Pergamon

0731-7085(94)00053-0

Journal of Pharmaceutical & Biomedical Analysis, Vol. 12, No. 9, pp. 1075–1078, 1994

0731-7085/94 $7.00 + 0.00

Excretion of 3-hydroxy-diflunisal as a monosulphate conjugate — identification using ESI-MS

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Abstract: Electrospray-ionization mass spectrometry was used to identify a novel, highly polar metabolite of diflunisal isolated from Gunn rat urine. Negative ion spectra were obtained of the sulphate conjugate of diflunisal and the new metabolite, which was identified as a sulphate conjugate of 3-hydroxydiflunisal.

Keywords: *Diflunisal; hydroxylation; sulphation; electrospray-ionization mass spectrometry (ESI-MS).*

Introduction

The salicylate anti-inflammatory agent diflunisal (DF, Fig. 1) is metabolized in humans and rats primarily by conjugation to its acyl glucuronide, phenolic glucuronide and sulphate conjugates [1, 2]. In initial investigations of the disposition of DF in Sprague–Dawley rats, the presence of an unidentified, highly-polar metabolite was reported in plasma and urine samples [3]. This metabolite was subsequently found to be much more important quantitatively in the urine of DF-dosed homozygous Gunn rats [4], and was shown to be a conjugate of 3-hydroxy-DF, which is a recently-identified Phase I metabolite of this drug [5, 6]. 3-Hydroxy-DF was also found to be a minor metabolite of DF in humans [5, 6].

Attempts to isolate the intact conjugate of 3-hydroxy-DF by extraction with diethyl ether under acidic conditions were unsuccessful due to its facile hydrolysis. This property of solvent-catalysed hydrolysis, manifested also by DF sulphate [3], suggested that the unknown conjugate of 3-hydroxy-DF was a sulphate, or possibly a disulphate. The present study identifies the conjugate as a monosulphate using electrospray-ionization mass spectrometry (ESI-MS).

Experimental

Materials

DF was a gift from Merck, Sharp & Dohme (Sydney, Australia). Authentic samples of DF sulphate and 3-hydroxy-DF were obtained from earlier studies [5, 7].

Isolation of 3-hydroxy-DF conjugates

Urine samples from an earlier study in which Gunn rats had been given 50 mg kg^{-1} DF i.v. [4] were thawed and pooled. A sample (15 ml, containing *ca.* 3 mg 3-hydroxy-DF as its conjugate) was evaporated to *ca.* 1.5 ml at 35°C under reduced pressure on a rotary evaporator, cooled in ice and centrifuged. Aliquots (50 μl) of the supernatant were injected into the HPLC system used for routine analysis of

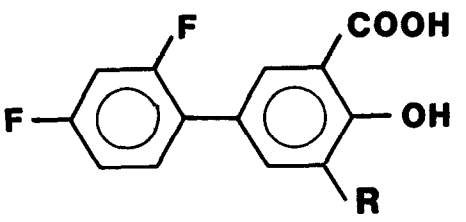

Figure 1
Chemical structure of diflunisal (DF) (R = H) and its Phase 1 metabolite, 3-hydroxy-DF (R = OH).

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DF and its metabolites [3]. This system employs a 4 μm Novapak C_{18} cartridge in a RCM-100 radial compression module (Waters Associates, Milford, MA, USA) with mobile phase pumped at 2 ml min^{-1}. The mobile phase was methanol–buffer (52:48, v/v), with the buffer prepared by adjusting 0.01 M NaH_2PO_4 to pH 2.7 with H_3PO_4 and dissolving Na_2SO_4. $10H_2O$ to 4% w/v. The peak corresponding to 3-hydroxy-DF conjugate eluted at *ca.* 2.6 min under these conditions. Fractions containing this peak were collected on ice, combined (*ca.* 30 ml) and stored at −20°C overnight. Precipitated Na_2SO_4 (a constituent of the mobile phase) was removed by centrifugation and decantation of the mother liquors. The volume was then reduced to *ca.* 1 ml under reduced pressure on a rotary evaporator at 35°C. Aliquots (50 μl) of the residue were injected into a second HPLC system comprising a μ-Bondapak C_{18} semi-preparative column (Waters Associates) with mobile phase methanol–acetic acid (0.1 M) (40:60, v/v) pumped at 3 ml min^{-1} (acetic acid was included in this mobile phase to optimize chromatographic behaviour). The peak corresponding to 3-hydroxy-DF conjugate eluted at *ca.* 18 min. Fractions containing this peak were collected on ice, combined (*ca.* 150 ml), and evaporated to near dryness under reduced pressure at room temperature on a rotary evaporator. The pH was then adjusted to 5 with 0.1 M NaOH. The resultant sample (*ca.* 1 ml) contained *ca.* 0.7 mg of 3-hydroxy-DF conjugate (as measured by hydrolysis to 3-hydroxy-DF) and was contaminated with <5% 3-hydroxy-DF, but with no other DF-related species, as shown by injection into the analytical HPLC system. The sample was then evaporated to dryness and stored at 4°C.

Mass spectrometry

ESI-MS was performed on a Hewlett–Packard (Palo Alto, CA, USA) 5988A quadrupole mass analyser fitted with a Vestec (Houston, TX, USA) electrospray source, a Phrasor (Duarte, CA, USA) high energy dynode detector and a Teknivent (St Louis, MO, USA) data system. The instrument was operated in anion mode, scanning the *m/z* range 50–1000 in 6 s and using an oxygen gas flow. A Harvard (South Natick, MA, USA) syringe pump was used to deliver a phase of methanol–water (50:50, v/v) at a flow rate of 10 μl min^{-1} to a loop injection apparatus. DF, DF sulphate, 3-hydroxy-DF and 3-hydroxy-DF conjugate were dissolved to *ca.* 1 μg μl^{-1} in methanol–water (50:50, v/v), and 5 μl samples injected into the system.

Results and Discussion

The unknown conjugate of 3-hydroxy-DF was obtained from the urine of Gunn rats given DF and purified using two different HPLC systems. The final sample contained <5% unconjugated 3-hydroxy-DF but no other DF-related species, and was known to be contaminated with sodium acetate. In order to characterize the conjugate, ESI-MS was employed using unconjugated 3-hydroxy-DF as well as DF and DF sulphate as reference standards.

The anionic ESI-MS spectrum obtained for DF showed an abundant molecular ion $[M-H]^-$ at *m/z* 249 as base peak and a lower intensity fragment at *m/z* 205, corresponding to $[(M-H)-CO_2]^-$ (Fig. 2A). This in-source fragmentation is an inducible effect in the atmospheric pressure electrospray ionization process [8, 9]: thus, decreasing the repeller voltage in the source from −20 to −10 V eliminated the DF fragment ion, whilst increasing the repeller voltage to −30 V gave enhanced fragmentation. ESI-MS analysis of 3-hydroxy-DF gave similar results, with ions detected at *m/z* 265 for $[M-H]^-$ and at *m/z* 221 for $[(M-H)-CO_2]$ (Fig. 2B).

The sample of DF sulphate was slightly contaminated (<5%) with DF itself, as shown by HPLC analysis. Negative ion ESI-MS analysis gave the DF sulphate molecular ion $[M-H]^-$ at *m/z* 329, though the base peak was at *m/z* 249, corresponding to $[(M-H)-SO_3]^-$, i.e. the DF anion (Fig. 2C). A peak at *m/z* 205 was also detected for $[(M-H)-SO_3-CO_2]^-$. There was no evidence for formation of doubly charged ions for DF sulphate. As noted above for DF, the extent of in-source fragmentation could be altered as a function of the repeller voltage. The relative distribution of the ions suggested that DF sulphate undergoes partial desulphation in the electrospray process, so that, with the contribution of the DF impurity, the DF ion was the base peak in the spectrum.

The sample of 3-hydroxy-DF conjugate, known to contain sodium acetate, exhibited a more complex ESI-MS pattern (Fig. 2D). All peaks marked with an asterisk emanated from the sodium acetate contaminant, as verified by

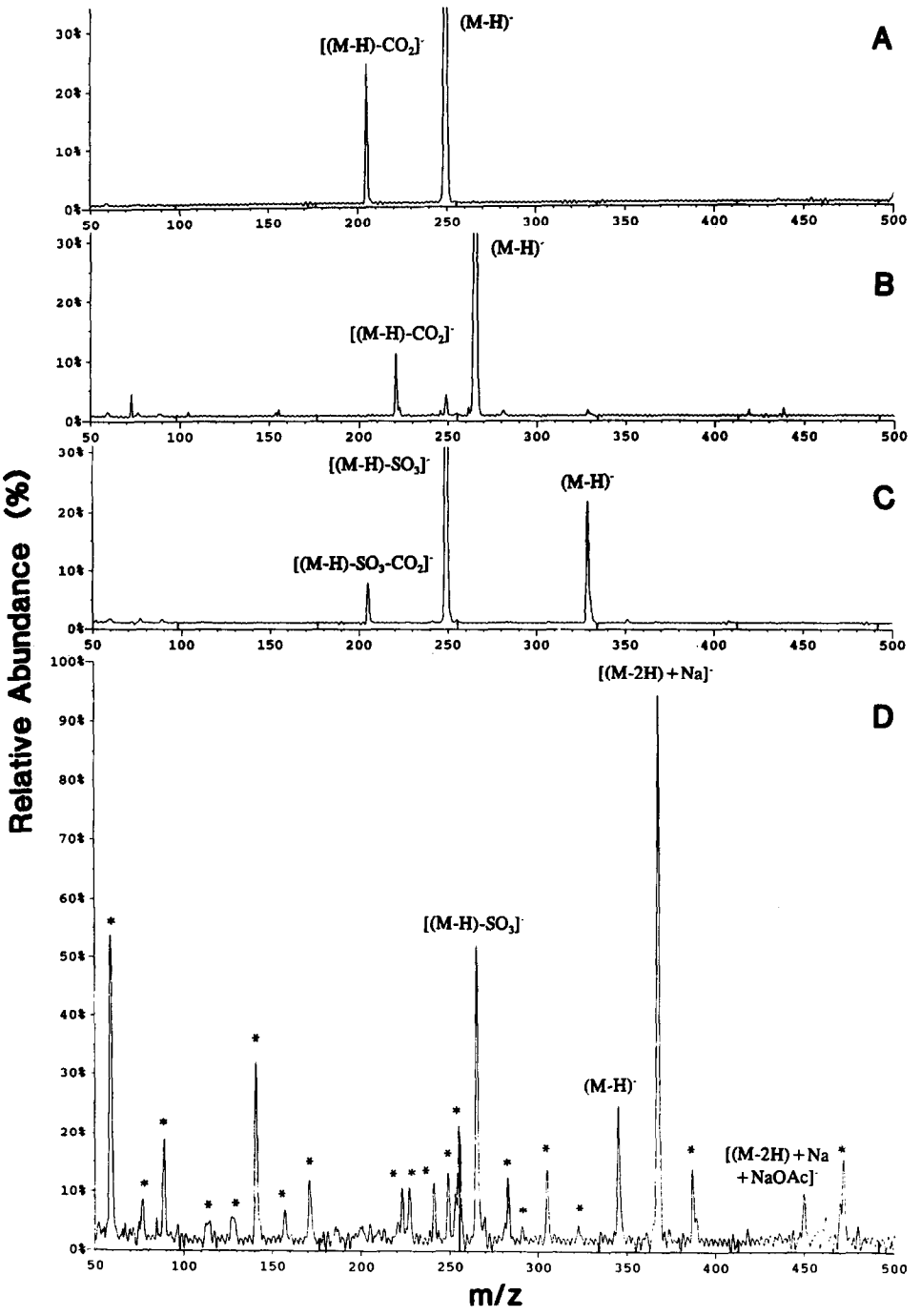

Figure 2
Electrospray ionization mass spectrometry of the samples of diflunisal (DF) (panel A), 3-hydroxy-DF (panel B), DF sulphate (panel C) and 3-hydroxy-DF conjugate (panel D) obtained at a source repeller voltage of −20 V. The peaks marked * in panel D were shown to emanate from the sodium acetate present in the sample.

running the spectrum of sodium acetate itself in methanol–water under the same conditions. It was apparent that many of these peaks occurred in a pattern of 82 *m/z* increments, corresponding to clustering of sodium acetate on ions such as CH_3COO^- at *m/z* 59. It is known that the presence of sodium ions in mass spectrometry samples results in counter-ion formation with the analyte. Solvent clusters and adducts have also been observed in ESI-MS which have been attributed to the de-solvation properties of ESI [10]. Therefore, it is not unusual for a concentrated sodium acetate solution to produce cluster ions with

abundant anionic species, including the acetate anion itself.

Ignoring this chemical background, a peak corresponding to the anion of a monosulphate conjugate of 3-hydroxy-DF, i.e. $[M-H]^-$, was seen at *m/z* 345, with the base peak at *m/z* 367 corresponding to the anion of its sodium salt, i.e. $[(M-2H) + Na]^-$. The peak at *m/z* 265 could be assigned as $[(M-H)-SO_3]^-$, analogous to that seen in the spectrum of DF sulphate. The peak at *m/z* 449 was assigned as a sodium acetate adduct of $[(M-2H) + Na]^-$, i.e. $[(M-2H) + Na + CH_3COONa]^-$. Finally, these assignments were probed by running the ESI-MS spectrum of DF sulphate to which sodium acetate had been added. Prominent anions attributable to the sodium salt $[(M-2H) + Na]^-$ at *m/z* 351 and to a sodium acetate adduct $[(M-2H) + Na + CH_3COONa]^-$ at *m/z* 433 were obtained. Thus, it was concluded that ESI-MS provided definitive evidence that the unknown conjugate was a monosulphate.

The present investigation thus illustrates the utility of ESI-MS in structure determinations of highly polar and potentially unstable drug conjugates, and shows that the 3-hydroxy Phase I metabolite of DF is excreted in the urine of the Gunn rat as a monosulphate conjugate. Which of the 2-hydroxy group (already present in the DF molecule) and 3-hydroxy group (generated *in vivo* by Phase I metabolism) becomes sulphated has not been elucidated. Furthermore, it should be noted that the conjugated form of 3-hydroxy-DF found in human urine need not be the same as that found in rat urine, though this is perhaps likely given the chromatographic evidence [5].

Glucuronosyltransferase-deficient homozygous Gunn rats, unlike Wistar and Sprague–Dawley rats, do not form DF phenolic glucuronide and have diminished capacity to form DF acyl glucuronide [4]. It is now confirmed that, in such rats, about three-quarters of i.v. DF doses are excreted as sulphate conjugates, i.e. about one-half as DF sulphate and about one-quarter as 3-hydroxy-DF sulphate. This result underlines the importance of sulphate conjugation in the *in vivo* disposition of DF, a question which has been the subject of some discussion in the literature [11, 12].

Acknowledgements — This work was supported by project grants from the National Health and Medical Research Council of Australia and the National Institutes of Health of the USA.

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[Received for review 11 February 1994; revised manuscript received 11 April 1994]