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Short communication

Analysis of diclofenac and its metabolites by high-performance liquid chromatography: relevance of *CYP2C9* genotypes in diclofenac urinary metabolic ratios

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Abstract

In humans, diclofenac is metabolised to 4′-hydroxy (OH), 3′-OH and 5-OH metabolites. The polymorphic CYP2C9 is involved in the metabolism of diclofenac to 4′-OH diclofenac and 3′-OH diclofenac. The aim of the present study was to develop a high-performance liquid chromatographic method to simultaneously measure diclofenac and its metabolites in urine, suitable for metabolic studies. After liquid–liquid extraction the compounds were separated in a reversed-phase column and measured by ultraviolet absorption at 282 nm. For all compounds intra-day and inter-day variations were less than 7%, and the limits of quantitation were 0.25 mg/l. No analytical interference with endogenous compounds was found. The relationship between diclofenac metabolic ratios among different *CYP2C9* genotypes is reported. The *CYP2C9*3/*3* subject had the highest diclofenac/4′-OH ratios. However no difference was found between *CYP2C9*2/*2* and **1/*1* genotypes. The chromatographic method developed was sensitive and reliable for the measurement of diclofenac and its metabolites simultaneously in human urine, and is suitable for use in diclofenac metabolism studies.

Keywords: Diclofenac; Hydroxydiclofenac; *CYP2C9* genotypes

1. Introduction

Diclofenac sodium is a widely used non-steroidal anti-inflammatory drug (NSAID) of the phenylacetic acid class. On the basis of current information it can be estimated that an average 256 000 inhabitants are treated every day with diclofenac in primary care in Spain. It has been shown to be effective in the treatment of symptoms of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, etc. Diclofenac is extensively metabolized in humans to a number of hydroxylated metabolites which are found in plasma and urine in free and conjugated forms. The main diclofenac metabolite in plasma and urine is 4′-hydroxy (OH) diclofenac and minor monohydroxy metabolites are 3′-OH diclofenac and 5-OH diclofenac [1]. The main enzyme responsible for the 4′-hydroxylation and 3′-hydroxylation of diclofenac

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seems to be CYP2C9, while the 5-hydroxylation is mediated by CYP3A4 and other CYP2C enzymes (CYP2C8, CYP2C18 and CYP2C19) in vitro [2–4]. The polymorphic CYP2C9 has been reported to catalyse the metabolism of many clinically important drugs, such as phenytoin, *S*-warfarin, tolbutamide, losartan and several NSAIDs [5–7]. Allelic variants of the *CYP2C9* gene causing decreased enzyme activity have been identified, such us *CYP2C9*2*, and *CYP2C9*3* [8,9].

The activity of CYP2C9 has been evaluated in vitro and in healthy volunteer studies by using the diclofenac/4′-OH diclofenac ratio [10–12]. Several analytical methods have been developed for the measurement of diclofenac and its metabolites in biological fluids. However, the simultaneous detection of diclofenac and its monohydroxylated metabolites in urine with high-performance liquid chromatography (HPLC) has only been reported in three studies with several limitations [13–15]. For use in human metabolic studies after the administration of a low single oral dose, the method should be precise, accurate, and the limit of quantification must be optimised. The aim of this study was to develop a HPLC method to assay diclofenac and its metabolites in human urine. As an application the relationship between diclofenac urinary metabolic ratios among different *CYP2C9* genotypes is reported.

2. Experimental

2.1. Chemicals

Diclofenac metabolites (3′-OH diclofenac, 4′-OH diclofenac and 5-OH diclofenac) were kindly supplied by Novartis (Basel, Switzerland). Diclofenac sodium, flurbiprofen as an internal standard (I.S.), and ascorbic acid were purchased from Sigma (Madrid, Spain). HPLC-grade acetonitrile, methanol, tetrahydrofuran, hydrochloric acid, disodium hydrogenphosphate dihydrate and potassium dihydrogenphosphate were obtained from Merck (Darmstadt, Germany). Water was Milli-Q quality prepared by the water purification system Millipore (Millipore, Bedford, MA, USA).

2.2. Instrumentation

The HPLC system (Beckman, Fullerton, CA, USA) consisted of a solvent pump (Model 110 B) and a programmable UV detector (Module 166) set at 282 nm. Data were collected and analysed by an IBM compatible personal computer with the Beckman System Gold program (version 7.11, Beckman, Fullerton, CA, USA). All chromatographic separations were performed at ambient temperature on a reversed-phase 250×4.6 mm, 5 μm particle size Hypersil ODS column (Sharlau Science, Madrid, Spain). The mobile phase, a mixture of acetonitrile–methanol–tetrahydrofuran–water (22:10:3:65, v/v), included disodium hydrogenphosphate dihydrate (4.5 g/l) and potassium dihydrogenphosphate (2.3 g/l). The mobile phase was filtered through a 0.22 μm GS-filter (Millipore). The flow-rate was maintained at 0.8 ml/min.

2.3. Extraction

The deconjugation of diclofenac was carried out with alkaline hydrolysis [16]. To 500 μl of urine, 100 μl of ascorbic acid (400 mg/l) and 100 μl of sodium hydroxide (5 *M*) were added. The tubes were vortexed for 30 s and then incubated at 72 °C for 1 h. Then 50 μl of flurbiprofen (125 mg/l) as I.S. and 1 ml of hydrochloric acid (2 *M*) were added. The extraction was carried out with 5 ml of diisopropylether. The tubes were then vortexed vertically for 10 min, and centrifuged at 4000 *g* for 10 min at 15 °C. The organic layer was transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen for 30 min. The residue was resuspended in 200 μl of mobile phase, and, after vortexing for 60 s, 20 μl was injected into the HPLC column with a manual injector.

2.4. Subjects

Diclofenac metabolism was studied among 15 subjects selected from a population previously genotyped for *CYP2C9* [17]. *CYP2C9* genotypes were **1/*1* for 10, **2/*2* for four and **3/*3* for one subject. They were eight male and seven female,

white European, healthy volunteers, aged 24 to 41 years (mean±SD: 30±7 years). To study the diclofenac metabolism, the subjects were given a single oral dose of 50 mg of enteric-coated diclofenac (Voltaren, Novartis, Switzerland) in the evening before going to bed. The subjects refrained from eating for 2 h before drug intake. All urine was collected over 8 h. After the measurement of urine volume, two samples of 20 ml each were stored at −20 °C until analysis. The subjects were mostly students and staff from the University of Extremadura, Badajoz, Spain. A routine clinical examination was performed and the medical history was taken before the study. Volunteers with antecedents of serious somatic diseases or adverse drug effects and those with any drug intake in the preceding 2 weeks before the study were excluded from the study. The subjects were informed about the aims of the study and gave their consent to participate. The study was performed according to the Helsinki Declaration and was approved by the Ethics Committee at the Extremadura University Hospital.

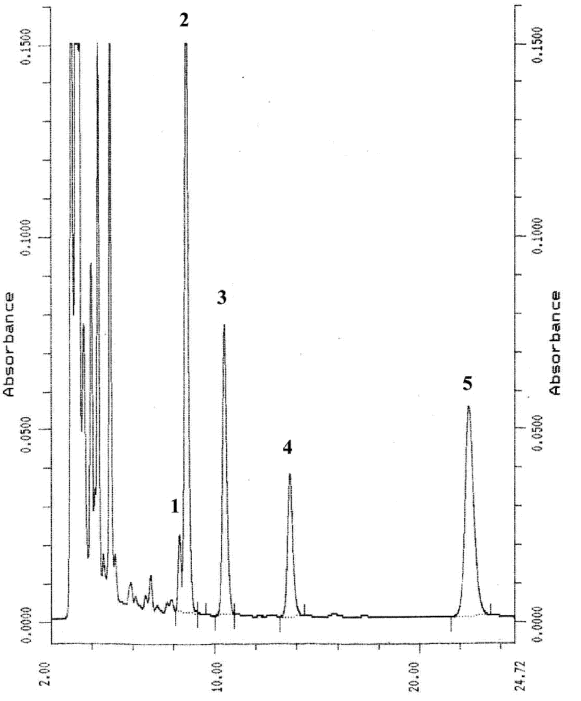

Fig. 1. Representative HPLC chromatogram of diclofenac and its metabolites in healthy volunteer urine after a 50 mg oral dose. The numbers refer to the following compounds: 1=3′-OH diclofenac; 2=4′-OH diclofenac; 3=5-OH diclofenac; 4=internal standard; 5=diclofenac.

3. Results

3.1. Chromatography

Fig. 1 shows a HPLC chromatogram of a volunteer's sample after a single oral dose of 50 mg diclofenac. The retention times for 3′-OH, 4′-OH, 5-OH and diclofenac were 8.4, 8.7, 10.5 and 22.5 min, respectively. Quantification of the peaks was carried out by measuring the peak heights of each compound and of the I.S. Calibration curves were drawn for duplicates, using five concentrations between 2 and 16 mg/l. The curves were linear for all the four compounds within this concentration range (r^2>0.99).

3.2. Precision, accuracy, recovery and limit of quantitation

The limit of quantitation was 0.25 mg/l for diclofenac and its metabolites, and the limits of detection defined by a minimum signal-to-noise ratio of three were 0.18, 0.08, 0.07 and 0.16 mg/l for diclofenac, and the 4′-OH, 3′-OH, and 5-OH metabolites, respectively.

The recovery was determined by comparing the peak height of extracted standards to the peak height of standards injected directly into the HPLC system. The recovery of diclofenac following the extraction was found to be on average 57% over the range of the three concentrations studied (4, 8 and 12 mg/l). The recovery was between 58 and 89% for the metabolites.

The intra-day and inter-day RSDs were less than 7% for all substances. The mean accuracy was greater than 99% for diclofenac, and 98, 99 and 97% for the 3′-OH, 4′-OH and 5-OH metabolites, respectively, over the 4 to 12 mg/l range (Table 1).

Table 1
Intra-day, inter-day assay variations and accuracy for diclofenac and its metabolites in urine

Analyte	Concentration (mg/l)	Intra-day variation (RSD, %)*	Inter-day variation (RSD, %)†	Accuracy (%)
Diclofenac	4	0	1.72	99
	8	2.66	2.50	100
	12	4.75	4.46	100
3′-OH	4	1.54	1.78	96
	8	2.57	2.86	99
	12	3.54	3.12	100
4′-OH	4	0.72	1.94	99
	8	2.16	2.68	100
	12	4.46	3.64	100
5-OH	4	3.93	6.21	99
	8	0.82	3.55	94
	12	5.66	6.70	99

* Intra-day variations are the mean of three independent measurements. † Inter-day variations were determined over a period of 1 month ($n=6$).

3.3. Diclofenac metabolism among different CYP2C9 genotypes

Diclofenac/4′-OH diclofenac ratio was 3.6 times higher in the *CYP2C9*3/*3* subject compared to *CYP2C9*1/*1* and *CYP2C9*2/*2* groups. No differences were found between diclofenac/3′-OH, diclofenac/4′-OH or diclofenac/5-OH urinary ratios between *CYP2C9*2/*2* and *CYP2C9*1/*1* subject subjects (Table 2, Fig. 2). No side effects during the diclofenac phenotyping test were observed.

4. Discussion

The present study has described an analytical method for the simultaneous quantification of diclofenac and three of its metabolites (3′-OH, 4′-OH and 5-OH) in human urine by HPLC. The method was found to be sensitive, accurate and precise. Several methods have been developed for the determination of diclofenac in human urine by HPLC. However only a few of them studied simultaneously diclofenac and its monohydroxy metabolites [13–15]. The present method enables the successful separation of 3′-OH and 4′-OH metabolites, which had been a problem in some of the previous HPLC methods [13,14]. It has been reported that 5-OH is unstable during analysis: in the present method as in an earlier reported method [15] the addition of ascorbic acid to the sample during preparation served to enhance the stability of this metabolite [14]. The presence of endogenous peaks which interfered with the analysis of the metabolites was observed in that previous method [15]. Nevertheless in the present method there were no interferences with diclofenac

Table 2
Diclofenac urinary metabolic ratios among 15 healthy volunteers with different *CYP2C9* genotypes

Mean±S.D.	*CYP2C9* genotype groups		
	**1/*1* ($n=10$)	**2/*2* ($n=4$)	**3/*3* ($n=1$)
Diclofenac/4′-OH diclofenac	0.5±0.2	0.5±0.1	1.8
Diclofenac/3′-OH diclofenac	6.4±2.5	5.0±0.8	7.1
Diclofenac/5-OH diclofenac	1.2±0.5	1.6±0.6	1.5

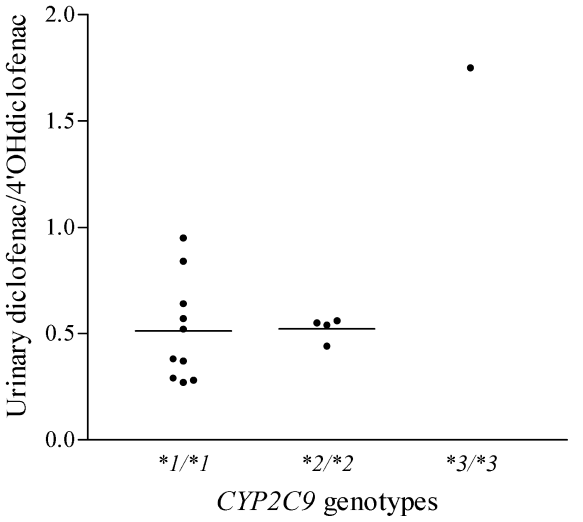

Fig. 2. Urinary metabolic ratios of diclofenac/4′-OH diclofenac among 15 healthy Spanish subjects in relation to their *CYP2C9* genotypes.

or metabolites. In addition the intra-day and inter-day variations of diclofenac and its metabolites (less than 7% for all substances) and the limits of quantitation were found in the present study to be better than in the previously published HPLC methods [13–15].

The *CYP2C9*3/*3* subject had the highest diclofenac/4′-OH ratio, which agrees with previous reports showing that the clearance of diclofenac to 4′-OH diclofenac tended to be lower in subjects heterozygous for *CYP2C9*3* compared to those homozygous for *CYP2C9*1* [12]. Moreover no significant differences of diclofenac 4′-hydroxylation has been observed between *CYP2C9*1/*1* and *CYP2C9*2/*2* genotypes as it has been shown previously [11]. Recently, in human liver microsomes the involvement of CYP2C8 in the 4′-hydroxylation of diclofenac-glucuronide has been reported [18], which may affect the determination of CYP2C9 activity by the diclofenac/4′-OH diclofenac ratio in vivo. Thus further research on this question is needed. The present data and previous ones [9,19,20] indicate that *CYP2C9*3/*3* subjects might have a decreased enzyme capacity. These subjects may have a higher than expected plasma concentration of CYP2C9 substrates, and be prone to a higher risk of potentially dangerous drug interaction if two or more CYP2C9 substrates are given concomitantly (warfarin, phenytoin, tolbutamide, etc.) [21–23]. The reported HPLC–UV method had the precision and reliability for use in the analysis of diclofenac and monohydroxylated metabolites, and is thus useful for metabolic studies.

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