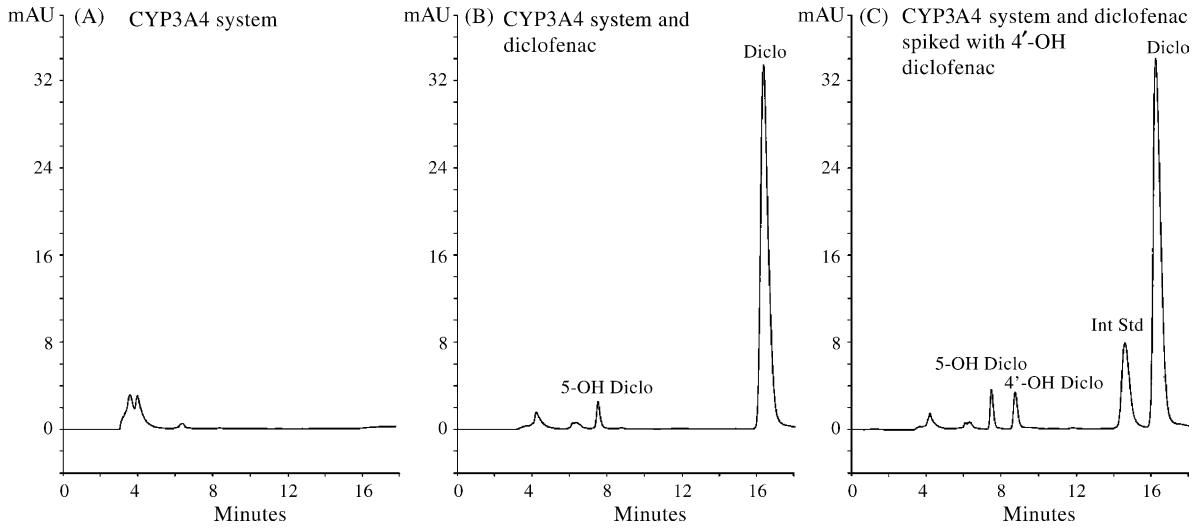

Fig. 2. Representative HPLC profiles from in vitro system containing: (A) just CYP3A4 system; (B) CYP3A4 system incubated with diclofenac; and (C) CYP3A4 system incubated with diclofenac (diclo) and then spiked with 4′OH-diclofenac (4′-OH-diclo) standard and internal standard (Int Std).

3.4. Extraction efficiency

Efficiency of extraction by our diethyl ether solvent system was assessed in acidified serum samples from control rats that were spiked with 0.78, 1.56, 3.125, or 6.25 μg/ml of diclofenac or 4′-hydroxydiclofenac and compared to calibration curves constructed as described in Section 2.8. Extraction efficiencies were similar across the four concentrations and averaged 79.91% for diclofenac and 76.88% for 4′-hydroxydiclofenac. These extraction efficiencies compare well with the 82% average extraction efficiency of the internal standard.

3.5. Application to serum from rats treated with a range of diclofenac doses

We applied the HPLC method developed in this study to serum collected from rats sacrificed at 3 h after treatment with 3, 10 or 50 mg diclofenac/kg. This dosage range was selected as meaningful for experimental studies because the low doses have reported pharmacological efficacy for analgesia and inflammation [23] while the highest dose reproducibly produces substantial enteropathy [24]. Three hours was selected as a time point meaningful for experimental studies because blood levels of diclofenac are reported to exhibit an apparent plateau

Table 1
Accuracy and precision of diclofenac and 4′-hydroxydiclofenac determination

Spiked concentration (μg/ml)	Observed concentration (μg/ml)	CV (%)	Percent of spiked concentration
Diclofenac within-day ($n = 4$)			
0.39	0.40 ± 0.02	5.2	102.6
0.78	0.81 ± 0.02	2.8	103.8
3.13	3.23 ± 0.19	5.2	103.4
6.25	6.35 ± 0.28	4.5	101.6
Diclofenac between-days ($n = 5$)			
0.39	0.38 ± 0.03	7.9	97.4
0.78	0.78 ± 0.04	5.2	100.0
3.13	3.16 ± 0.26	7.8	100.9
6.25	6.48 ± 0.21	3.2	103.7
4′-OH-Diclofenac within-day ($n = 4$)			
0.39	0.38 ± 0.01	2.5	97.5
0.78	0.73 ± 0.05	6.9	93.6
3.13	3.54 ± 0.35	9.8	113.0
6.25	6.74 ± 0.26	3.9	107.8
4′-OH-Diclofenac between-days ($n = 4$)			
0.39	0.35 ± 0.02	4.3	89.8
0.78	0.76 ± 0.05	6.7	97.5
3.13	3.21 ± 0.17	5.8	102.6
6.25	6.63 ± 0.19	2.2	106.1

Observed concentrations are expressed as mean ± S.D. for the indicated n values.

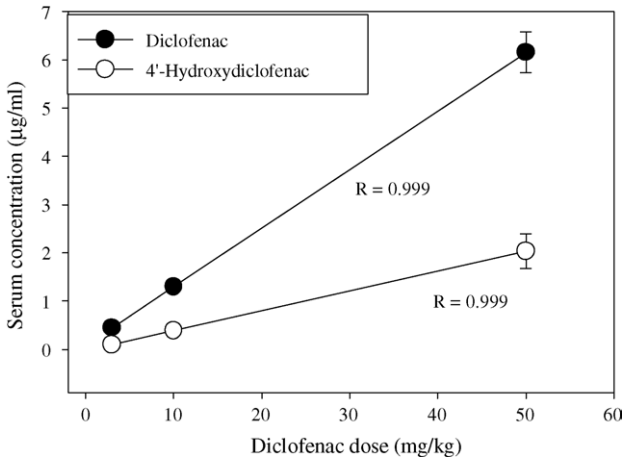

Fig. 3. Dosage effect on serum concentrations of diclofenac and its 4′-hydroxydiclofenac metabolite at 3 h after diclofenac treatment. Values are means ± S.D. of four animals given 3 mg/kg, five animals given 10 mg/kg, and eight animals given 50 mg/kg. When S.D. bars are not shown, the S.D. lies within the symbol. The correlation coefficients (*R*) are 0.999.

between 2 and 4 h after treatment with pharmacological doses of diclofenac [23]. Observed concentrations of diclofenac and 4′-hydroxydiclofenac were calculated from standard curves for diclofenac or 4′-hydroxydiclofenac and then corrected for the extraction efficiency of the internal standard as described in Section 2.8.

Serum concentrations of both diclofenac and 4′-hydroxydiclofenac increased in a linear manner when plotted against the administered drug dosage with *R*-values of ≥0.999 (Fig. 3). However, the slope for the metabolite was shallower than the slope for the parent compound which suggested some kind of dosage-dependent difference. To probe the nature of this difference, we assessed the influence of dosage on the percentage of administered drug recovered in serum as parent compound or metabolite using a formula for estimating the serum volume of each rat based on body weight [36]. As shown in Fig. 4, dose had no apparent effect on the estimated percentage of administered drug recovered as the 4′-hydroxydiclofenac metabolite over the 3–50 mg/kg range of drug dosage. In contrast, the estimated percentage of administered drug recovered as the parent compound showed a dose-dependent decrease from 0.45 to 0.37% over this dosage range (Fig. 4). The relatively low percentages of dosage that we recovered in serum for parent compound and 4′-hydroxydiclofenac metabolite are not due to poor absorption from the GI tract. In fact, diclofenac is known to be rapidly absorbed, distributed to multiple tissues including into presumptive target sites for efficacy for rheumatoid arthritis, namely synovial membrane, articular cartilage and bone [18,37], and extensively biotransformed by the liver to metabolites which are eliminated via bile or urine [16].

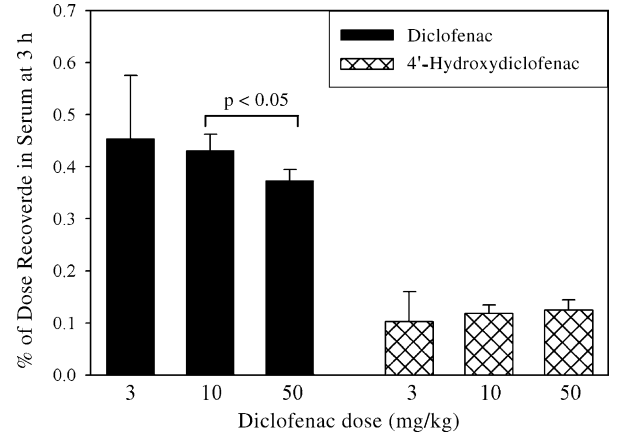

Fig. 4. Effect of diclofenac dosage on the recovery (as percentage of dose given) of diclofenac and its 4′-hydroxydiclofenac metabolite at 3 h after drug treatment. Values are mean ± S.D. of four animals given 3 mg/kg, five animals given 10 mg/kg, and eight animals given 50 mg/kg.

4. Conclusions

We have developed a simple and rapid method for determining serum diclofenac and its major 4′-hydroxy metabolite with a single extraction step and isocratic HPLC separation. No derivatization and or complex instrumentation is needed. This reliable method affords good sensitivity for monitoring diclofenac and its major 4′-hydroxy metabolite in small volumes of serum from rodents treated with an experimentally useful range of diclofenac dosages.

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