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Detection of non-steroidal anti-inflammatory drugs in equine plasma and urine by gas chromatography–mass spectrometry

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Abstract

A gas chromatographic–mass spectrometric (GC–MS) procedure for the detection of seventeen non-steroidal anti-inflammatory drugs (NSAIDs) in equine plasma and urine samples is described. The extraction of the compounds from the biological matrix was performed at acidic pH (2–3) with diethyl ether. Ethereal extracts were washed with a saturated solution of sodium hydrogencarbonate (urine) or treated with a solid mixture of sodium carbonate and sodium hydrogencarbonate (plasma). The ethereal extracts were dried and derivatized by incubation at 60°C with methyl iodide in acetone in the presence of solid potassium carbonate. Mono- or bismethyl derivatives of the NSAIDs were obtained. After derivatization kinetic studies, 90 min was the incubation time finally chosen for screening purposes for adequate methylation of all the compounds under study. For individual confirmation analyses, shorter incubation times can be used. The chromatographic analysis of the derivatives was accomplished by GC–MS with a run time of 13 min. In general, extraction recoveries ranged from 23.3 to 100% in plasma and from 37.5 to 83.8% in urine samples. Detection limits from less than 5 to 25 ng/ml were obtained for both plasma and urine samples using selected-ion monitoring. The procedure was applied to the screening and confirmation of NSAIDs in routine doping control of equine samples.

1. Introduction

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is a common practice in equine medicine for numerous musculo-skeletal inflammatory conditions. These drugs are also misused in competing horses to mask signs of inflammation and pain. For this reason, NSAIDs are considered prohibited substances in competition by different veterinary regulations and they constitute the main doping agents found in horse samples [1]. Hence, sensitive screening procedures to detect their presence in equine plasma or urine samples are required in doping control.

Most of the reported procedures for NSAIDs are optimized to detect one particular drug and its metabolites in biological fluids using gas chromatography–mass spectrometry (GC–MS) [2–7] or high-performance liquid chromatography (HPLC) [8–20]. A few screening procedures have also been reported. Donike et al. [21] described a GC–MS comprehensive screening procedure for NSAIDs using trimethylsilylation. Owen et al. [22] described a screening procedure for a limited number of NSAIDs in plasma using HPLC with detection limits from 0.05 to 2 μg/ml. De Jong et al. [23] reported a sensitive method to determine five NSAIDs in equine urine using GC with tandem MS.

In this paper, a method for the simultaneous

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detection of seventeen NSAIDs in horse plasma and urine is described. The procedure is based on a liquid–liquid extraction under acidic conditions, methylation and GC–MS analysis.

2. Experimental

2.1. Chemicals and reagents

The compounds were supplied by the following pharmaceutical manufacturers: flurbiprofen and ibuprofen (Laboratorios Liade, Madrid, Spain); ibuproxam (Laboratorios Novag, Barcelona, Spain); indomethacin (Laboratorios Uriach, Barcelona, Spain); and phenylbutazone (Laboratorios Miquel, Barcelona, Spain). The other compounds were supplied by Sigma Química (Alcobendas, Madrid, Spain). Flufenamic acid (Sigma Química) was used as an internal standard. 7-Ethyltheophylline was used as an internal or external standard in some experiments and was synthesised from theophylline (Sigma Química) and ethyl iodide (Merck, Darmstadt, Germany) in alkaline medium. Methyl iodide was purchased from Merck. The water used was of Millio-Q purity (Millipore Ibérica, Barcelona, Spain). Diethyl ether was of analytical-reagent grade and distilled before use. Other reagents were of analytical-reagent grade.

2.2. Standard solutions

Stock standard solutions were prepared by dissolving the compounds in methanol (1 mg/ml). Working standard solutions were prepared by 1:10 and 1:100 dilution of the stock standard solutions with methanol (100 and 10 μg/ml, respectively). The concentration was checked by UV spectrophotometry using the solution of 10 μg/ml. All solutions were stored at −20°C.

2.3. Gas chromatography–mass spectrometry

GC–MS was performed with a Model 5890A Series II gas chromatograph coupled to a Model 5970 electron impact mass-selective detector via a direct capillary interface (Hewlett-Packard, Palo Alto, CA, USA). An HP 7673A automatic sampler was used (Hewlett-Packard). The instrument was linked to an HP 9000/345 workstation (HP-UX) (Hewlett-Packard).

The column was a 25 m × 0.2 mm I.D. fused-silica cross-linked methylsilicone with a 0.11-μm film thickness (Hewlett-Packard). Helium was used as the carrier gas at 0.65 ml/min. The injection port and detector temperatures were 280°C. The oven temperature was increased from 100 to 200°C at 25°C/min and then to 300°C at 15°C/min, with a final hold time of 2.3 min; the total run time was 13 min. The injection volume was 2 μl and a splitting ratio of 10:1 was used.

Screening analyses were performed in the selected-ion monitoring (SIM) acquisition mode, monitoring three characteristic ions for each compound (see Table 1). In some experiments and for confirmation purposes, the scan acquisition mode (m/z 70–600) was used.

2.4. Sample extraction

Urine

A 2-ml urine sample was pipetted into a centrifuge tube and 50 μl of internal standard working solution (flufenamic acid, 10 μg/ml) were added. The urine was adjusted to pH 2–3 with 1 *M* hydrochloric acid and extracted with 8 ml of diethyl ether. After mixing (rocking at 40 movements/min for 20 min) and centrifugation (10 min at 1500 *g*), the aqueous phase was discarded. A 1-ml volume of a saturated solution of sodium hydrogencarbonate was added to the organic layer; after vortex mixing (20 s) and centrifugation (5 min at 1500 *g*), the organic layer was separated and evaporated to dryness under a nitrogen stream at 40°C.

Plasma

A 2-ml plasma sample was pipetted into a centrifuge tube and 50 μl of internal standard working solution (flufenamic acid, 10 μg/ml) were added. The plasma was adjusted to pH 2–3 with 1 *M* hydrochloric acid and 2 ml of water were added. After vortex mixing (10 s), the sample was extracted twice with 6 ml of diethyl

ether. After mixing (rocking at 40 movements/min for 20 min) and centrifugation (10 min at 2000 *g*), the organic layers were combined and treated with 200 mg of sodium carbonate–sodium hydrogencarbonate buffer (2:1). After vortex mixing for 1–2 min and centrifugation (5 min at 2000 *g*), the organic layer was separated and evaporated to dryness under a stream of nitrogen at 40°C.

2.4. Derivatization

The dry residue was dissolved in 150 μl of acetone. Methylation was performed by adding 50 μl of methyl iodide and 50 mg of anhydrous potassium carbonate and heating the mixture in a dry bath at 60°C. After cooling at room temperature, 2 μl of derivatization mixture were analysed by GC–MS.

The derivatization kinetics of some NSAIDs were studied. A 10-μg amount of the compound and 10 μg of 7-ethyltheophylline (internal standard) were evaporated to dryness. After adding the derivatization reagents, the mixture was incubated at 60°C for 0, 30, 60, 90, 120, 180 or 240 min. Each derivatization time was studied in triplicate. The samples were analysed by GC–MS in the scan acquisition mode.

2.5. Recovery studies

Blank urine and plasma horse samples spiked with anti-flammatory drugs (400 ng/ml) were subjected to the extraction procedure in triplicate. Before evaporation of the organic phase, 50 μl of a methanolic solution of 10 μg/ml of 7-ethyltheophylline (external standard) were added to the organic phase. The organic phase was then evaporated to dryness, derivatized and analysed by GC–MS in the scan mode. For salicylic acid, blank human urine and plasma samples spiked with 10 and 6.5 μg/ml, respectively, were studied.

The extraction recoveries were calculated by comparing the ratio of the peak areas of the compound and external standard obtained from the extracted samples with those from unextracted standard mixtures at concentrations corresponding to 100% recovery. For NSAIDs, the chromatographic peak area of one of the main ions was used to calculate the extraction recovery; for the external standard, the peak area in the chromatogram at *m/z* 208 was used.

2.6. Detection limits

Samples spiked with different concentrations of the compounds were subjected to the extraction and derivatization procedures. The extracts were analysed by GC–MS in the SIM acquisition mode. The detection limit was taken as the concentration with a signal-to-noise ratio between 3 and 5, calculated in the chromatogram of the base ion, except for niflumic acid (*m/z* 263), mefenamic acid (*m/z* 255), ketoprofen (*m/z* 209), tolfenamic acid (*m/z* 243), phenylbutazone (*m/z* 322) and suxibuzone (*m/z* 264).

3. Results and discussion

Although metabolic biotransformation has been described for some NSAIDs in horses, most of them can be found as unchanged compounds in equine plasma and urine [2,3,6–8,11,12,16,24–27]. Therefore, procedures to screen for NSAIDs in equine samples can be designed to detect the suspected parent compounds. The detection of most NSAIDs by GC requires prior derivatization to obtain good chromatographic behaviour; propyphenazone was the only compound under study that could be directly measured by GC. Trimethylsilylation [21] and alkylation with a variety of reagents [2–7,23,24] are the derivatization procedures currently used to determine these drugs by GC. In this work, methylation with methyl iodide in acetone and the presence of potassium carbonate was studied. All NSAIDs, except propyphenazone, were easily derivatized. The methylation occurs in the carboxylic acid functions or in the enolic acid groups (phenylbutazone and oxyphenbutazone); phenolic groups of oxyphenbutazone and salycilic acid are also derivatized to form methyl ethers. Ibuproxam is bis- or monomethylated in the hydroxyamide group. The

Table 1
Derivatives of NSAIDs formed, absolute and relative retention times and recoveries and detection limits in plasma and urine

No.	Compound	Derivative	M_r[a]	m/z (SIM)[b]	t_R[c] (min)	RRT[d]	Plasma		Urine	
							Recovery (%) (mean ± S.D.)	LOD[e] (ng/ml)	Recovery (%) (mean ± S.D.)	LOD[e] (ng/ml)
1	Salicylic acid	Bismethyl	166.03	77, 135, 166	2.64	0.49	61.3 ± 1.1	NS[f]	5.0 ± 1.7	NS
2	Ibuprofen	Monomethyl	220.13	161, 177, 220	3.65	0.68	97.7 ± 2.4	5	75.9 ± 7.7	10
3	Ibuproxam	Bismethyl	249.15	91, 161, 188	4.78	0.89	100.0 ± 6.8	5	81.3 ± 7.3	5
4	Flufenamic acid	Monomethyl	295.07	263, 295	5.37	1	80.9 ± 4.3	NS	70.7 ± 2.1	NS
5	Flurbiprofen	Monomethyl	258.09	178, 199, 258	5.48	1.02	71.0 ± 2.4	5	73.0 ± 1.3	10
6	Propyphenazone	Underivatized	230.15	77, 215, 230	5.63	1.05	71.3 ± 6.4	5	83.8 ± 2.7	5
7	Niflumic acid	Monomethyl	296.06	236, 263, 295	5.75	1.07	86.2 ± 1.2	5	76.9 ± 1.7	5
8	Naproxen	Monomethyl	244.10	170, 185, 244	5.94	1.10	73.9 ± 1.2	5	52.2 ± 2.0	5
9	Flunixin	Monomethyl	310.08	263, 295, 310	6.10	1.13	86.9 ± 1.4	<5	79.6 ± 3.5	<5
10	Mefenamic acid	Monomethyl	255.11	208, 223, 255	6.41	1.19	23.3 ± 2.5	25	75.4 ± 3.1	10
11	Ketoprofen	Monomethyl	268.10	105, 209, 268	6.51	1.21	50.8 ± 4.9	10	37.5 ± 3.1	10
12	Tolfenamic acid	Monomethyl	275.06	208, 243, 275	6.80	1.26	79.2 ± 6.4	5	81.1 ± 1.7	5
13	Diclofenac	Monomethyl	309.02	214, 242, 309	6.96	1.29	75.2 ± 1.3	5	75.4 ± 0.5	5
14	Meclofenamic acid	Monomethyl	309.02	242, 277, 309	7.43	1.38	72.5 ± 2.1	5	80.6 ± 6.1	5
15	Phenylbutazone	Monomethyl	322.16	183, 266, 322	7.48	1.39	51.2 ± 3.7	25	67.8 ± 3.4	10
16	Oxyphenbutazone	Bismethyl	352.15	213, 296, 352	8.79	1.63	41.8 ± 5.2	10	42.9 ± 5.1	25
17	Indomethacin	Monomethyl	371.08	139, 312, 371	10.42	1.94	46.1 ± 3.6	5	60.9 ± 4.7	5
18	Suxibuzone	Monomethyl	452.18	183, 264, 452	10.96	2.04	56.8 ± 7.8	10	70.1 ± 1.4	25

[a] Molecular mass.
[b] m/z monitored in SIM acquisition mode.
[c] Retention time.
[d] Relative retention time.
[e] Limit of detection.
[f] Not studied.

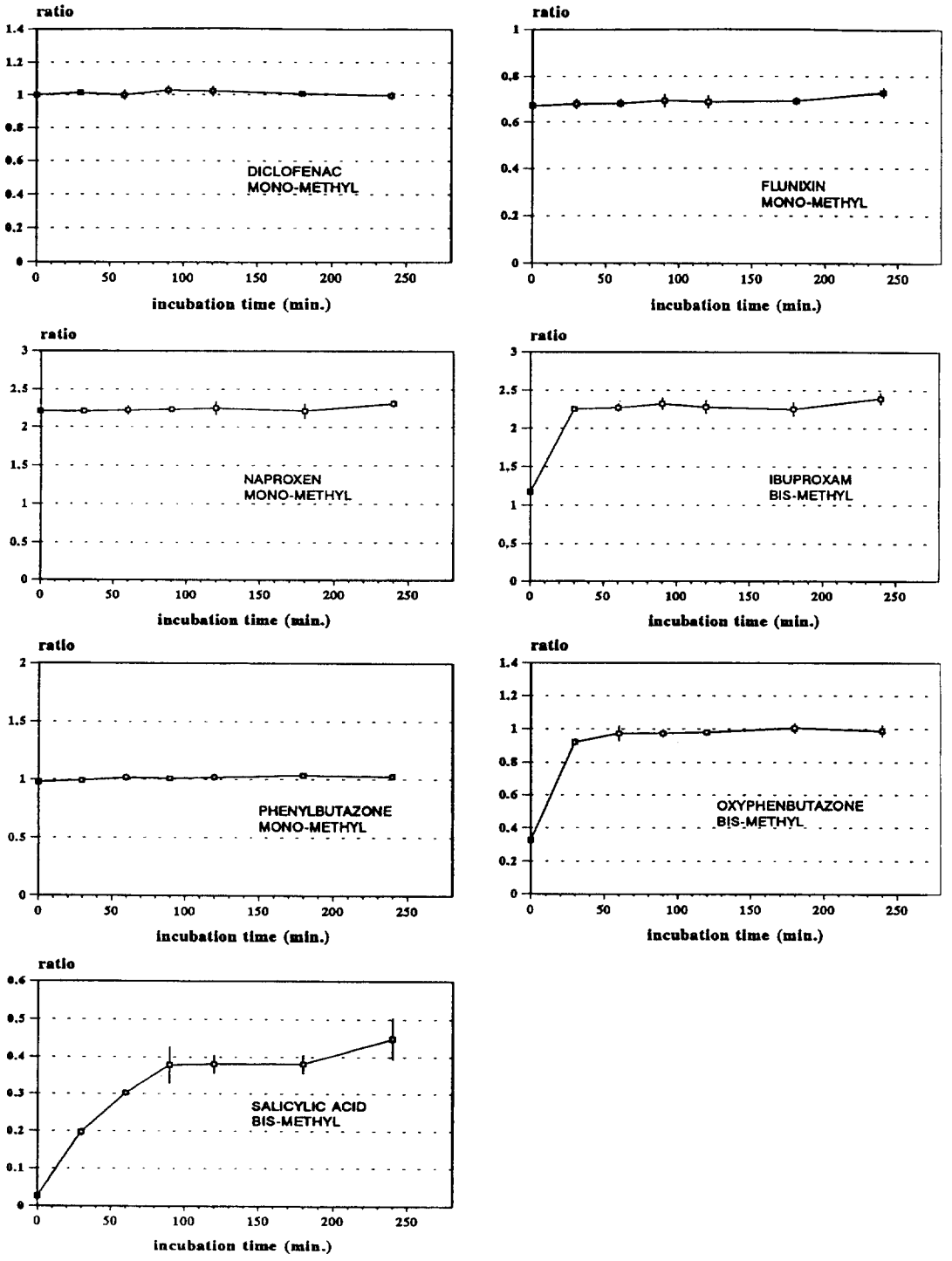


Fig. 1. Methylation kinetics of some NSAIDs. The ratio between the base peak area of the derivative and the m/z 208 peak area of the internal standard is plotted against incubation time.

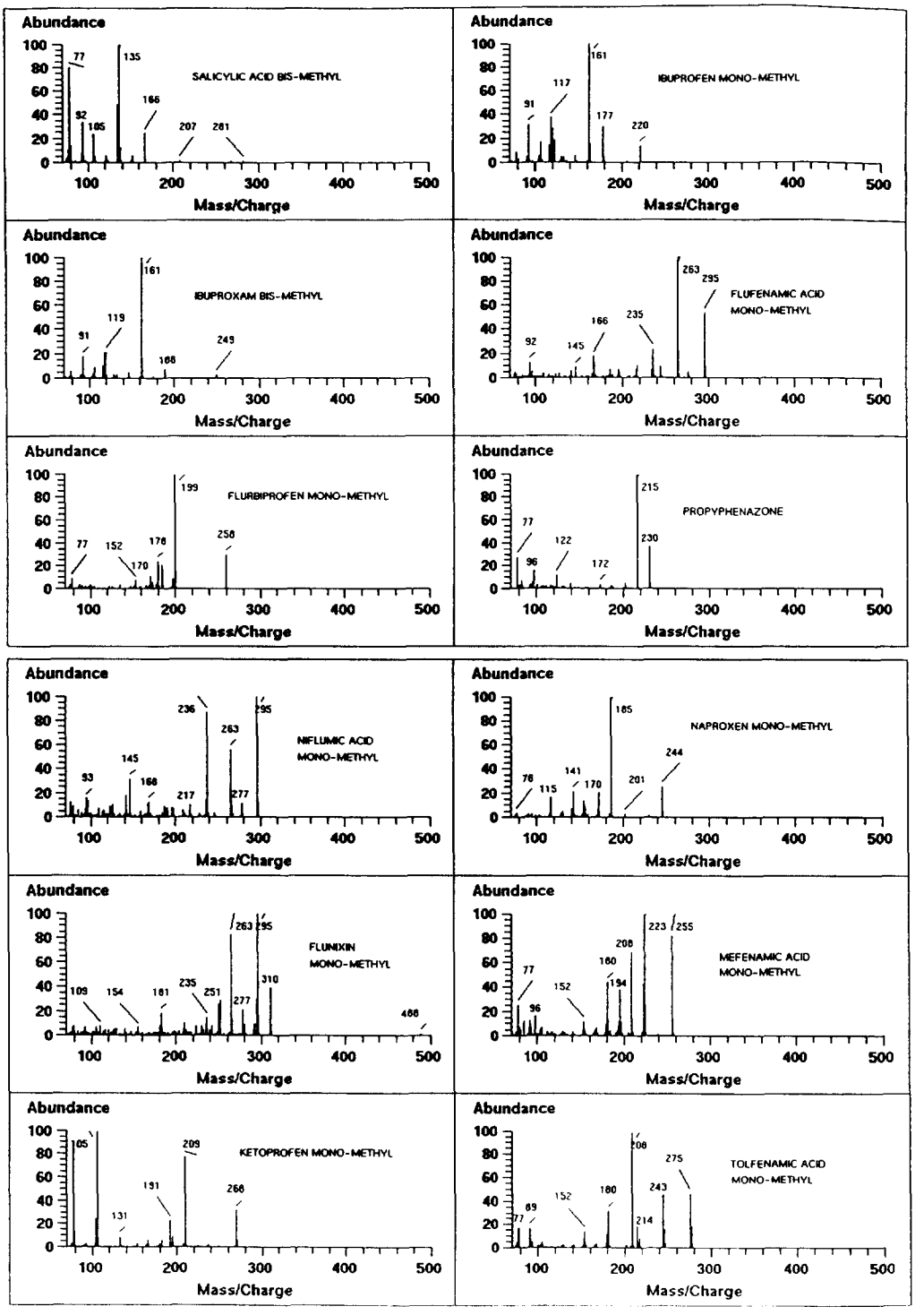

Abundance
SALICYLIC ACID BIS-METHYL
77
135
92
105
166
207
281
Mass/Charge
Abundance
IBUPROFEN MONO-METHYL
161
117
91
177
220
Mass/Charge
Abundance
IBUPROXAM BIS-METHYL
161
119
91
188
249
Mass/Charge
Abundance
FLUFENAMIC ACID
MONO-METHYL
263
295
166
235
92
145
Mass/Charge
Abundance
FLURBIPROFEN MONO-METHYL
199
77
152
176
170
258
Mass/Charge
Abundance
PROPYPHENAZONE
215
77
122
96
172
230
Mass/Charge
Abundance
NIFLUMIC ACID
MONO-METHYL
236
295
263
145
93
168
217
277
Mass/Charge
Abundance
NAPROXEN MONO-METHYL
185
244
141
76
115
170
201
Mass/Charge
Abundance
FLUNIXIN
MONO-METHYL
263
295
310
109
154
161
235
251
277
460
Mass/Charge
Abundance
MEFENAMIC ACID
MONO-METHYL
223
255
208
77
160
152
194
96
Mass/Charge
Abundance
KETOPROFEN MONO-METHYL
105
209
131
191
268
Mass/Charge
Abundance
TOLFENAMIC ACID
MONO-METHYL
208
275
243
152
180
89
77
214
Mass/Charge

Fig. 2.

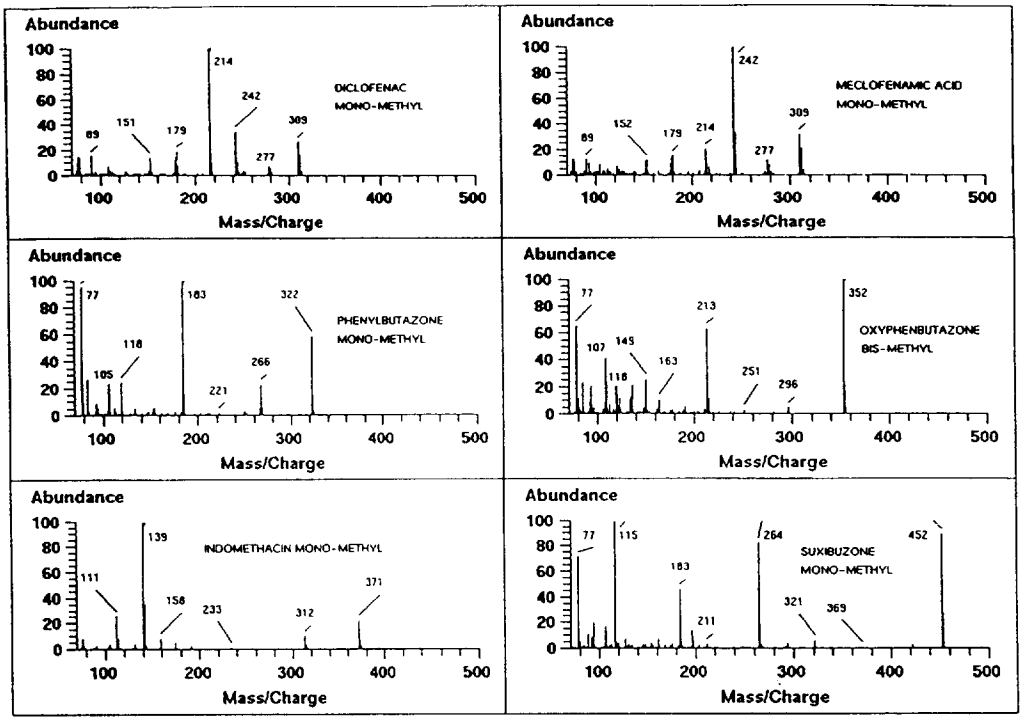


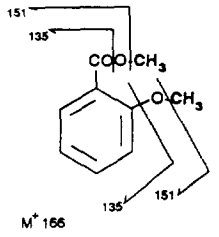
Fig. 2. Electron impact mass spectra.

secondary amine functions present in some of the compounds (flufenamic acid, niflumic acid, flunixin, mefenamic acid, tolfenamic acid, diclofenac and meclofenamic acid) are not derivatized under the conditions used.

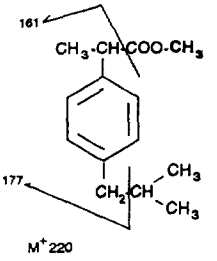
The methyl derivatives obtained and their retention times are listed in Table 1. For most of the compounds, the mono- or the bismethyl derivative was formed; only ibuproxam gave a small proportion of the monomethyl derivative in addition to the main bismethyl derivative. The formation of side-products, reported for other methylation procedures [4], was not observed. The problem of instability for the phenylbutazone and oxyphenbutazone derivatives reported when using trimethylsilylation [21] was not observed; methyl derivatives were stable for at least 1 week after derivatization.

The derivatization kinetics of some compounds representative of the group was studied (Fig. 1). The results obtained suggest that the kinetics of the methylation depend on the nucleophilic properties of the reacting functional group. For compounds with only carboxylic acid or enolic acid groups (diclofenac, flunixin, naproxen and phenylbutazone), complete derivatization is achieved without incubation at 60°C. The derivatization of phenolic or hydroxyamide groups (oxyphenbutazone, salicylic acid and ibuproxam) requires some incubation at 60°C. Owing to the derivatization kinetics of salicylic acid, an incubation time of 90 min was finally chosen for screening purposes. Confirmation analyses can be performed using a shorter incubation time depending on the specific analyte compound.

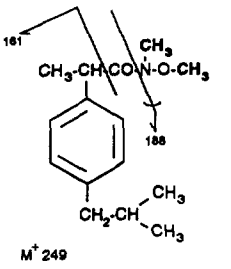
Electron impact mass spectra of the compounds are shown in Fig. 2. Tentative fragmentation profiles are proposed in Fig. 3. Some common fragmentation pathways are observed. The loss of a methoxycarbonyl group (59 u) from the molecular ion is observed for ibuprofen, flurbiprofen, naproxen, flunixin, ketoprofen, diclofenac and indomethacin methyl derivatives at



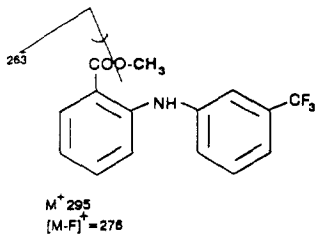
1 SALICYLIC ACID bis-methyl



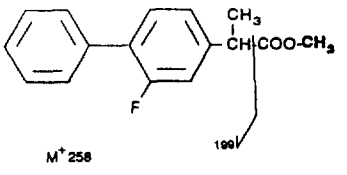
2 IBUPROFEN mono-methyl



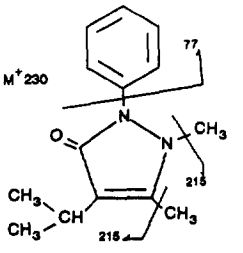
3 IBUPROXAM bis-methyl



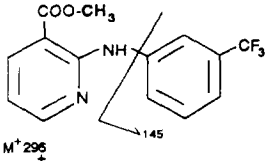
4 FLUFENAMIC ACID mono-methyl



5 FLURBIPROFEN mono-methyl

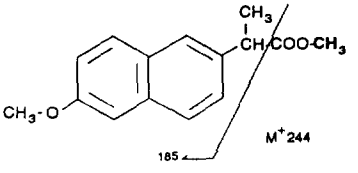


6 PROPYPHENAZONE

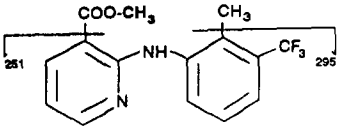


M+ 296
[M-H]+ = 295
[M-F]+ = 277
[295-32]+ = 263
[295-59]+ = 236

7 NIFLUMIC ACID mono-methyl

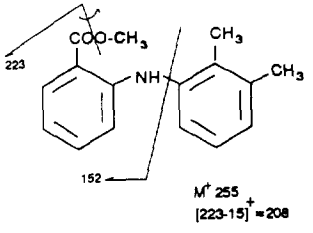


8 NAPROXEN mono-methyl

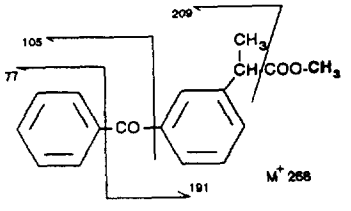


M+ 310
[295-32]+ = 263
[295-F]+ = 277

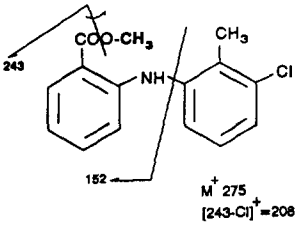
9 FLUNIXIN mono-methyl



10 MEFENAMIC ACID mono-methyl



11 KETOPROFEN mono-methyl



12 TOLFENAMIC ACID mono-methyl

Fig. 3.

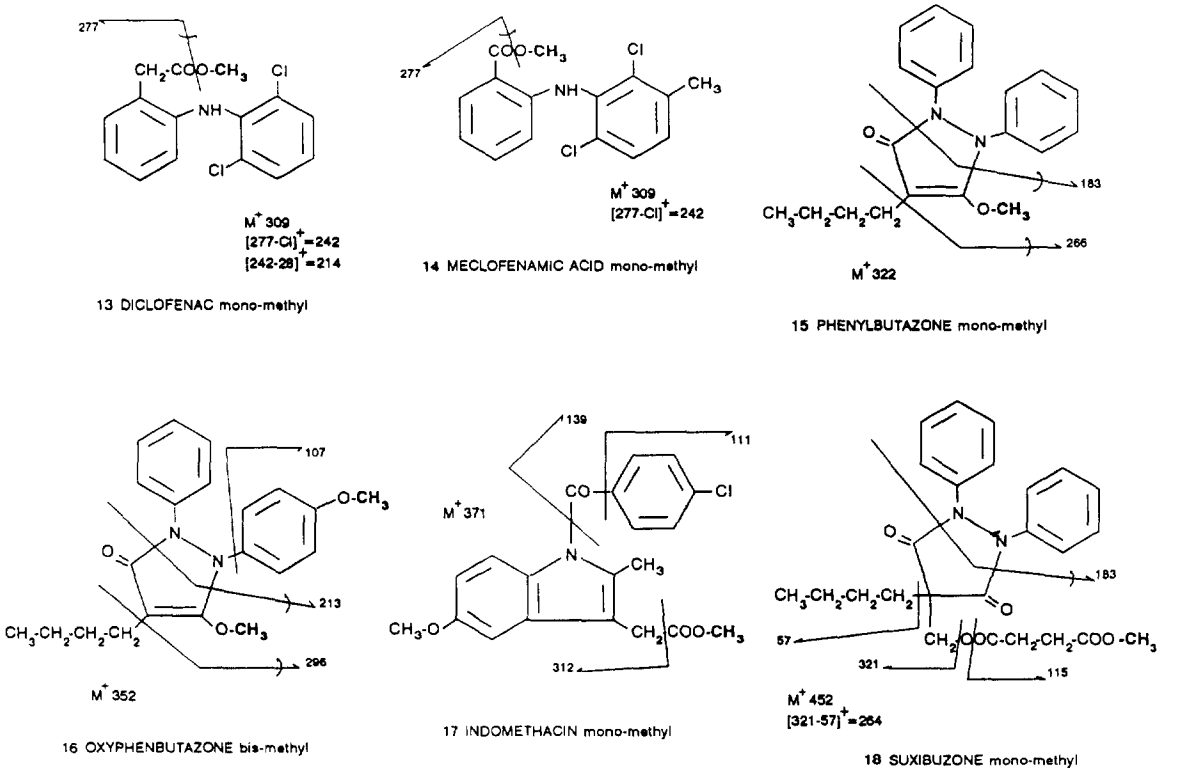


Fig. 3. Tentative fragmentation profiles.

m/z 161, 199, 185, 251, 209, 250 *and* 312, respectively. For niflumic acid monomethyl derivative, the loss of the methoxycarbonyl group from the deprotonated molecular ion (*m/z* 295) can result in an ion at *m/z* 236.

The loss of CH_3OH (32 u) in derivatives with methyl esters and secondary amine functions in the *ortho*-position on a benzene ring, as reported for the methyl derivatives of flunixin and tolfenamic acid metabolites [2,6] is also observed. For flufenamic acid, mefenamic acid, tolfenamic acid, diclofenac and meclofenamic acid this loss occurs from the molecular ion (*m/z* 263, 223, 243, 277 and 277, respectively). For niflumic acid and flunixin, the loss of CH_3OH (*m/z* 263 for both compounds) occurs from the ion at *m/z* 295, corresponding to the deprotonated and the demethylated molecular ions, respectively.

The loss of halogen atoms is also observed in some mass spectra. In the mass spectra of tolfenamic acid, diclofenac and meclofenamic acid, ions at *m/z* 208, 242 and 242, respectively, could be formed by the loss of chlorine from the $[M-32]^{+\cdot}$ ion. The loss of fluorine from the molecular ion is observed in the mass spectra of flufenamic acid and niflumic acid (*m/z* 276 and 277, respectively); for flunixin, the ion at *m/z* 277 could be also due to the loss of fluorine from the demethylated molecular ion.

For the pyrazole derivatives phenylbutazone, oxyphenbutazone and suxibuzone, the ions at *m/z* 183, 213 and 183, respectively, could be formed after the cleavage of two C–N bonds in the pyrazole ring with H rearrangement.

As indicated previously, dealkylation under electron impact ionization is also observed for some compounds: losses of methyl groups for salicylic acid bismethyl derivative, propyphenazone and flunixin monomethyl derivative to form ions at *m/z* 151, 215 and 295, respectively; loss of an isopropyl group for ibuprofen monomethyl derivative and propy-

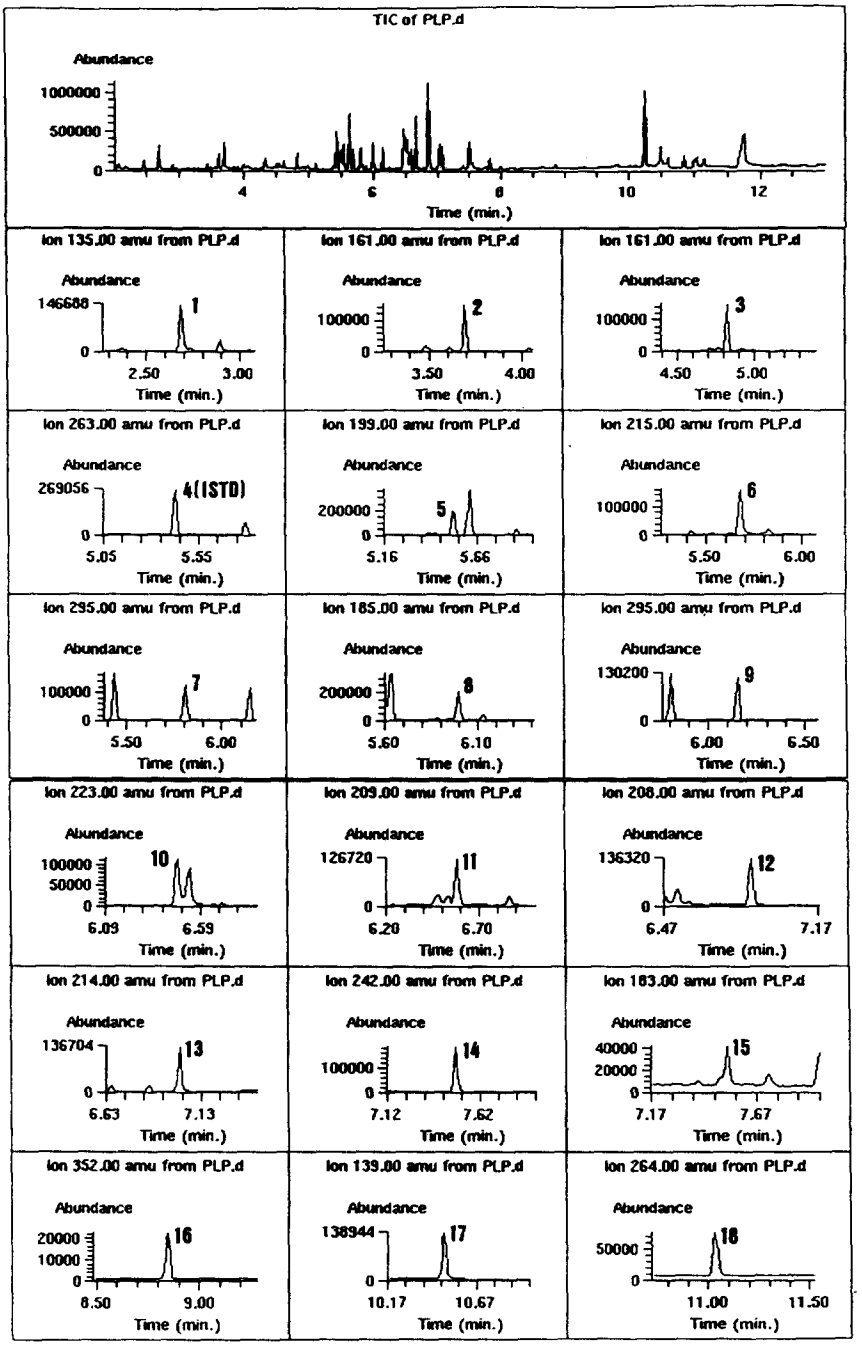


Fig. 4. Results obtained for the analysis of a horse plasma spiked with 200 ng/ml of NSAIDs. Peaks: 1 = salicylic acid bismethyl; 2 = ibuprofen monomethyl; 3 = iboproxam bismethyl; ISTD = flufenamic acid monomethyl (internal standard); 5 = flurbiprofen monomethyl; 6 = propyphenazone; 7 = niflumic acid monomethyl; 8 = naproxen monomethyl; 9 = flunixin monomethyl; 10 = mefenamic acid monomethyl; 11 = ketoprofen monomethyl; 12 = tolfenamic acid monomethyl; 13 = diclofenac monomethyl; 14 = meclofenamic acid monomethyl; 15 = phenylbutazone monomethyl; 16 = oxyphenbutazone bismethyl; 17 = indomethacin monomethyl; 18 = suxibuzone monomethyl derivative.

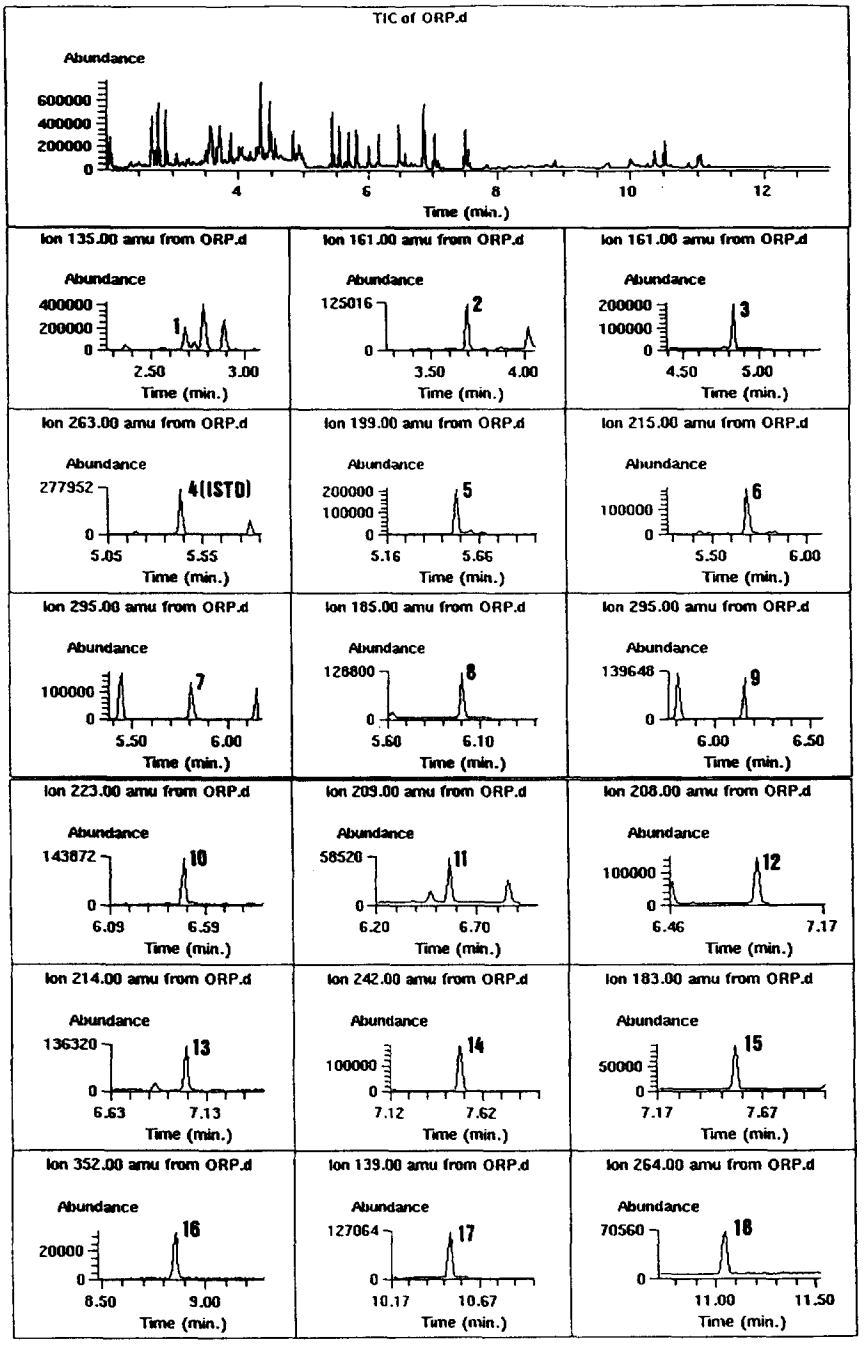


Fig. 5. Results obtained for the analysis of a horse urine spiked with 200 ng/ml of NSAIDs. Peaks: 1 = salicylic acid bismethyl; 2 = ibuprofen monomethyl; 3 = iboproxam bismethyl; ISTD = flufenamic acid monomethyl (internal standard); 5 = flurbiprofen monomethyl; 6 = propyphenazone; 7 = niflumic acid monomethyl; 8 = naproxen monomethyl; 9 = flunixin monomethyl; 10 = mefenamic acid monomethyl; 11 = ketoprofen monomethyl; 12 = tolfenamic acid monomethyl; 13 = diclofenac monomethyl; 14 = meclofenamic acid monomethyl; 15 = phenylbutazone monomethyl; 16 = oxyphenbutazone bismethyl; 17 = indomethacin monomethyl; 18 = suxibuzone monomethyl derivative.

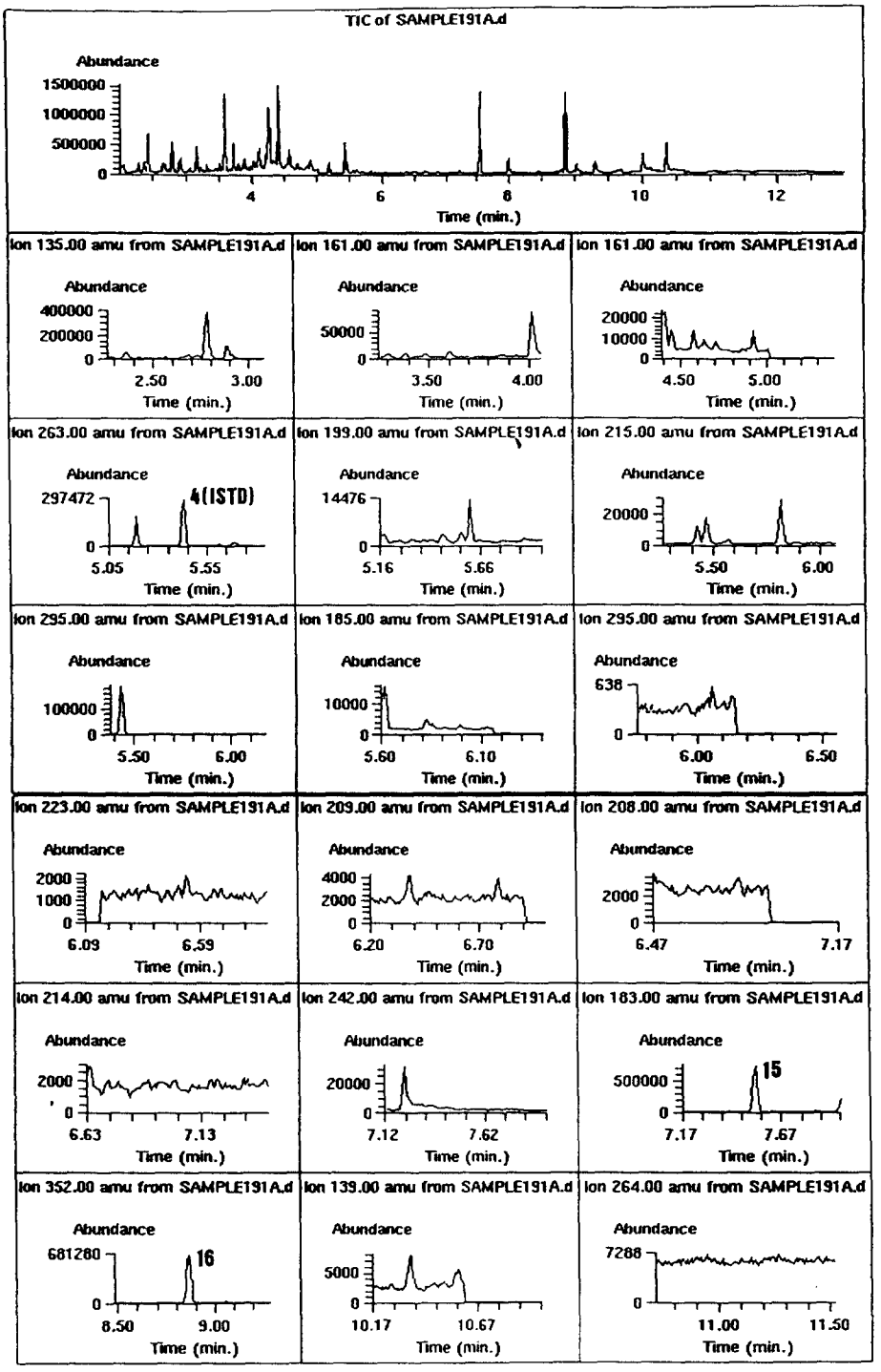


Fig. 6. Results obtained for the analysis of a horse urine containing 5 μg/ml of phenylbutazone and 10 μg/ml of oxyphenbutazone. Peaks: ISTD = flufenamic acid monomethyl (internal standard); 15 = phenylbutazone monomethyl; 16 = oxyphenbutazone bismethyl derivative.

phenazone to form ions at m/z 177 and 172, respectively. For phenylbutazone and oxyphenbutazone derivatives, the fragments at m/z 266 and 296 could be formed by loss of the butyl chain with H rearrangement.

The common acidic nature of NSAIDs allows the extraction of these compounds from the biological matrix by liquid–liquid procedures under acidic conditions. The extraction recoveries from plasma and urine are given in Table 1. Recoveries from plasma samples ranged from 23.3% for mefenamic acid to 100.0% for ibuproxam. For urine samples, recoveries from 37.5% (ketoprofen) to 83.8% (propyphenazone) were obtained, except for salicylic acid (only 5%). Nevertheless, as salicylic acid is normally present in horse samples [1], cut-off concentrations for this compound in equine plasma (6.5 μg/ml) and urine (750 μg/ml) have been established by the Fédération Equestre Internationale [25]. Therefore, at these levels salicylic acid is easily detected using the procedure described. For the accurate determination of salicylic acid in urine, other specific procedures must be used. Chromatograms obtained in the analysis of spiked equine plasma and urine samples are presented in Figs. 4 and 5.

Detection limits, calculated using the SIM acquisition mode, ranged from less than 5 to 25 ng/ml for both plasma and urine samples (Table 1). The sensitivity achieved is better than that reported for previous screening procedures [21,22], and similar to those described using more sophisticated instrumentation [23]. The detection limits obtained ensure the detection of all of the compounds after administration of normal doses of these drugs to horses [3,8,11, 12,16,20,26–29].

The methodology developed has been widely applied in equine doping control for screening (SIM acquisition mode) and for confirmation purposes (scan acquisition mode) with total reliability of the results. An example of the results for a real doping sample which contains oxyphenbutazone and phenylbutazone (10 and 5 μg/ml, respectively) is presented in Fig. 6.

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