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The determination of non-steroidal anti-inflammatory drugs by GC–MS–MS in equine urine*

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Abstract: Results are given for a more sensitive screening procedure for non-steroidal anti-inflammatory drugs using GC–MS–MS. By monitoring a selected characteristic reaction for each drug very low detection limits are reached even in a difficult biological matrix such as equine urine. Detection down to 5 ng ml^{-1} for ibuprofen, ibufenac, alclofenac, fenoprofen, ketoprofen, naproxen and diclofenac is possible in contrast to the 0.5 $\mu g\ ml^{-1}$ limit for normal GC–MS detection. Examples are given of real positive cases for diclofenac and ibuprofen.

Keywords: *Analgesics; tandem mass spectrometry; solid-phase extraction; doping.*

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are the mainstay of drug therapy for the inflammation and pain associated with various forms of arthritis. However, they are also increasingly being used in equine medicine as a means of doping during competition. Because of the high potency of the modern NSAIDs the necessary dosage is low and the levels in blood and urine are consequently very low. This makes it difficult for the anti-doping laboratories to detect the illegal use of these analgesics.

Current screening procedures are based on liquid–liquid extractions followed by a high-performance liquid chromatographic (HPLC) determination [1]. Unfortunately, most papers deal only with the detection of one particular drug [2] or with screening at high concentrations [3]. More recently, a screening of a limited number of NSAIDs in plasma was published using HPLC [4] with detection limits at 0.2–2.0 $\mu g\ ml^{-1}$.

However, for doping control of horses it is necessary to use urine mostly, for which the published methods are not suitable. Moreover, the detection limits are not good enough as these drugs are being used at sub-therapeutic dosages. Besides that, the HPLC screening will always need a separate confirmation as the retention time is insufficient proof for litigation. Mass spectrometric confirmation therefore is mandatory. Currently the NSAIDs are being analysed by GC–MS after methylation.

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In this paper results of an investigation into more sensitive detection methods for these drugs which maintain the specificity of mass spectrometry is described. The use of tandem mass spectrometry (GC–MS–MS) is shown to realise these objectives [5, 6]. By selecting the (molecular) ion of interest with the first mass filter, all the background ions of the very complex matrix of equine urine can be eliminated. A collision gas is used to fragment the selected ion, and the daughter ions produced are detected by the second mass filter resulting in a very characteristic spectrum called "collisional activated daughter" or CAD spectrum. Not only is the specificity increased by this method but also the sensitivity increases dramatically as will be demonstrated [7, 8].

Experimental

Stock solutions

Stock solutions containing 2 μg ml^{-1} in methanol of the following NSAIDs were made: ibufenac, ibuprofen, fenoprofen, alclofenac, naproxen, ketoprofen and diclofenac. Spiked equine urines were prepared by adding different amounts of each of these stock solutions (or dilutions) to 1 ml of blank urine. Blank equine urine was obtained from the veterinary clinic of the University of Utrecht. In this way spiked urines containing all seven of the above mentioned drugs in concentrations of 100, 50, 25, 10, 5 and 1 ng ml^{-1}, respectively were prepared. Reference mixtures of these drugs were made by adding the appropriate amount into a vial and evaporating off the methanol. Mixtures with 100, 10 and 1 ng, respectively of each of these drugs were prepared. All glassware was silylated before use.

Sample preparation

The extraction was performed using Analytichem Bond-Elut C18 columns (1 ml). The columns were conditioned with 2 × 1 ml of methanol, 2 × 1 ml water and 1 ml buffer (pH 5). To 1 ml of hydrolysed equine urine, 1 ml 1 M of $NaH_2PO_4 \cdot H_2O$ (pH 3.5) was added resulting in a buffer at pH 5. The sample was applied to the column and sucked through under a vacuum.

After washing the columns with 2 × 1 ml water 200 μl *n*-hexane was added to dry the column. The analytes were eluted with 600 μl of dichloromethane. The extract was evaporated under nitrogen at 50°C. The residue was redissolved in 200 μl of acetone. Methylation was performed by adding 20 μl of methyl iodide and some K_2CO_3 crystals and heating the mixture to 60°C for 1 h. After evaporation under nitrogen the derivatized extracts were taken up in 50 μl of acetone of which 1 μl was injected into the GC–MS–MS system.

Mass spectrometry

All measurements were performed by means of a Finnigan MAT TSQ-45 GC–MS–MS system. Chemical ionization was with either methane (0.40 torr) or ammonia (0.25 torr) as reagent gas. Argon was the collision gas at a pressure of 1.5 mtorr. Other conditions were as follows: emission current, 0.20 mA; electron multiplier voltage, 2400 V in MS–MS mode; manifold temperature, 135°C; ion source temperature, 120°C. The selective reaction monitoring was by means of the Multi-Experiment program in the SuperIncos software. GC conditions were as follows: the column used was a J&W DB-5 fused-silica capillary column (25 m × 0.25 mm, i.d.; film thickness, 0.25 μm); injector temperature, 250°C; temperature programme 50°C (1 min) then ramp at 40°

min^{-1} to 280°C (10 min). Injections were by means of the splitless mode with the split/sweep valves closed for 30 s after injection.

Results and Discussion

Because solid-phase extraction is faster and cleaner than liquid–liquid extractions, different column packings were evaluated with ibufenac and ibuprofen as model compounds. Best results were obtained using the C18 columns with recoveries of 60–70%. Before starting the MS–MS experiments two different reagent gases were evaluated using the normal GC–MS full scan mode. Both methane and ammonia appeared to be suitable with comparable responses. The CI–methane spectra all have the $(M - 59)^+$-ion as base peak with abundant $(M + 1)^+$-ions present as well. As expected the $(M + 18)^+$-ions dominate the CI-ammonia spectra. Both gases produce CAD spectra with one or more abundant daughter ions.

These CAD spectra are very characteristic because the background ions from the urine matrix cannot pass the first quadrupole mass filter. The daughter ions generated in the second quadrupole filter by collisions with argon are detected by the third quadrupole mass filter. Because these daughter ions can only arise from the selected parent ion the resulting CAD spectra are highly specific. Although the transmission through more mass filters decreases the absolute signal the detection limits are improved because the noise decreases at an appreciably faster rate resulting in higher signal-to-noise ratios.

The highest sensitivities are obtained by selecting a specific parent–daughter transition. The first mass filter selects the parent ion and the second mass filter the daughter ion of the selected fragmentation. This so-called selective reaction monitoring (SRM) results in very good detection limits.

From the daughter spectra of the NSAIDs it is possible to select a characteristic fragmentation for each of these compounds. For each compound it is possible to describe an experiment in the Finnigan SuperIncos software determining the parent ion, the daughter ion, scan time, cycle time and positive or negative ion detection. These individual experiments are then linked together to a multi-experiment routine which switches these experiments on time basis. More than one reaction can be monitored in one experiment for compounds with similar retention times as in the case of ibuprofen and ibufenac. In this way it is possible to set up a screening procedure for NSAIDs on the known retention times.

Different possible reactions were monitored for these compounds with either methane or ammonia as reagent gas and the selected reactions are given in Table 1. To compare the sensitivities of GC–MS confirmation and the SRM analysis reference mixtures of the

Table 1
Selected reactions used in the multi-experiment for the screening of NSAIDs

	CI–Ammonia	CI–Ammonia	CI–Methane
Ibufenac	224–147	224–207	207–147
Ibuprofen	238–161	238–221	221–161
Alclofenac	258–181	258–241	241–181
Fenoprofen	274–197	274–257	257–197
Naproxen	262–185	262–245	245–185
Ketoprofen	286–209	286–269	269–209
Diclofenac	310–278 + 250	310–278 + 250	310–278 + 250

NSAIDs were measured first. As can be seen in Fig. 1 the absolute detection limit for a full scan GC–MS analysis is 2 ng. Using the MID mode this can be improved to 100–200 pg, while with the SRM technique 2–20 pg of each of the analgesics can be detected. Examples of SRM measurements are given for naproxen and fenoprofen in Fig. 2.

Equine urine is one of the most difficult biological matrixes. The detection limit for these compounds using GC–MS is 0.5 μg ml^{-1}. Of course this can be improved by using MID but background ions hamper the identification of the peaks at the 100 ng ml^{-1} level. Detection below these levels is therefore not possible without further sample clean-up.

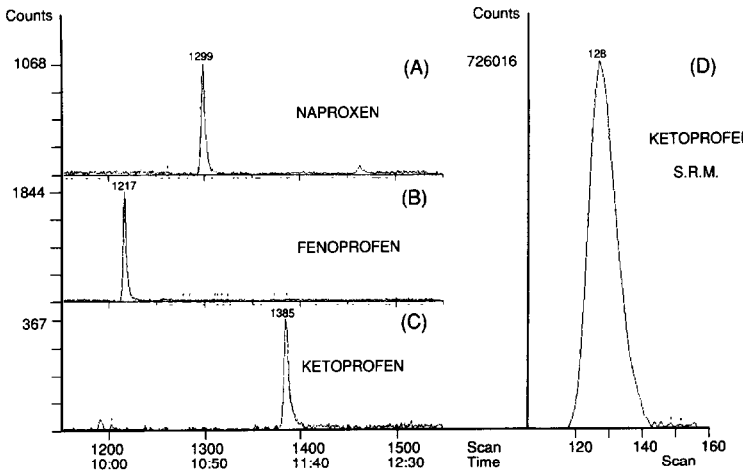

Figure 1
Comparison of full-scan GC–MS data (A, B and C) with GC–MS–MS using the SRM technique (D). For a 2 ng injection of reference compounds the mass chromatograms are given for naproxen (A, *m/z* 262), fenoprofen (B, *m/z* 274) and ketoprofen (C, *m/z* 286). Scan conditions: 200–350 in 0.45 s with ammonia as reagent gas. The signal for ketoprofen after injection of the same mixture using the reaction monitoring of *m/z* 286–269 is given in (D).

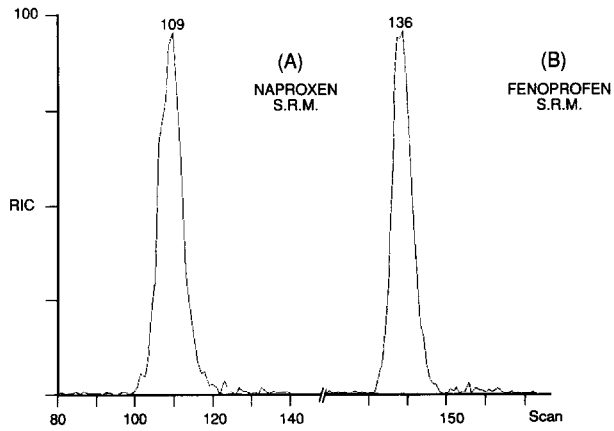

Figure 2
Mass chromatograms obtained after injection of 20 pg of NSAIDs references using SRM. (A) naproxen: parent ion, 262; daughter ion, 185. (B) fenoprofen: parent ion, 274; daughter ion, 197.

With the proposed SRM screening procedure it is possible to measure spiked urines down to at least 5 ng ml^{-1}, and for some compounds detection at the 1 ng ml^{-1} level is still possible.

There were no remarkable differences in sensitivity between the different possible selected reactions although there are definite differences in the abundances of the $(M + 18)^+$-ions in the ammonia–CI spectra compared with the $(M + 1)^+$-ions in the methane–CI spectra. The reaction $(M + 18)^+$ to $(M + 1)^+$ is less specific than the reaction monitoring of the fragmentation to the $(M - 59)^+$-ion. This resulted in better signal-to-noise ratios for ibufenac, alclofenac and ketoprofen. The best chromatograms were obtained using the methane–CI and some examples of the obtained peak intensities at 25 ng ml^{-1} are given in Fig. 3.

As examples of real positive samples Fig. 4(A) shows the result of an equine urine sample containing diclofenac. The method also may be used for human samples as is

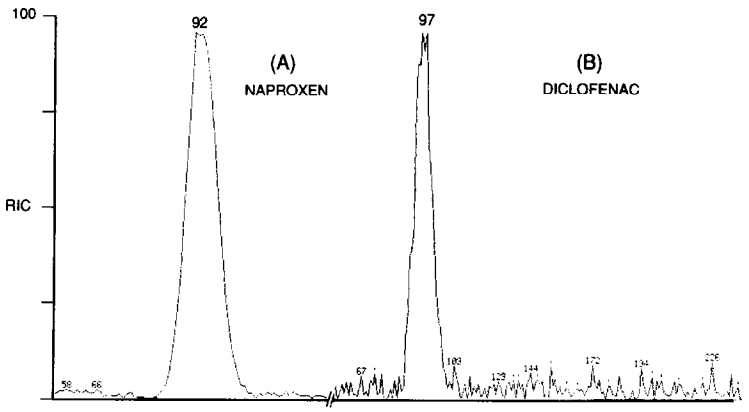

Figure 3
Examples of mass chromatograms obtained using the SPE and SRM detection of equine urine spiked with 25 ng ml^{-1} of the NSAIDs. Methane is used as reagent gas. (A) naproxen: parent ion, 245; daughter ion, 185; (B) diclofenac: parent ion, 310; daughter ions, 278 and 250.

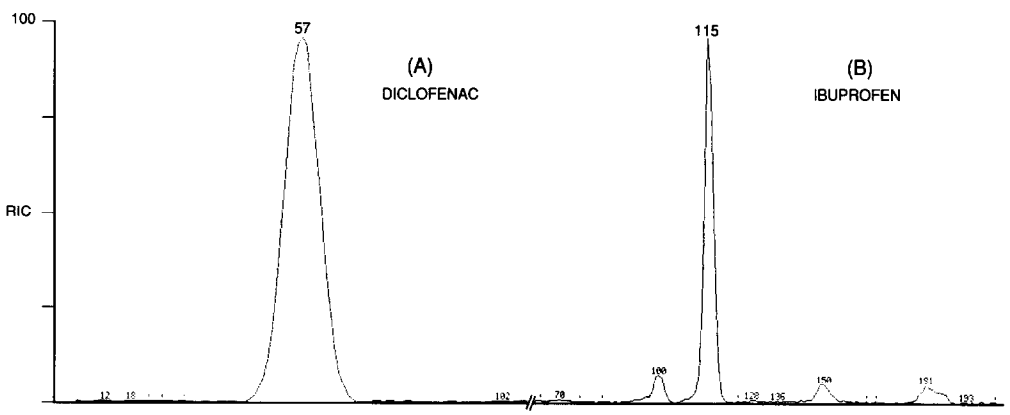

Figure 4
Results for real urine samples. (A) equine urine found positive for diclofenac. (B) human urine taken 50 h after the intake of 200 mg ibuprofen.

shown in Fig. 4(B) for a urine sample taken 50 h after a single intake of 200 mg ibuprofen.

Conclusions

The use of GC–MS–MS in the SRM mode clearly has lowered the possible detection limits for non-steroidal anti-inflammatory drugs considerably. Detection limits down to 5 ng ml^{-1} are now no problem and still improvements can be made.

Even with GC–MS–MS there is a peak at the ibufenac retention time in blank equine urine corresponding to a concentration of ± 3 ng ml^{-1} ibufenac. Although the recoveries of the SPE appear to be sufficient they can be increased considerably. It is the authors' intention to evaluate other more specific extractions to increase the recoveries and to eliminate the contamination present at the ibufenac peak.

Negative ion detection also will be studied further as preliminary results show better detection limits for at least the chloro-containing analgesics. Of course the use of other derivatives could help in this respect.

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