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# Detection and disposition of tolmetin in the horse

P. Van Eenoo, F.T. Delbeke *, K. Roels, K. Baert

*Doping Control Unit, Department Pharmacology, Pharmacy and Toxicology, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium*

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**Abstract**

Non-steroidal anti-inflammatory drugs (NSAIDs) are prohibited by the International Federation of Horse Racing Authorities but are commonly used in veterinary practice. Plasma and urinary concentrations of the NSAID tolmetin were determined by a high-performance liquid chromatographic procedure with UV detection following oral administration of a dose of 1 g to six fasted untrained standard bred mares. With a limit of quantitation (LOQ) of 0.05 μg/ml tolmetin was present in plasma for 9–12 h post-administration. Maximum concentrations of 2.1 ± 0.89 μg/ml were found after 0.7 ± 0.25 h. The elimination half-life was 2 ± 1.25 h. Plasma protein binding at concentrations of 0.25 and 2.5 μg/ml was 92 ± 4.9 and 84 ± 4.2%, respectively. As early as 1 h after dosage, tolmetin could be detected in unhydrolysed urine and remained detectable up to 48 h (LOQ = 0.5 μg/ml). The maximum concentrations occurred 1.8 ± 0.4 h after administration. The percentage of the dose excreted as unchanged tolmetin within 12 h was 58 ± 7.9%. Neither conjugates nor metabolites could be detected under the experimental conditions studied. For confirmatory analysis in doping control, an LC–MS method was developed. Analysis was performed on an ion trap LC–MS system equipped with an ESI probe in positive $MS^2$ mode.


*Keywords:* Tolmetin; Doping control; Horse; Pharmacokinetics

## 1. Introduction

Several human drug preparations have been reported as illicit substances in body fluids of horses at racecourses and at jumping events. A Norwegian study revealed that 43% of all prescriptions in veterinary medicine are human drug preparations. About 19% of those prescriptions is disposed for horses [1].

Results of administration of human drug preparations to horses have been described for indomethacin [2], fencamfamine [3], alclofenac [4], fenoprofen [5], oxprenolol [6] and tiaprofenic acid [7].

Preliminary results on the excretion of tolmetin in the horse were previously presented [8]. The present study focuses on the disposition of orally administered tolmetin, a human non-steroidal anti-inflammatory drug (NSAID), in the horse.

* Corresponding author. Tel.: +32-9-2647347; fax: +32-9-2647497.
*E-mail address:* frans.delbeke@rug.ac.be (F.T. Delbeke).

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Owing to the polar nature of NSAIDs, screening for these substances in doping control of horses is most often done using HPLC–UV [9]. Suspected positive samples need to be confirmed with mass spectrometry [10,11]. Because GC–MS requires a derivatisation step, LC–MS is a less time-consuming option [9]. Hence, an LC–MS method for the confirmation of tolmetin was developed.

## 2. Material and methods

### 2.1. Reagents

Tolmetin was obtained from Janssen-Cilag (Berchem, Belgium). The internal standard (prenazone) was from Boehringer-Ingelheim (Brussels, Belgium). HPLC water was obtained with the MILLI-Q water purification system from Millipore (Brussels, Belgium). HPLC grade acetonitrile was from BDH Laboratory Supplies (Poole, UK). The enzyme preparation β-glucuronidase (EC 3.2.1.31) *Escherichia coli* was obtained from Boehringer Mannheim (Brussels, Belgium). All other chemicals were analytical grade.

### 2.2. Animal experiments

Six untrained standard bred mares were fasted 12 h before oral administration of 1 g tolmetin, i.e. five tablets Tolectin® (Janssen-Cilag, Berchem, Belgium) containing tolmetin sodium salt in an amount equivalent to 200 mg of tolmetin-free acid. The drug was mixed with a small amount of pelleted cubes. The horses were allowed water ad libitum but did not have access to hay or straw during the experiment. Pelleted food was offered 8 h after the administration of tolmetin.

Heparinised blood samples (10 ml) were collected by direct venipuncture of a jugular vein. Sampling times were 0 (control), 0.33, 0.66, 1, 2, 3, 4, 6, 9, 12, 24 and 48 h after the administration. Blood samples were centrifuged at $1700 \times g$ for 10 min and plasma was removed and stored at −20 °C awaiting analysis. A balloon-tipped catheter was placed in the bladder of each mare. The total urine production was collected over a 12 h period following drug administration using the blood collection schedule from the 1 h sample onwards. After this 12 h period, aliquots of urine were taken every 12 h until 48 h after dosing. Urinary volume was measured. A portion was frozen (−20 °C) to await analysis within 1–2 days of collection. The samples were analysed in duplicate and if necessary, dilutions were made with blank urine.

### 2.3. Instrumental analysis

#### 2.3.1. Quantitative analysis (HPLC–UV)

The HPLC system (TSP, Fremont, CA) consisted of a P-4000 pump, model AS 3000 autosampler and a focus forward optical scanning detector set at 310 nm (tolmetin) and 245 nm (internal standard). A $100 \times 3$ mm i.d. reversed-phase column packed with octadecyl silica (5 μm, Nucleosil Chrompack, Antwerpen, Belgium) with an appropriate guard column was used. The loop volume was 20 μl. The mobile phase comprised 37% acetonitrile and 63% water–acetic acid (99:1, v/v). The flow rate was 0.8 ml/min. The column temperature was 35 °C.

#### 2.3.2. Qualitative analysis (LC–MS)

The HPLC instrument was the same as for the qualitative analysis, but the flow of the mobile phase was altered and reduced as required for MS analysis (0.3 μl/min).

LC–MS and LC–$MS^2$ experiments were performed on a LCQ-DECA® ion trap mass spectrometer (Finnigan MAT, San Jose, CA). Full scan ESI and APCI mass spectra in positive and negative mode were obtained. The different parameters (electrospray voltage, tube lens offset, capillary voltage, auxiliary and sheet gas flow, first and secondary octapole offset, intra-octapole lens) were optimised for tolmetin (precursor ion: $m/z$ 258.3) using a built-in syringe pump. A concentration of 10 μg/ml was continuously infused at a flow of 10 μl/min in the eluent flow (37% acetonitrile and 63% water–acetic acid (99:1, v/v), 0.3 ml/min). The tune file was used for further MS analysis. For $MS^2$ experiments, $m/z$ 258.3 and 321.2 were chosen as precursor ions in the positive ESI mode for tolmetin and internal standard, respectively. The relative collision energy for

both compounds was set at 29.5 and 30%, respectively.

### 2.4. Analytical procedures

#### 2.4.1. Plasma

Samples were prepared in duplicate by pipetting 2.0 ml into a 15 ml screw-capped tube. Subsequently, 50 μl of internal standard (50 μg/ml), 250 μl of 1 M HCl and 5 ml of diethylether were added. Extraction was performed by rolling during 20 min. After centrifugation ($1100 \times g$, 5 min), the organic layer was transferred to a clean screw-capped tube and evaporated under nitrogen at 40 °C. After evaporation of the organic extracts, the extraction step was repeated and the combined residue was redissolved in 200 μl of the mobile phase, briefly vortexed and 20 μl was injected.

#### 2.4.2. Urine without hydrolysis

Duplicate samples (1 ml) were acidified with 250 μl of 1 M HCl and 50 μl internal standard (50 μg/ml) added. Extraction was performed by rolling with 5 ml diethylether (20 min). After centrifugation (5 min, $1100 \times g$), the organic layer was removed into another tube and washed by vortexing (2 min) with 1 ml of a freshly prepared $NaHCO_3$ solution (1%). After centrifugation (2 min, $1100 \times g$), diethylether layer was separated and subsequently treated as described for plasma.

#### 2.4.3. Enzymatic hydrolysis of urine

Urine samples (1 ml) were buffered with 100 μl of 0.2 M phosphate buffer, pH 7, and 50 μl β-glucuronidase added. Hydrolysis was performed at 56 °C for 3 h. After cooling, hydrolysate was extracted as described above.

#### 2.4.4. Alkaline hydrolysis

Urine (1 ml) was treated with 50 μl of 1 M NaOH for 30 min at room temperature. The hydrolysate was then acidified with 300 μl of 1 M HCl, the internal standard added and subsequently extracted.

#### 2.4.5. Quantification

Standard curves were obtained by subjecting spiked plasma and urine to the appropriate extraction method in triplicate at each concentration. The prepared tolmetin concentrations were 0.05, 0.10, 0.25, 0.5, 1, 2.5 and 5 μg/ml for plasma and 0.5, 1, 2.5, 5, 7.5 and 10 μg/ml for urine. The precision of the assay was measured at three different concentrations 2.5, 0.50, and 0.10 μg/ml in plasma and 7.5, 5.0 and 0.5 μg/ml in urine.

Recovery in plasma and urine was tested at the same concentrations by analysing samples spiked with tolmetin prior to extraction and after extraction.

Selectivity and specificity were tested by analysing 10 blank urine and plasma samples and analysing 20 other NSAIDs (Table 1).

#### 2.4.6. Plasma protein binding in vitro

The binding of tolmetin to horse plasma protein was determined by ultrafiltration. The plasma was obtained from a fresh-pooled heparinised blood sample. Solutions of tolmetin (2.5 and 0.25 μg/ml) were prepared with plasma and allowed to equilibrate for 30 min. Quintuplicate samples (1 ml) were centrifuged in the micropartition apparatus

Table 1
Retention time (RT) and relative retention time (RRT) of NSAIDs

| Substance | RT (min) | RRT |
|---|---|---|
| Tolmetin | 3.48 | 0.26 |
| Piroxicam | 4.36 | 0.33 |
| Alclofenac | 6.21 | 0.47 |
| Flunixin | 6.30 | 0.48 |
| Tiaprofenic acid | 6.37 | 0.48 |
| Ketoprofen | 6.86 | 0.52 |
| Naproxen | 7.18 | 0.55 |
| Meloxicam | 7.84 | 0.60 |
| Nimesulide | 9.50 | 0.72 |
| Fenoprofen | 10.19 | 0.78 |
| Flurbiprofen | 11.07 | 0.84 |
| Carprofen | 11.42 | 0.87 |
| Diclofenac | 12.27 | 0.93 |
| Ibuprofen | 12.44 | 0.94 |
| Indomethacin | 12.55 | 0.95 |
| Prenazone | 13.14 | 1.00 |
| Flufenaminic acid | 15.58 | 1.19 |
| Vedaprofen | 18.89 | 1.44 |

(model MPS-I, Amicon) at $1500 \times g$ for 20 min. YMT membrane disks with a molecular weight cut-off (MW = 776) were used in combination with the MPS micropartition device. Tolmetin concentrations were measured by the HPLC–UV method.

### 2.5. Pharmacokinetic analysis

Non-compartmental pharmacokinetic parameters [12] including elimination constant ($k_e$) and elimination half-life ($t_{1/2}$) were determined using appropriate equations. The time at which peak concentration occurred ($t_{max}$) and the peak concentration ($C_{max}$) were estimated from the experimental data points. The percentage of the dose eliminated after 12 h was determined from the urinary data.

### 2.6. Statistical analysis

Differences between values were compared using Student's $t$-test for paired data, and were considered to be significant at the 95% probability level.

## 3. Results and discussion

### 3.1. HPLC–UV method

Under the described HPLC conditions, the peaks corresponding to tolmetin and the internal standard were well-resolved, sharp and symmetrical. No endogenous compounds co-eluted with these peaks. The retention time for tolmetin and internal standard was 3.5 and 13.2 min, respectively (Fig. 1). For both urine and plasma assays, no interferences were found with other NSAIDs mainly used in veterinary practice.

Linear calibration graphs ($r^2 \geq 0.995$) were obtained in the 0.05–5 μg/ml range for plasma and in the 0.5–10 μg/ml range for urine. The limit of quantitation (LOQ) was defined as the lowest concentration at which acceptable accuracy and repeatability was obtained. The limit of quantification in equine plasma and urine was 0.05 and 0.5 μg/ml, respectively. The accuracy and repeatability of the method was tested at three levels for each matrix and is shown in Table 2. Acceptable tolerances (%) for repeatability can be calculated from $RSD_{max} = 2^{(1-0.5 \log C)}$. The maximum allowed tolerance for repeatability is $\frac{2}{3}RSD_{max}$ [13]. Tolerances for accuracy can be expressed as mean error [14]. The accuracy is also shown in Table 2 and is within the acceptable deviation of 15% [14].

Extraction recovery from tolmetin in urine and plasma is given in Table 3. No interferences were detected in blank urine or plasma samples at the retention time of the internal standard and tolmetin. As shown in Table 1, the retention time of other NSAIDs analysed with the same method differed from the retention time of tolmetin and the internal standard.

### 3.2. Plasma protein binding in vitro

Tolmetin plasma protein binding in the horse was examined at two different plasma concentrations, i.e. 0.25 and 2.5 μg/ml. Studies in men have shown that tolmetin is highly bound (>99%) to plasma proteins [15,16]. From this work, it appears that plasma protein binding in the horse is somewhat lower. Values of, respectively, $92 \pm 4.9$ and $84 \pm 4.2$% at 0.25 and 2.5 μg/ml were found.

### 3.3. Pharmacokinetics

A typical semi-logarithmical plasma concentration–time curve is shown in Fig. 2. The absorption takes place in the first hour. The calculated plasma half-life of elimination for tolmetin in the horse is $120 \pm 75$ min; this is comparable to the plasma half-life of 2 h in human [17].

Other divergent plasma half-lives of 50–60 min [15,18] and even up to approximately 5 h [19,20] were also measured in human. As shown in Fig. 2, maximum concentrations ($2.1 \pm 0.89$ μg/ml) were reached $0.7 \pm 0.25$ h post-administration. Tolmetin was detectable in plasma for 9–12 h post-administration.

In men, the principal elimination routes of tolmetin are oxidation of the methyl group on

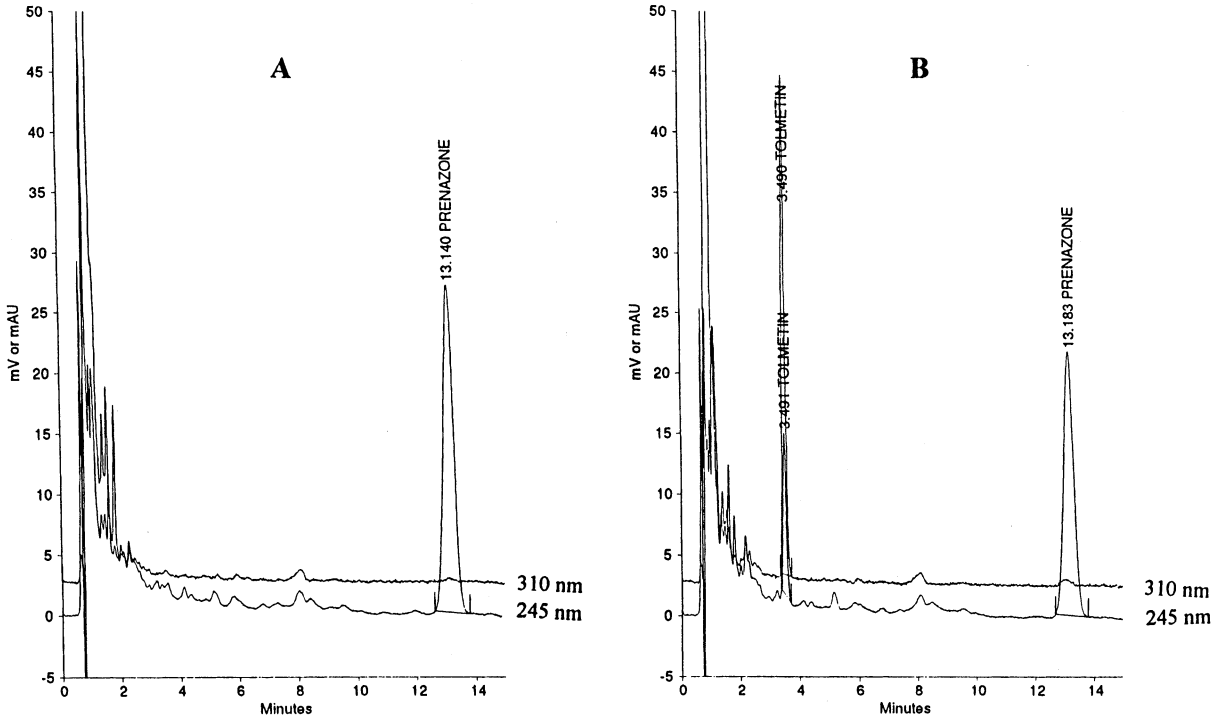


Fig. 1. HPLC chromatograms (245 and 310 nm) of (A) blank plasma extract and (B) plasma extract 6 h after the oral administration of 1 g tolmetin to horse 3.

the phenyl moiety and conjugation with glucuronic acid, leading to 1-β tolmetin glucuronide [20]. Therefore, detection of tolmetin and/or its metabolites in the horse might be improved by enzymatic or alkaline hydrolysis of the urine [2,5]. However, no significant increase in equine urinary concentrations of tolmetin was measured after enzymatic or alkaline hydrolysis.

As shown in Fig. 3, nearly 60% of the administered drug is excreted unchanged in the first 12 h. This percentage is higher than the percentages found after the administration of other human NSAIDs to the horse, including indomethacin [2],

Table 2
Accuracy and repeatability (RSD) of the method for plasma and urine at three levels and the maximum allowable deviation for repeatability ($\frac{2}{3}RSD_{max}$)

| Concentration (μg/ml) | Plasma | | | Urine | | |
|---|---|---|---|---|---|---|
| | 0.1 | 0.5 | 2.5 | 0.5 | 5.0 | 7.5 |
| RSD (%) | 2.7 | 3.6 | 0.6 | 3.9 | 5.4 | 3.0 |
| Accuracy (%) | −11.2 | −1.7 | 2.3 | 1.6 | 1.6 | 1.3 |
| $\frac{2}{3}RSD_{max}$ (%) | 15.1 | 11.8 | 9.3 | 11.8 | 8.4 | 7.9 |

Table 3
Extraction recoveries for tolmetin in plasma and urine at three levels

| Concentration (μg/ml) | Plasma | | | Urine | | |
|---|---|---|---|---|---|---|
| | 0.5 | 1.0 | 2.5 | 1.0 | 2.0 | 5.0 |
| Recovery (%) | 97.3±6.8 | 97.3±6.8 | 97.3±6.8 | 96.8±5.0 | 100.1±4.2 | 97.8±1.9 |

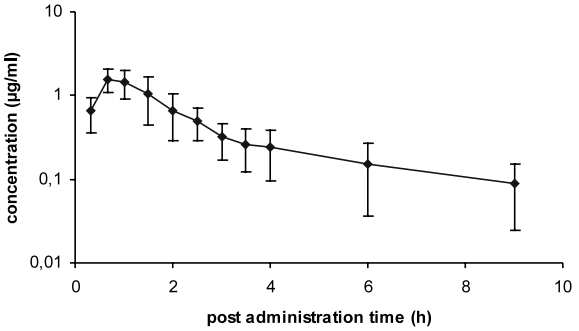


Fig. 2. Plasma concentration time curve (mean and standard deviation of all horses) after oral administration of 1 g tolmetin.

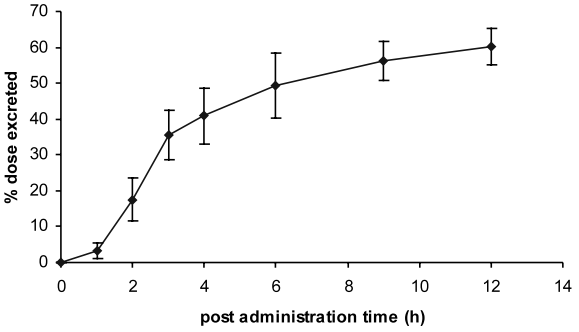


Fig. 3. Urinary cumulative excretion profile of unchanged drug after oral administration of tolmetin (mean and S.D. of all horses).

alclofenac [4], fenoprofen [5] and tiaprofenic acid [7] where values of, respectively, 12, 1, 13 and 38% were noticed. A maximum urinary concentration up to 1673 μg/ml was reached after 1.8±0.4 h post-administration. No metabolites of tolmetin in the horse have been detected. Using the described HPLC method, tolmetin was detectable in urine for 24–48 h post-administration.

### 3.4. LC–MS method

For doping control purposes, a positive screening result needs to be confirmed by mass spectrometry in order to unequivocally identify the presence of a prohibited substance [11]. The polar nature of NSAIDs makes these substances particularly suitable for LC–MS.

Via direct infusion, the full scan ESI and APCI mass spectra of tolmetin in positive and negative mode were determined. Best sensitivity was obtained in the positive ESI mode. The full scan mass spectrum of tolmetin in positive ESI mode consisted mainly of the ion $[M+1]$. For identification of a prohibited substance in doping control, at least three diagnostic ions should be present in a mass spectrum [21,22] if only one kind of mass spectrometric technique is used. Therefore, $MS^2$ conditions were determined and the collision energies were determined in such a way that the precursor ion would still be present in the product spectrum with a relative intensity of approximately 30%, as shown in Fig. 4.

## 4. Conclusions

A quantitative HPLC–UV method was developed and validated for the detection of tolmetin in horse urine and plasma. The LOQ in equine plasma and urine were 0.05 and 0.5 μg/ml, respectively. The calculated plasma half-life was 120±75 min. Plasma protein binding was 92±4.9% at 0.25 μg/ml and 84±4.2% at 2.5 μg/ml.

Using the described HPLC–UV method, tolmetin was detectable for 9–12 and 24–48 h post-administration in plasma and urine, respectively. Up to 60% of tolmetin was excreted unchanged during the first 12 h post-administration.

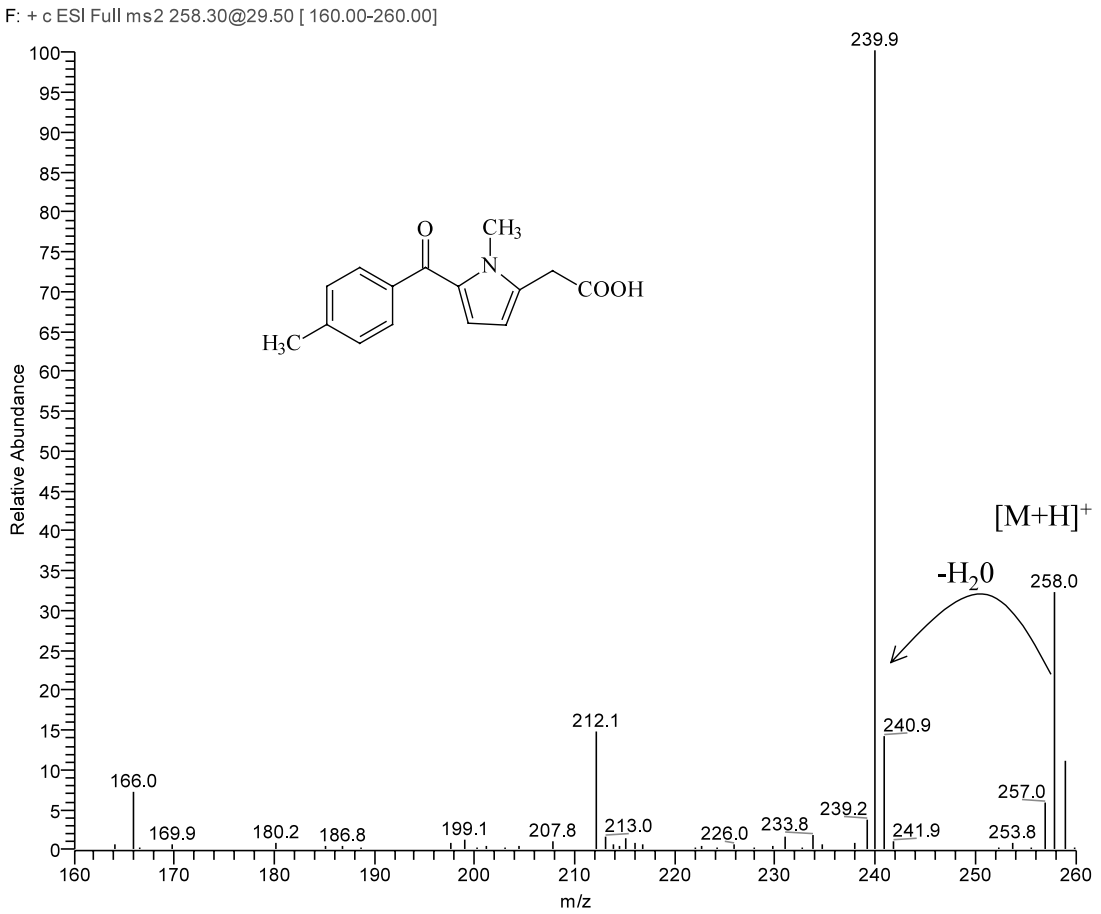


Fig. 4. Product spectrum ($m/z$ 258.3) of tolmetin in a urine sample spiked at 5 μg/ml.

Although the HPLC–UV method was used for quantitative purposes in this work, it can also be used as a screening method for tolmetin in doping control. For confirmatory purposes, suitable LC–$MS^2$ conditions were determined.

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