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Isolation of meclofenamic acid and two metabolites from equine urine — a comparison between horse and man*

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Abstract: Two metabolites of meclofenamic acid have been isolated from equine urine. Both metabolites are found to be monohydroxylated forms of meclofenamic acid by gas chromatography–mass spectrometry after extractive alkylation.

The parent drug and the metabolites are separated by reversed-phase liquid chromatography on a Spherisorb ODS column, using methanol–phosphate buffer eluents and UV detection at 280 nm. The structure of the metabolites is discussed on the basis of LC, TLC and GC–MS data.

Keywords: *Meclofenamic acid; metabolites; horses; gas chromatography–mass spectrometry; liquid chromatography.*

Introduction

Meclofenamic acid, 2-[(2,6-dichloro-3-methylphenyl)amino] benzoic acid belongs to the group of non-steroidal anti-inflammatory drugs. It has been in use for less than 10 years, and is marketed in the form of its sodium salt (Meclomen®) for the treatment of rheumatoid arthritis and similar conditions in humans [1], or in the form of the free acid (Arquel®) for the treatment of various inflammatory diseases of the musculoskeletal system of the horse [2].

Meclofenamic acid was included in a study on the effect of anti-inflammatory drugs on the performance of horses [3]. In a previous paper a method for the determination of meclofenamic acid in equine plasma by reversed-phase liquid chromatography has been described [4]. In the present paper, the structure and chromatographic behaviour of two metabolites of meclofenamic acid found in equine urine are discussed. Both metabolites have previously been identified in human urine [5]; the structures are shown in Fig. 1.

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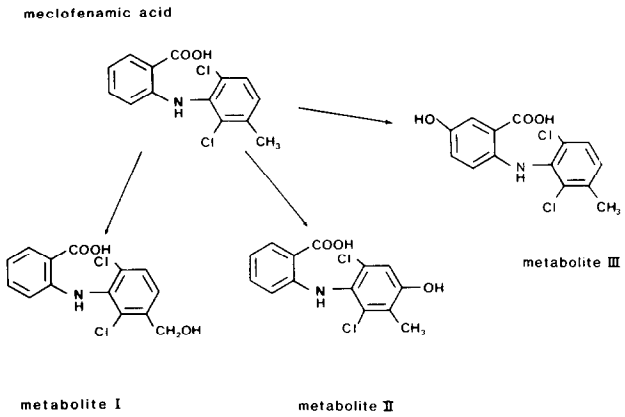

Figure 1
Metabolic pathway for meclofenamic acid in man.

Experimental

Chemicals and reagents

Analytical grade solvents were used without further purification. Fast blue salt B for microscopy (Fluka AG, Buchs, Switzerland) and Folin–Ciocalteus Phenolreagenz (Merck) were used.

Meclofenamic acid, 2-[(2,6-dichloro-3-methylphenyl)amino]benzoic acid, used as a reference compound and metabolite III (2-[(2,6-dichloro-3-methylphenyl)amino]-5-hydroxybenzoic acid) were kindly supplied by Parke, Davis & Co. (Pontypool, UK). Diclofenac sodium, 2-[(2,6-dichlorophenyl)amino]benzene-acetic acid monosodium salt, was a gift from Ciba–Geigy AG (Basle, Switzerland).

Tetrabutylammonium iodide from Eastman–Kodak (Rochester, N.Y., USA) was converted into hydroxide by shaking an aqueous solution with silver oxide. Methyl iodide (Fluka AG, purum quality) was redistilled before used. Pentafluoropropionic anhydride and pentafluoropropanol were obtained from Reagenta (Uppsala, Sweden).

Citrate buffers were used in the batch experiments whereas phosphate buffers were used in the chromatographic experiments. All buffers were prepared with an ionic strength of 0.1.

Apparatus

Thin-layer chromatography (TLC) was carried out on pre-coated TLC-plates, silica gel 60 F-254, 20 × 20 cm (Merck, Darmstadt, FRG).

Liquid chromatography (LC) was performed with a Constametric I pump (Milton Roy Company, Riviera Beach, FL, USA), an automatic sample processor, WISP Model 710 (Waters Associates, Milford, MA, USA) with a LDC SpectroMonitor III ultraviolet variable wavelength detector operated at 280 nm and Shimadzu Chromatopac C-RIB integrator (Shimadzu Corporation, Kyoto, Japan).

The chromatographic columns were constructed from 316 stainless steel (100 mm × 3.0 mm i.d.) equipped with zero volume Swagelok unions and column end fittings and 2 μm stainless steel frits from Altex Scientific. The columns were packed with 10-μm Spherisorb ODS (Phase Separations Ltd, Queensferry, UK).

Gas chromatography–flame ionization detection (GC–FID) was carried out with a Shimadzu GC-7A instrument equipped with glass columns (1.5 m × 2.0 mm i.d.) packed with 3% OV 17 on Gas Chrom Q 100/120 mesh. Nitrogen was used as carrier gas at a flow rate of 55 ml min^{-1}.

The injector was maintained at 250°C and the column oven maintained at 150°C for 2 min prior to programming at a heating rate of 16°C min^{-1} to 260°C and held at this temperature for 8 min. The detector oven was maintained at a temperature 20°–50°C higher than the column oven.

Gas chromatography–mass spectrometry (GC–MS). All experiments were carried out on a Finnigan 4000 quadrupole mass spectrometer (Finnigan Corp., Sunnyvale, CA, USA) equipped with the standard unmodified EI/CI ion source and fitted with the INCOS data system. The GC conditions were as above with the exception that helium was used as carrier gas at a flow rate of 30 ml min^{-1}. Electron-impact (EI) mass spectra were recorded at 70 eV, at an ion chamber temperature of 270°C.

Drug administration

Horse. A 10% m/v solution of sodium meclofenamate monohydrate prepared in polyethylene glycol 400–water (25:75, v/v) was injected into the right jugular vein of the horse. The intravenous bolus corresponded to 2.2 mg kg^{-1} b.w. of meclofenamic acid. Blood and urine samples were collected. The urine sample was spontaneously received 5 h after the administration of sodium meclofenamate.

Man. Meclofenamic acid was given orally to one healthy volunteer, 3.5 mg kg^{-1} b.w. Urine samples were collected before and 4 h after the administration of meclofenamic acid.

Hydrolysis and extraction of urine samples

Horse. The urine, 150 ml, was added to 14 ml of 10 M sodium hydroxide to hydrolyse any conjugated species present. Glucuronide conjugation is a common metabolic pathway for non-steroidal anti-inflammatory drugs [6]. After 20 min at room temperature phosphoric acid was added to pH 3 and the urine was extracted twice with dichloromethane. The combined dichloromethane extracts were washed with citrate buffer of pH 5.2 and extracted with 0.1 M sodium hydroxide. The alkaline aqueous phase was adjusted to pH 5.2 with phosphoric acid and extracted with heptane. Equal volumes of aqueous and organic phases were used throughout the whole extraction procedure. The heptane phase was evaporated and the residue dissolved in methanol.

Man. Urine, 2 ml, was treated as given above. The residue after evaporation of the heptane was dissolved in 200 µl chromatographic eluent and a 7 µl aliquot was injected.

Separation of meclofenamic acid and metabolites

Aliquots of the equine urine extract and samples of meclofenamic acid, diclofenac sodium and metabolite III were applied to TLC plates. The plates were developed using the lower phase of a mixture of chloroform–methanol–citrate buffer pH 4.6 (3:2:1, v/v) or chloroform–methanol–water (3:2:1, v/v). The plates were viewed under UV-radiation at 254 nm and parts of the plates were sprayed with two reagents for phenols, either 0.5% aqueous Fast Blue salt B solution followed by 0.1 M sodium hydroxide solution or

20% aqueous sodium carbonate solution followed by Folin–Ciocalteu reagent diluted 1:3 with water [7, 8]. Spots, separated on the plates and detected by UV-radiation, were scraped off, eluted with methanol and analysed by LC and GC–MS.

Meclofenamic acid and the metabolites were separated by reversed-phase liquid chromatography on a Spherisorb ODS column with methanol–phosphate buffer (45:55, v/v) eluents [4]. The eluent was filtered through a 0.2 μm membrane filter and treated in an ultrasonic bath for some minutes before use. The flow rate was 0.85 ml min^{-1}, giving a pressure of 3500 kPa. The capacity ratios, k', were calculated from $k' = (V_R - V_m)/V_m$, where V_R is the retention of the compound and V_m the void volume of the column determined by injecting sulfathiazole.

Structural determination of metabolites

GC and GC–MS were performed after extractive alkylation of the compounds [9]. Samples, both extracts and compounds isolated by TLC, were mixed with 2.0 ml 0.1 M sodium hydroxide and 50 μl 0.5 M tetrabutylammonium hydroxide. The solution was equilibrated with 5.0 ml 0.5 M methyl iodide or ethyl iodide in dichloromethane for 30 min at 50°C. The organic phase was evaporated and the residue dissolved in methanol–toluene–cyclohexane (4:6:0.5, v/v) before analysis by GC and GC–MS.

GC–MS analyses were also performed after derivatization of the samples with pentafluoropropionic anhydride and pentafluoropropanol (3:1, v/v) for 20 min at 70°C. This solution was taken to dryness and the remaining material was dissolved in 50 μl of chloroform before analysis.

Results and Discussion

Column chromatography

Liquid chromatographic separations of extracts from horse and man urine, respectively, are shown in Fig. 2. In the chromatograms peak 3 is the parent compound whereas peaks 1 and 2 are two metabolites of meclofenamic acid present in urine from both horse and man. Peaks with the same retention as components 1 and 2 were also found in equine plasma. No peaks were obtained that corresponded to metabolite III (see Fig. 1).

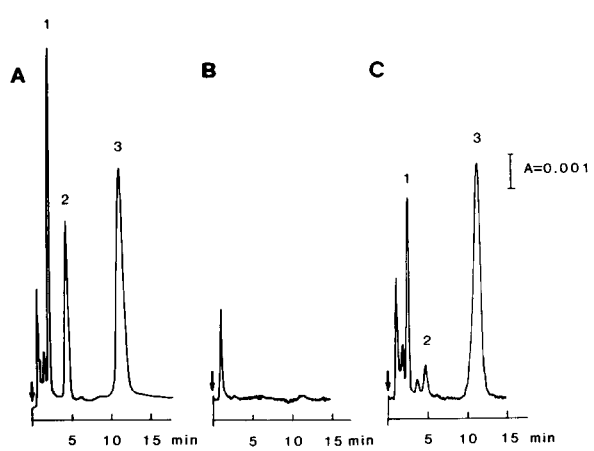

Figure 2
Liquid chromatograms from urine extracts: (A) horse; (B) blank from man; (C) man. Eluent: methanol–phosphate buffer pH 6.1 (45:55, v/v). Key: 1 = metabolite I; 2 = metabolite II; 3 = meclofenamic acid.

The LC retentions of the compounds were found to be influenced by the pH of the phosphate buffer in the eluent, as shown in Fig. 3. The highest retention was obtained at acidic pH, when the compounds are present in unionized form (meclofenamic acid, pk_{HA} = 3.76 [10]). The retention of all compounds decreases between pH 5.5 and 8 when the proportion of the ionized form of the acids in the eluent increases. The slope was, however, smaller than 1, indicating that the acids are retained on the column both in unionized form and as ion-pairs with sodium (cf. [11]). The retention of the metabolites was lower compared to meclofenamic acid and this is in accordance with the addition of one aliphatic or one aromatic hydroxyl group to the parent drug, as found in the mass spectrometric experiments discussed below.

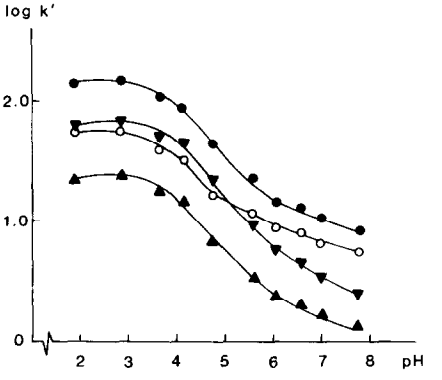

Figure 3
Relationship between retention, as log k', of the analytes and pH of the phosphate buffer in the eluent. Key: ● = meclofenamic acid; ▼ = metabolite II; ○ = diclofenac; ▲ = metabolite I. Eluent: methanol–phosphate buffers (45:55, v/v).

No change in selectivity between meclofenamic acid and the metabolities was observed. A change in retention order was, however, obtained between diclofenac and metabolite II at a pH of about 5. Diclofenac sodium was used as the internal standard in the previously reported determination of meclofenamic acid in equine plasma [4].

Thin-layer and column chromatography

Three narrow bands were observed under UV-radiation after thin-layer chromatographic separation of aliquots of the equine urine extract. The bands, with R_f-values 0.85, 0.74 and 0.70, were separately scraped off, eluted with methanol and analysed by LC.

Meclofenamic acid and metabolite II seem to undergo some degradation or rearrangement processes on the TLC-plate. The spot on the TLC-plate representing meclofenamic acid gave two peaks, with k' 13.9 and 17.4, when analysed by LC. The sum of the peak areas was estimated to be the same as the area under the peak with k' 13.9 obtained when the urine extract was injected directly on the LC column. Similarly metabolite II gave two peaks with k' 2.4 and 4.9 after separation of the urine extract on the TLC-plate and one peak with k' 4.9 on direct injection of the urine extract. With citrate buffer in the developing solvent for TLC, the degree of degradation or rearrangement was lower. The additional peak represented about 15% of the total amount of drug when citric buffer was used in the developing solvent compared with 50% when pure water was used. This stabilization effect of citric acid on TLC plates has earlier been reported for phenylbutazone [12] and was assumed to be due to complexation of Fe^{2+} ions on the plates.

A second part of the TLC plate was sprayed with Fast Blue salt B reagent and a third part was sprayed with Folin–Ciocalteu reagent. The middle of the three bands on the plate turned red–brown or blue, respectively, indicating the presence of a phenolic compound.

The TLC and LC retention data are given in Table 1.

Table 1
Chromatographic retention data for meclofenamic acid, diclofenac and metabolites of meclofenamic acid

Compound	R_f TLC	k' LC
Meclofenamic acid	0.85	13.9
Diclofenac	0.79	8.0
Metabolite II	0.74	4.9
Metabolite I	0.70	1.9
Metabolite III	0.66	4.6

LC: Column: 10 μm Spherisorb ODS; Eluent: methanol–phosphate buffer pH 6.1 (45:55, v/v).
TLC: Silica gel 60 F-254; Eluent: chloroform–methanol–citrate buffer pH 4.6 (3:2:1, v/v), lower phase.

Gas chromatography

A gas chromatographic separation of meclofenamic acid and the two metabolites from equine urine was performed after extractive alkylation of the compounds. A typical chromatogram is given in Fig. 4. The peaks just before and unresolved from peaks 1 and 2, respectively, are normally constituents from equine urine.

Mass spectra of meclofenamic acid and metabolites

In the GC–MS analysis, the first peak in the gas chromatogram has a mass spectrum that corresponds to methyl meclofenamate (Fig. 5). The mass spectra of the two other peaks, shown in Figs 6 and 7, show a peak distribution at the molecular ion which indicate the presence of two chlorine atoms in each of the compounds.

In the case of the spectrum shown in Fig. 6, the molecular ion is increased by 30 mass units compared to methyl meclofenamate. This can be explained by a metabolic hydroxylation of one of the aromatic rings and further methylation during the derivatization. This is in accordance with the findings on the TLC plate, as this mass spectrum corresponds to the phenolic compound found on the TLC plate.

The molecular ion of the metabolite shown in Fig. 7 shows an increase of 16 mass units compared to methyl meclofenamate. This indicates that another metabolic oxidation of the molecule has occurred. The fragmentation pattern of this mass spectrum shows similarities to that of the parent drug. Therefore it is possible that the position for the phase I metabolic process is oxidation of the methyl group as shown in Fig. 1.

Further evidence that this compound was an alcoholic metabolite of meclofenamic acid was that, when isolated by TLC, it did not form a coloured derivative when treated with phenolic reagents.

A comparison of the mass spectrum of meclofenamic acid with another anti-inflammatory drug tolfenamic acid, 2-[(3-chloro-2-methylphenyl)amino]benzoic acid, reveals the same fragmentation pathway for the two acids [13].

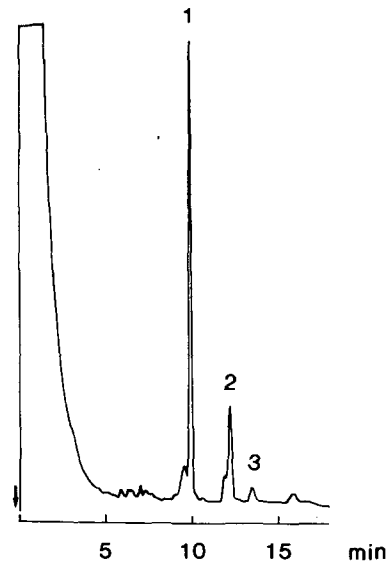

Figure 4
Gas chromatogram from equine urine after extractive alkylation of the compounds with methyl iodide. Key: 1 = meclofenamic acid; 2 = metabolite II; 3 = metabolite I.

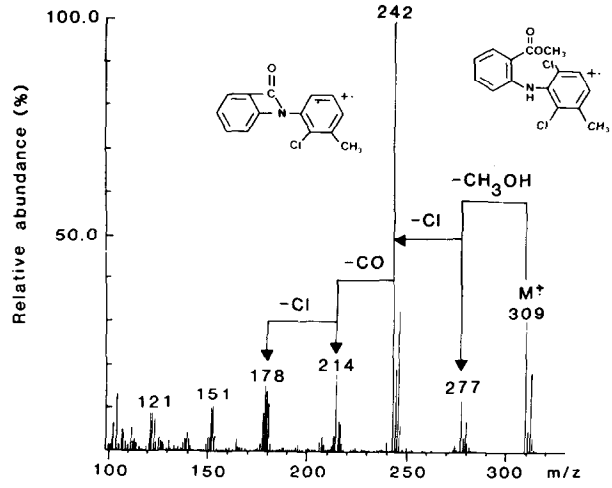

Figure 5
Electron-impact mass spectrum of component 1; which is identical to that of authentic methyl meclofenamate. The fragmentations shown are speculative.

A fragment ion, earlier reported in the mass spectrum for tolfenamic acid [14], containing both rings, also seems to be produced in the case of meclofenamic acid, which has a base peak *m/z* 242 (inset in Fig. 5). An analogous base peak fragment also seems to be formed by the two metabolites with *m/z* 272 and 258, respectively. The corresponding fragmentation pattern was obtained independent of the previous treatment of the compounds, i.e. methylated, ethylated or acylated. This fragment ion strongly supports the first two degradation steps in all three analyses, i.e. loss of CH_3OH and Cl, as indicated in Fig. 5.

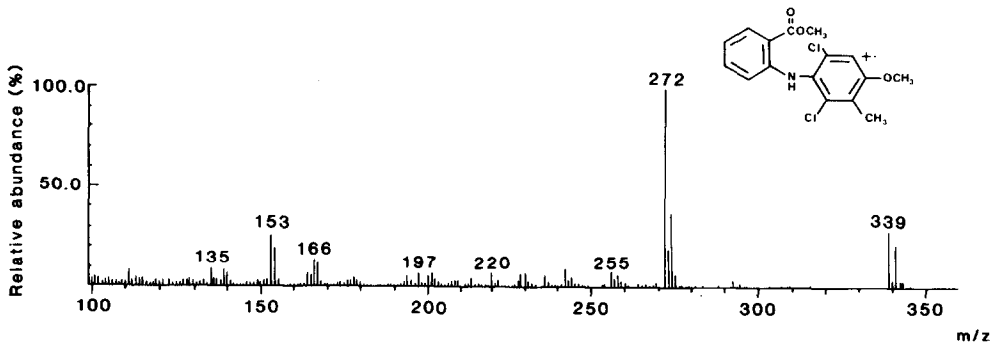

Figure 6
Electron-impact mass spectrum of component 2; dimethylated metabolite II.

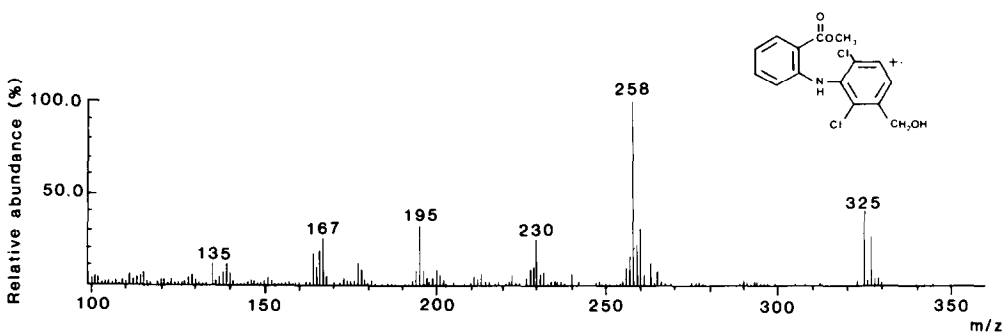

Figure 7
Electron-impact mass spectrum of component 3; methylated metabolite I.

Metabolites of meclofenamic acid found in man

There are many possible positions for a phenolic hydroxyl group in meclofenamic acid molecule, and the correct position cannot be ascertained from the present mass spectrometric data. We believe that the position of the phenolic group is the same as in metabolite II found in man [5], (see Fig. 1). This is supported by the TLC- and LC-data presented.

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