Journal of Pharmaceutical & Biomedical Analysis
Vol. 9, No. 7, pp. 585–588, 1991
Printed in Great Britain

0731–7085/91 $3.00 + 0.00

Short Communication

Isolation and identification of a new metabolite of diflunisal

S.H. HANSEN,* C. CORNETT, J. HANSEN-MØLLER, L.L. LARSEN and S. VAABEN

PharmaBiotec Research Centre, The Royal Danish School of Pharmacy, 2 Universitetsparken, DK-2100 Copenhagen, Denmark

Keywords: *Diflunisal; diflunisal metabolite; chromatographic and spectroscopic characterization.*

Introduction

The metabolism of diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid) was first described in 1975 by Tocco *et al.* [1]. They found that diflunisal was excreted in the urine as an acyl glucuronide (DAG) and a phenolic glucuronide (DPG). The acyl glucuronide was later shown to be highly unstable [2] as well as reactive towards plasma proteins *in vivo* [3].

After multiple dose administration of diflunisal a new metabolite, the sulphate conjugate (DS), was isolated from human urine by Loewen *et al.* [4] in 1986. The amount of DS excreted accounted for less than 10–20% of the administered dose. There has been some discussion in the literature concerning the role of sulphate conjugation for the elimination kinetics of diflunisal in the rat [5, 6]. In humans the sulphate conjugate is found after single dose administration of diflunisal [7]. Dickinson and King [personal communication] have identified some "diglucuronides" of diflunisal originating from positional isomers of DAG after *i.v.* administration of DAG and isomers of DAG.

Despite the similarity of diflunisal to salicylic acid (salicylic acid is partly excreted as an ortho- and a para-hydroxylated phase I metabolite [8]) there have been no reports of phase I metabolism occurring in the case of diflunisal.

In the present paper the isolation and identification of a hydroxylated metabolite of diflunisal is reported.

Experimental

Apparatus

A HPLC consisting of a Waters 6000 A pump (Milford, MA, USA), a Rheodyne 7125 loop injector equipped with either a 20 or 1000 μl loop (Cotati, CA, USA), a Kontron SFM-23 fluorescence detector (München, Germany) operated with an excitation wavelength of 264 nm and emission at 420 nm and a Carlo Erba DP 700 Data Processor (Milano, Italy) was used. The columns were thermostated at 38°C in a Shimadzu CTO-6A column oven (Tokyo, Japan).

Chemicals

Diflunisal (+99.5%) was a gift from Dumex Ltd (Copenhagen, Denmark). DAG and DPG were isolated from human urine as described earlier [2]. DS was synthesized as described previously [9]. β-Glucuronidase from Helix Pomatia 100.000 ie/ml, dodecyltrimethylammonium bromide (DTMA) and *D*-1,4-saccharo lactone were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and were supplied by Merck (Darmstadt, Germany).

Chromatography

A pre-separation of the metabolite from human urine (2800 ml) was performed using a Pharmacia (Uppsala, Sweden) column 700 × 33 mm i.d. packed with Amberlite XAD-4 as described previously [2]. The fractions

* Author to whom correspondence should be addressed.

collected containing the unknown metabolite were pooled and concentrated by roto-evaporation followed by freeze drying. The freeze dried product was dissolved in 50 ml methanol–water (50:50, v/v) and preparative HPLC was performed using a Knauer column (Berlin, Germany) 250 × 8 mm i.d. packed with Spherisorb octyl 5 μm (Phase Separations, Queensferry, Clwyd, UK) and an eluent consisting of methanol–0.1 M potassium citrate pH 3.5 (60:40, v/v) containing 8 mM of DTMA was used at a flow-rate of 4.0 ml min^{-1}. After roto-evaporation followed by freeze drying of the collected fractions, the product was dissolved in water.

This solution was passed through a 50 × 8 mm column packed with Dowex 50 W × 4 (200–400 mesh in the acid form). The collected aqueous solution was finally purified on a HPLC column 120 × 4.6 mm packed with 5 μm Chromasil C8 (Eka Nobel, Surte, Sweden) using a mobile phase consisting of methanol–water–glacial acetic acid (100:100:0.2, v/v).

The purity of the isolated metabolite was tested using an analytical HPLC system identical to the preparative system given above but using an analytical column with the dimensions 120 × 4.6 mm i.d.

Spectroscopy

^{1}H and ^{13}C-NMR spectra were obtained on a Bruker AMX 400 WB instrument. In order to remove exchangeable protons the metabolite was dissolved in deuterium oxide followed by evaporation. The metabolite was then dissolved in hexadeutero acetone (5 mg in 500 μl). ^{1}H and ^{13}C correlated spectra were obtained using standard pulse sequence from the AMX-pulse library. The correlation was optimized for a $^{1}J_{^{1}H^{13}C}$ of 130 Hz. A damping of 1 and 2 dB was used for the ^{1}H and ^{13}C channel, respectively. Fast atom bombardment mass spectrometry FAB-MS was performed on a Varian VG 12-250 instrument. The samples were dissolved in glycerol.

Collection of urine

Urine from one person (male, 34 years old, weight 68 kg) given 1 g of diflunisal was collected over a period of 48 h. A total volume of 2800 ml of urine was collected.

Results and Discussion

When urine samples from two patients receiving diflunisal were analysed according to the HPLC method in ref. 2 a minor peak co-eluting with DPG was observed (after enzym-

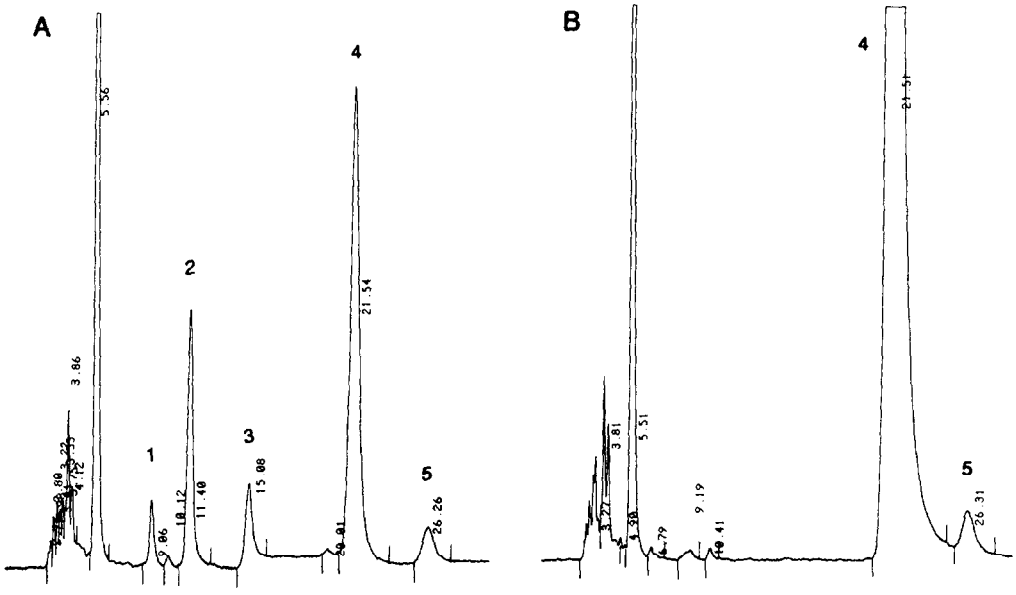

Figure 1
Chromatogram of urine sample from patient. (A) untreated sample, (B) sample treated with β-glucuronidase/sulphatase from *Helix pomatia*. Column: Spherisorb octyl 5 μm, 120 × 4.6 mm i.d. Eluent: Methanol–0.1 M potassium citrate pH 3.5 (60:40, v/v) with 8 mM of DTMA added. Flow-rate: 1.0 ml min^{-1}. Fluorescence detection: Ex. 264 nm and Em. 420 nm. Peaks 1, DAG; 2, DPG; 3, DS; 4, diflunisal and 5, unknown metabolite.

Table 1
^{1}H- and ^{13}C-data for diflunisal and the unknown metabolite

	^{1}H-NMR data observed for				^{13}C-NMR data		
	Diflunisal		Unknown metabolite		Observed for		
Carbon no.	δ-^{1}H	J	δ-^{1}H	J	Diflunisal $\delta^{13}C$	Unknown metabolite $\delta^{13}C$	Calculated for unknown metabolite $\delta^{13}C$
1	—	—	—	—	125	125	135.8
2	8.05	dd 2.4; 1.4	7.56	t < 1.5	129.9	120	123
3					112	112	119.7
4					160.8	149	143
5	7.06	d 8.7			116.7	145	142.7
6	7.70	dd 8.7; 2.4; 1.6	7.27	t* 1.9	135.4	120	121.2
7					170.7	170.7	172.6–175.6
1′					123	123	
2′					162	162	
3′	7.1	m	7.1	m	103.2	103.2	
4′					159	159	
5′	7.1	m	7.1	m	111	111	
6′	7.55	dt 9.7; 8.6	7.55	dt 9.7; 8.6	130.7	130.7	

*dd, but appears as (apparent J = 1.9) distorted triplet because of the linewidth.
J, coupling constant; d, doublet; dd, double doublet; t, triplet; dt, double triplet; m, multiplet.

atic treatment of the samples). Having a fluorescence similar to diflunisal it was presumed to be an unknown metabolite of diflunisal.

Recently, it was found that diflunisal, its known conjugates DAG, DPG and DS and an unknown metabolite could all be separated by reversed-phase HPLC using an octyl-silica column with an eluent containing a long chain quaternary ammonium ion as an ion-paring agent (Fig. 1). The unknown metabolite in this system was found to be identical to the peak coeluting with DPG in the above LC system.

The new conditions therefore were used for the preparative HPLC experiments despite the fact that it was necessary to remove the quaternary ammonium compound by ion-exchange and finally use HPLC in order to remove the buffers. The final product was found by HPLC analysis to yield a single peak corresponding to the unknown metabolite.

The retention time and size of the chromatographic peak of the metabolite was not changed when the urine was treated with β-glucuronidase/sulphatase, acetic acid or base indicating that the metabolite was neither glucuronic acid nor sulphate conjugate.

In the spectroscopic studies the isolated metabolite was compared to diflunisal. FAB-MS gave $MH^+ = 251$ and $MH^+ = 267$ for diflunisal and the unknown metabolite, respectively, the difference of 16 corresponding to an oxygen atom.

From NMR data given in Table 1 it is obvious that a modification of the phenolic ring has taken place. A proton in position 5 is missing, and assuming that it has been replaced by an extra phenolic group in position 5 ^{13}C-NMR data for this ring has been calculated. The calculated data are in good agreement with the observed values for the unknown metabolite.

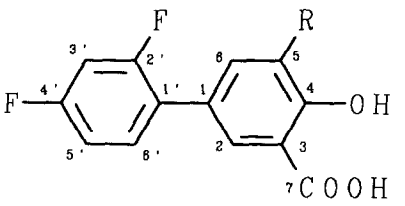

Figure 2
The chemical structure of diflunisal and the new metabolite. R = H: diflunisal. R = OH: metabolite.

The FAB-MS and the NMR data show that the unknown metabolite is 2′,4′-difluoro-4,5-dihydroxy-3-biphenylcarboxylic acid (Fig. 2). This metabolite is excreted as less than 2% of the ingested dose.

Acknowledgements — This work was supported by The Danish Medical Research Council grant no. 12-3045. FAB-MS spectra were performed by Dr Tore Vulpius, The Chemical Institute, The H.C. Ørsted Institute, University of Copenhagen. The grants to purchase the AMX spectrometer were kindly provided by The Alfred Benzon Foundation, The Technology Council and PharmaBiotec Research Centre.

References

[1] D.J. Tocco, G.O. Breult, A.G. Zacchei, S.L. Steelman and C.V. Perrier, *Drug Metab. Dispos.* **3**, 453–466 (1975).
[2] J. Hansen-Møller, L. Dalgaard and S. Honoré Hansen, *J. Chromatogr.* **420**, 99–109 (1987).
[3] G.E. McKinnon and R.G. Dickinson, *Res. Comm. Chem. Pathol. Pharmacol.* **66**, 339–354 (1989).
[4] G.R. Loewen, G. McKay and R.K. Verbeeck, *Drug Metab. Dispos.* **14**, 127–131 (1986).
[5] R.K. Verbeeck and R.G. Dickinson, *Drug Metab. Dispos.* **18**, 267–268 (1990).
[6] J.H. Lin, *Drug Metab. Dispos.* **18**, 268–269 (1990).
[7] G.R. Loewen, R.J. Herman, S.G. Ross and R.K. Verbeeck, *Br. J. Clin. Pharmacol.* **26**, 31–39 (1988).
[8] D.V. Parke, *The Biochemistry of Foreign Compounds*, p. 175. Pergamon, Oxford (1968).
[9] R.G. Dickinson, A.R. King and J. Hansen-Møller, *Xenobiotica*, **21**, 635–640 (1991).

[Received for review 15 February 1991; revised manuscript received 24 April 1991]