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Preparation of Deuterated Diclofenac for Use as an Internal Standard in Quantitative Measurement by Gas Chromatography Negative-ion Chemical Ionization Mass Spectrometry

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A procedure for the preparation of deuterated diclofenac is described. The resulting d_5-diclofenac was used as an internal standard for quantitative measurement of the drug. The benefits of deuteration versus oxygen-18 labeling are demonstrated. Enhanced stability of the isotope label resulted in greater flexibility in sample handling during the work-up steps of plasma samples.

Diclofenac [*o*-(2,6-dichlorophenyl)aminophenylacetic acid, the biologically active ingredient of Voltaren] is a potent, non-steroidal, anti-inflammatory and anti-rheumatic agent.[1–3] Several methods for the quantitative determination of this drug in biological materials have been described, with detection limits in the lower nanogram range.[4,5] In our laboratory, a method for trace level determination of diclofenac in human plasma has been developed, based on gas chromatography/negative-ion chemical ionization mass spectrometry[6] (GC/NICI-MS). The assay was applied to the pharmacokinetics of the drug in human volunteers, following topical epidermal administration of diclofenac gel. The use of the pentafluorobenzyl (PFB) ester derivative permitted detection of the low levels encountered, typically in the subnanogram range. In the above-mentioned study, quantitation was achieved by internal standardization via $^{18}O_2$-diclofenac. This labeled analogue, however, is prone to acid-catalyzed back-exchange of the isotope label, and hence a strict protocol for sample handling, regarding timetables and handling sequence, had to be obeyed in order to achieve reproducible results. It was thus highly desirable to look for alternative possibilities of standardization, still conserving the benefits of stable isotope dilution techniques, but displaying enhanced stability of isotope label. These criteria were largely met by the use of a deuterated analog.

EXPERIMENTAL

Materials

Diclofenac was purchased from Sigma (Vienna, Austria). Pentafluorobenzyl bromide was supplied by Regis (Morton Grove, IL, USA). CD_3OD_3, D_2SO_4, and D_2O were purchased from Fluka (Vienna, Austria). All other solvents and reagents of analytical grade were from Merck (Darmstadt, FRG) GC/MS equipment was from Fisons Instr., Vienna.

Gas chromatography/mass spectrometry

An 8000 gas chromatograph (Fisons Instruments, Vienna, Austria) coupled to a Fisons Trio 1000 mass spectrometer was used. The column was directly connected to the ion source of the MS. The GC was equipped with a DB-5MS fused silica capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness) from Fisons. The splitless Grob injector was kept at 290 °C. Helium was used as a carrier gas. Initial column temperature was 150 °C for 1 min, followed by an increase of 30 °C per min to 310 °C and an isothermal hold until elution was complete. The mass spectrometer transfer line was kept at 325 °C. Negative-ion chemical ionization was performed with methane as a moderating gas at an electron energy of 70 eV and an emission current of 0.135 A.

Derivatization

Pentafluorobenzyl (PFB) esters were formed using PFB bromide in acetonitrile (7% w/w, 50 μL) and diisopropylethylamine (10 μL) for 15 min at room temperature.[6]

Preparation of deuterated diclofenac

Diclofenac (10 mg) was dissolved in 0.5 mL CD_3OD, 600 μL D_2SO_4/D_2O (400/200, v/v) were added, and the mixture heated to 160 °C for 16 h. After cooling, the mixture was made alkali with 3 M sodium hydroxide. Then, after addition of 0.5 mL of ethanol, the mixture was kept at 100 °C for 3 h, cooled to room temperature, re-acidified with 6 M HCl and extracted with ethyl acetate. The ethyl acetate layer was dried over anhydrous sodium sulphate. After removing the solvent under a stream of nitrogen, the residue was dissolved in 100 mL of methanol and isotope incorporation checked by GC/NICI-MS.

Rabbit plasma sample preparation

50 μL of a solution of the internal standard, d_5-diclofenac (10 ng/50 μL methanol) were added to 50 μl/mL of plasma, mixed thoroughly and the mixture diluted with 2 mL of 0.2 M HCl. The sample was applied to a 3 cm^3 Bond Elut C_{18} column, previously conditioned with 4 mL methanol and 9 mL doubly distilled water. The adsorbed sample was washed with 9 mL of water and 9 mL of *n*-hexane and eluted with 2 mL of dichloromethane. The solvent was removed under a stream of nitrogen, the PFB derivatives formed as described above and again dried under nitrogen. The dry residue was dissolved in 400 μL of *n*-hexane and transferred to autosampler vials. An aliquot of 1.5 μL was subjected to GC/NICI-MS analysis.

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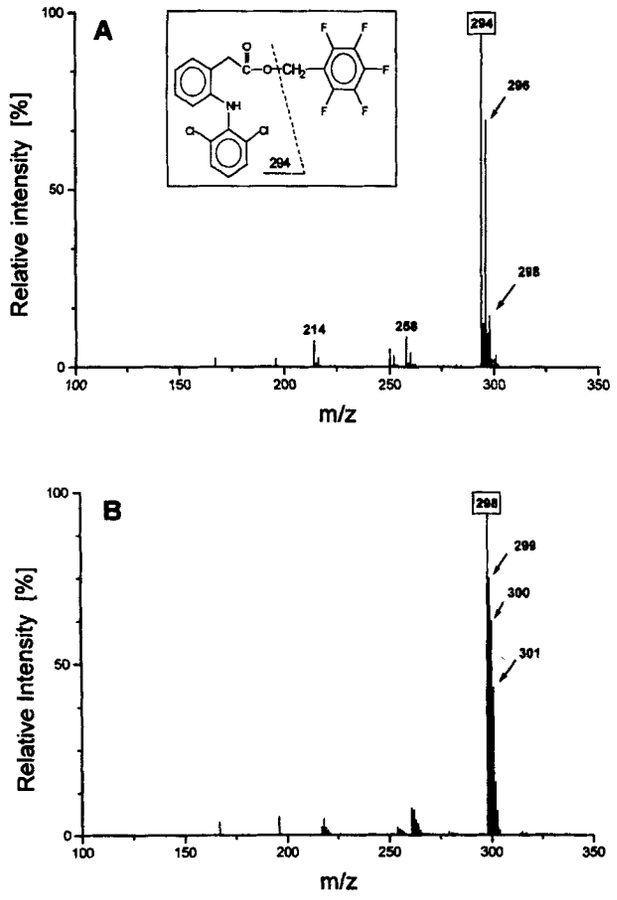

Figure 1. Partial NICI mass spectra (a) unlabeled and (b) deuterated diclofenac PFB ester.

RESULTS AND DISCUSSION

When diclofenac was subjected to a deuterium exchange reaction under acidic conditions, no target compound could be isolated. This was due to intramolecular ring closure to the indolone derivative and dimerization reactions. Saponification of the product with sodium hydroxide, however, led to the deuterated free acid, which could then be utilized as internal standard.

The NICI mass spectra of native and deuterated diclofenac PFB ester are shown in Fig. 1(a) and (b), respectively. No molecular ion is observed. The mass spectrum is dominated by the resonance-stabilized carboxylate anion formed from PFB ester derivatives under NICI conditions at m/z 294 (M − 181). Further fragmentation occurs only to a minor extent, with neutral loss of HCl and of carbon dioxide from the carboxylate anion being the main cleavage routes leading to m/z 258 (M − 181 − HCl), m/z 250 (M − 181 − CO_2), and m/z 214 (M − 181 − CO_2 − HCl). Minor fragment ions arise from the PFB ester moiety at m/z 196 and m/z 167. After the deuteration labeling procedure, the mass of the base peak has shifted to m/z 298, indicating exchange of four protons for deuterium (Fig. 1(b)). Although the abundance of the dichloro isotope intensities at m/z 300 and m/z 302 could confirm this, the high relative intensities at m/z 299 and m/z 301 in fact suggest a maximal incorporation of five deuterium atoms. The relative isotopic abundancies are listed in Table 1. From these data, m/z 298 should serve as a diagnostic ion for quantitative GC/MS of the drug, but due to the high natural isotopic abundance of native diclofenac at m/z 298, m/z 300 was chosen.

Table 1. Relative isotopic abundances of deuterated diclofenac

m/z	Relative abundance [%] deuterated diclofenac[a]
294	0.00
295	0.00
296	0.01
297	0.03
298	100.00
299	70.59
300	62.75
301	41.96
302	14.61
303	7.16

[a] Relative intensities were measured for the carboxylate ion of the PFB ester derivative under GC/NICI-MS conditions and are not corrected for natural isotopic abundances of ^{13}C and ^{37}Cl.

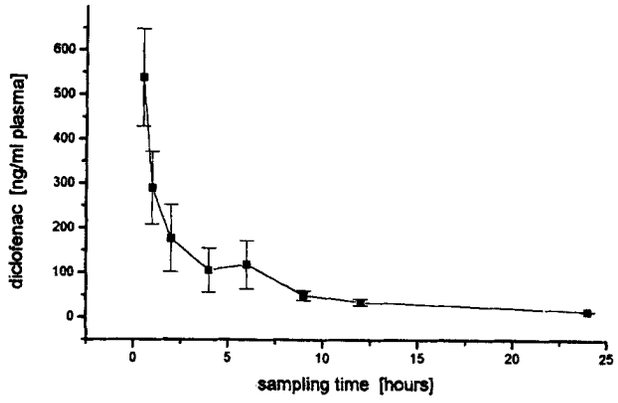

Figure 2. Rabbit plasma concentration versus time after topical epidermal administration of diclofenac. Values are expressed as means ± standard deviation for 5 animals.

Compared to a previously prepared $^{18}O_2$-labeled diclofenac standard, d_5-diclofenac was much more stable to isotope back-exchange. There was no measurable loss of label at alkaline pH or from storage in 0.2 M HCl (the medium used for analytical determinations) for 2 weeks at room temperature. The standard was used to measure diclofenac in rabbit plasma samples after topical epidermal application of the drug (Fig. 2). The standard curve generated was linear between 3 ng/mL plasma and 1200 ng/mL plasma. For the $^{18}O_2$-labeled analog, conditions had to be carefully controlled, especially regarding exposure to low pH values. A two-week storage under the same conditions as outlined above led to 23% back exchange in this case. Therefore, the deuterated standard described here is definitely superior to the previously described $^{19}O_2$-labeled diclofenac and is recommended for quantitative GC/MS determinations of the drug.

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