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Determination of Niflumic Acid in Human Urine by Gas Chromatography/Negative Chemical Ionization Mass Spectrometry

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A sensitive method has been developed for the detection and determination of niflumic acid (**NA**) in human urine. Samples were extracted with diethylether. Flunixin (**FN**) was added to the sample prior to extraction as an internal standard. Niflumic acid was converted to its methyl derivative and analyzed by capillary gas chromatography/negative chemical ionization mass spectrometry. Using selected ion monitoring (SIM), the levels of **NA** down to 5 pg/ml could be detected in 5 ml spiked urine sample. Calibration curve was linear over the range of 0.5 ppm-50 ppm. The recovery of niflumic acid from urine at 40 pg/ml was to be 91.7±3.8(n =3) and the coefficient of variation was 4.1%.

Key words : Niflumic acid, Metabolite, GC/MS, Negative chemical Ionization

INTRODUCTION

Niflumic acid is a potent non-steroidal anti-inflammatory drug with analgesic activity, especially used in equine medicine and it is also abused for horse doping. It's pharmacokinetic study (Lan *et al.*, 1973) has shown rapid absorption followed by metabolism, mainly hydroxylation and glucuro conjugation. Several analytical method have been developed, using spectrophotometry, gas chromatography (GC) and high-performance liquid chromatography (HPLC), but none of them have been able to provide the sensitivity level of 10 pg/ml determinations necessary for human studies (Boissier *et al.*, 1971; Cowen *et al.*, 1976; Houin *et al.*, 1981; Cohen *et al.*, 1974; Schumacher *et al.*, 1979; Hoshino *et al.*, 1979; Guechot *et al.*, 1984).

In this report a sensitive and specific procedure for the analysis of niflumic acid in human urine achieving is described. The method involves a O-methylated **NA** and separation on a 12 m cross-linked methylsilicon fused-silica capillary column. A closely related methyl analogue, flunixin (**FN**) was used as an internal standard (Fig. 1). Simultaneously selected ion monitoring(SIM) with the high mass negative ions by methane chemical ionization was then performed. Using this method urine level of **NA** in a volunteer ingested with 250 mg of niflumic acid was determined.

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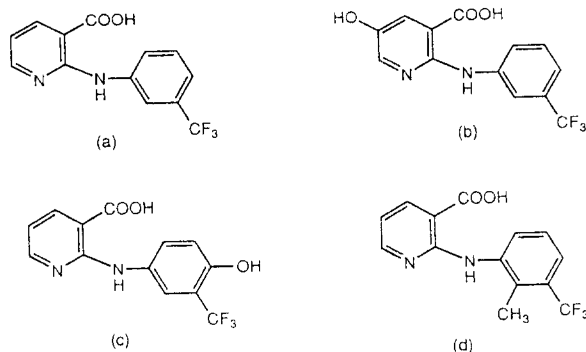

Fig. 1. Molecular structure of **NA** (a), **M1** (b), **M2** (c) and **FN** (d)

MATERIALS AND METHODS

Reagents and chemicals

Methyliodide (CH_3I) was obtained from Sigma Chemical Co. (St. Louis, MO). All the chemicals and reagents were of analytical reagent grade. Niflumic acid was obtained from Dong-A pharm. Co.(Seoul, Korea). Acetone and potassium carbonate were supplied by J. T. Baker Chemical Co. (Phillipsburg, NJ, USA).

Drug administration and sample collection

After oral administration of a capsule containing 250 mg of niflumic acid, urine samples were collected at the following times: 3, 6, 9, 12, 17, 21, 24, 27, 29, 32, 48 and 56 hours for first three days.

Extraction and urine samples

A 5 ml volume of urine was taken with a pipette and transfer to a centrifuge tube. 25 μl of methanolic solution of **FN** 1000 ppm were added to the tube, and the sample was homogeneously mixed on the Vortex-mixer and adjusted pH=2 with 6N-HCl. Samples prepared by the previous procedure were heated at 110°C using heating block for 20 min. After cooling, 5 ml of ether were added to each tube and the tubes were shaken at 130 cycles/min on a horizontal shaker for 20 min. It was centrifuged at 2500 rpm for 5 min and the resulting samples were freezed in refrigerating circulator at -30°C. Ethereal phase was transferred to the other tube and it was evaporated by the vacuum rotary evaporator.

Derivatization of samples (Myung *et al*, 1994)

The residue was dissolved in 180 μl of distilled acetone and the tube was vortex-mixed for a few second. After the addition of 20 μl of methyliodide and 40 mg of potassium carbonate, each sample was placed on an aluminum heating block at 60°C for 30 min. After cooling, the resulting solutions were injected into the GC/MS system using the autosampler under the conditions described below.

Gas chromatography/mass spectrometry

A Hewlett-Packard 5988A Mass Spectrometer coupled to a HP 5890A Gas Chromatograph, HP 59970C MS ChemStation and HP 7673A Autosampler was used. The mass spectrometer was operated in negative chemical ionization mode with a filament current of 300 μA and an electron energy of 230 eV. Methane reagent gas was introduced into the ion source through a transfer line after passing through a trap of activated charcoal and molecular sieve. The ion source was held at 0.6 Torr and at 120°C. Negative ions at m/z 296 and m/z 310 were selected in SIM mode for the determination of **NA** and **FN** (internal standard), respectively. A cross-linked methylsilicone capillary (12 m*0.2 mm I.D., with a film thickness of 0.3 μm) column was installed in the gas chromatograph and inserted directly into the ion source of the mass spectrometer. Helium (0.97 ml/min) was used as a carrier gas. The oven was held at 130°C for 0.5 min, programmed from 130°C to 230°C at 10°C/min, from 230°C to 300°C at 30°C/min, and finally held at 300°C for 3 min. Injector and transfer line temperature were at 290°C. Under these conditions **NA** eluted at 7.14 min and **FN** at 7.79 min.

RESULTS AND DISCUSSION

Mass spectrometry

Reactions of **NA**, **M1**, **M2** and **FN** with methyliodide under the condition described above proceed rapidly to form the [O-CH_3] derivative at the position of carboxylic acid and hydroxyl group. The derivatives appeared stable and gave a good chromatographic peak shape as shown in Fig. 2. The relative sensitivity of positive chemical ionization (PCI), electron impact ionization (EI), and negative chemical ionization (NCI) mode was determined by comparing the abundance of derivatized niflumic acid at the same electron multiplier value when monitoring the ions at m/z 296, 297 and 236 in the NCI, PCI and EI modes, respectively. By this method, the NCI mode was found to be 270 and 900-fold more sensitive than the EI and PCI mode, respectively.

The electron impact mass spectra of the methylated **NA** is shown in Fig. 3. The negative ion mass spectrum of the methylated **NA** is shown in Fig. 4. In contrast to the electron impact spectrum, other ions except molecular ion at m/z 296 were absent in that mass spectrum. Therefore, SIM using negative ion is

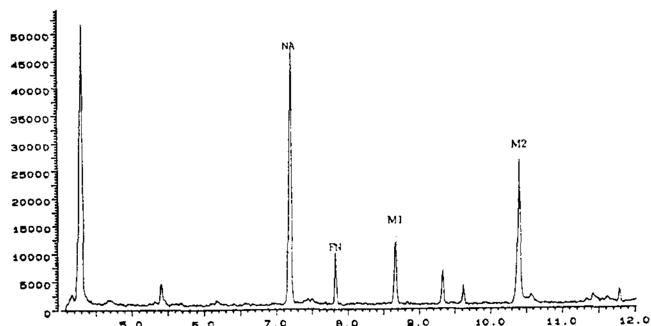

Fig. 2. NCI Mass chromatogram of the methylated **NA**, **M1**, **M2** and **FN**

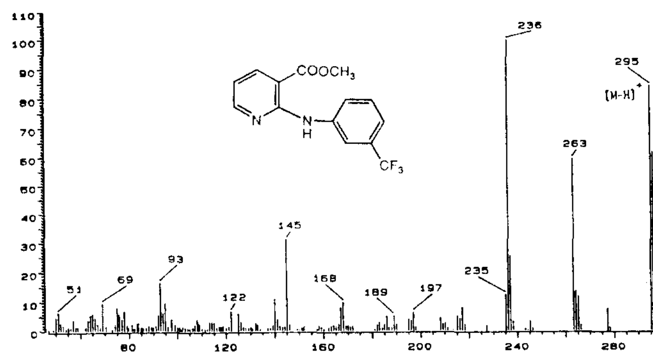

Fig. 3. EI mass spectrum of the methylated **NA**

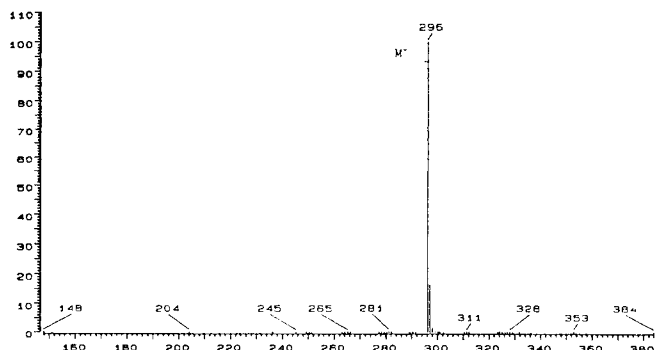

Fig. 4. NCI mass spectrum of the methylated **NA**

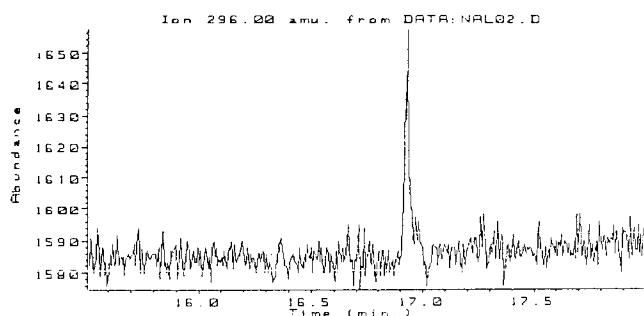

Fig. 5. Selected negative ion chromatogram for m/z 296 of 5pg/ml **NA**

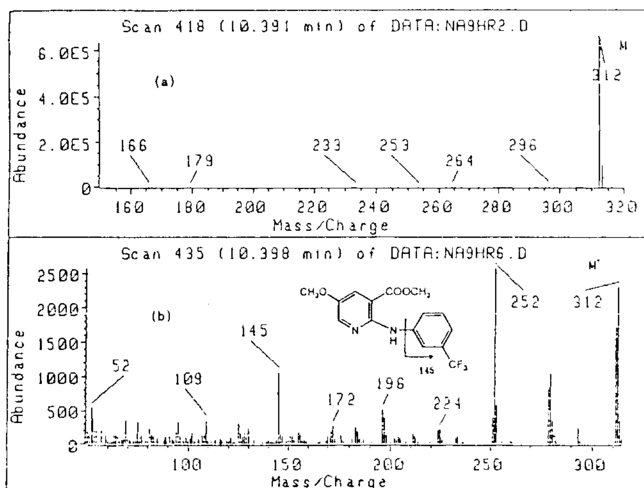

Fig. 6. NCI (a) and EI (b) mass spectra of the methylated **M1**

more effective than that using the positive ions of the electron-impact. Levels of **NA** down to 5 pg/ml (signal-to-noise ratio of 3:1) could be detected in spiked urine sample. Fig. 5 shows the selected ion current profile for 5 pg/ml of **NA**.

Fig. 6 and Fig. 7 are mass spectra of two metabolites (**M1** and **M2**) which are monohydroxylated derivatives of niflumic acid (Cohen *et al.*, 1974). The most abundant ions found in the spectrum of **M1** are 16 amu higher than the corresponding ions found for **NA**. The observed molecular ion peak m/z 312 in the

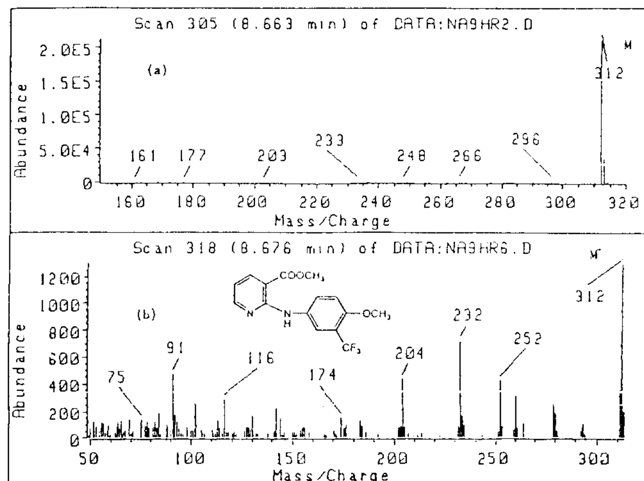

Fig. 7. NCI (a) and EI (b) mass spectra of the methylated **M2**

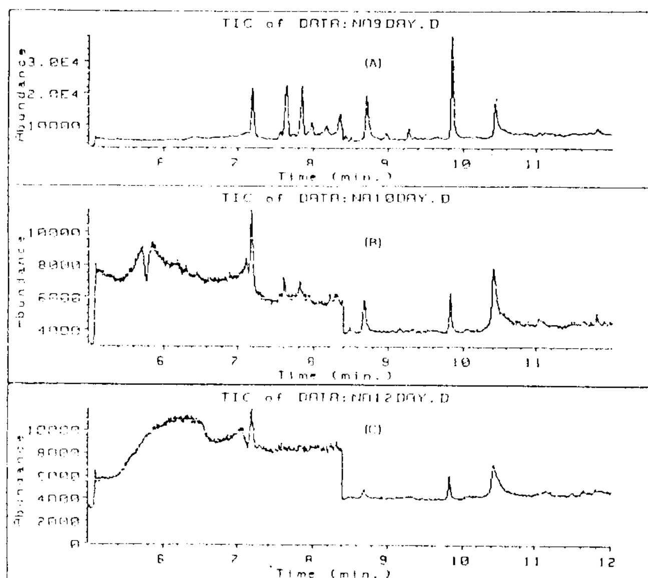

Fig. 8. NCI mass Chromatogram of the methylated **NA** and its metabolites for 9 days (A), 10 days (B) and 12 days (C) after dosing

spectrum of **M2** corresponds to the molecular weight of a methylated monohydroxylated niflumic acid.

NA and its two metabolites were also found 9 days, 10 days and 12 days after dosing (Fig. 8).

Calibration and calculation

Quantification was performed by comparing the integrated peak area for m/z 296 with that for the internal standard monitoring m/z 310. Extraction yields were 91.76±3.77% (n=3), 98.26±5.28% (n=3) and 111.3±5.75% (n=3) for NA at levels of 40 pg/ml, 500 ng/ml, 10 ug/ml, respectively. The calibration curve obtained by assaying spiked urine sample was linear in the measured range 0.5-50 μg/ml. The equation of

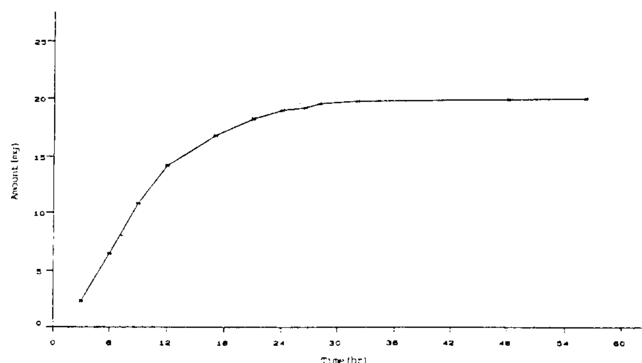

Fig. 9. Cumulative urinary excretion of **NA** following a 250 mg oral dose to a human subject.

the regression line was y=0.894x-0.878 (r=0.999). Cumulative urinary excretion of **NA** following a 250 mg oral-dose to a human subject is shown in Fig. 9. This graph is shown ca. 8% excretion of dose amount.

CONCLUSION

The GC/NCI MS method proved to be both selective and sensitive. Low picogram concentrations of niflumic acid could be quantified in urine.

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