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Mass spectrometric characterization of efaproxiral (RSR13) and its implementation into doping controls using liquid chromatography–atmospheric pressure ionization-tandem mass spectrometry

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Efaproxiral (2-[4-[[(3,5-dimethylanilino)carbonyl]methyl]phenoxyl]-2-methylpropionic acid, formerly referred to as RSR13) is prohibited in sports according to the World Anti-Doping Agency (WADA). The drug as well as structurally related compounds and a stable isotope-labeled derivative have been synthesized to elucidate the fragmentation pathway of efaproxiral, using electrospray ionization (ESI) and tandem mass spectrometry by employing a novel linear ion trap – orbitrap hybrid mass spectrometer – in positive and negative ionization modes. The elimination of 2-methyl acrylic acid (−86 u) has been identified as a major fragmentation process in both charge states. Negative ionization and collision-induced dissociation (CID) caused an additional release of carbon dioxide (−44 u), and positive ionization the loss of formic acid (−46 u). Efaproxiral was incorporated into an existing screening procedure for doping controls using solid-phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry, enabling a limit of detection of 2.5 ng/ml and interday precisions ranging from 7.9 to 13.0%. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: Orbitrap; fragmentation; doping; sport; screening

INTRODUCTION

The sophisticated structure, composition and versatility of hemoglobin (Hb), which consists of two α- and two β-chains forming a noncovalent tetrameric protein, is particularly responsible for oxygen transport in the human body.[1,2] The tetramer equilibrates between a deoxygenated T- and an oxygenated R-state, a fact that is significantly affected by so-called allosteric modifiers, the most important one of which is 2,3-diphosphoglycerate (2,3-DPG). A decrease of the oxygen affinity of hemoglobin results in an increased unloading of oxygen in target tissues, which is of considerable interest for various clinical conditions such as tissue hypoxia due to inadequate blood flow or impaired oxygen carrying capacity caused, for instance, by hemorrhage or dilutional anemia.[3] Moreover, an increased tissue oxygenation is essential, e.g. in case of myocardial ischemia or treatment of tumors, and may be accomplished by an artificial reduction of the oxygen affinity of hemoglobin.[4,5] For these purposes, numerous synthetic analogs of 2,3-DPG have been developed in the past.[6–8] The drug efaproxiral (2-[4-[[(3,5-dimethylanilino)carbonyl]methyl]phenoxyl]-2-methylpropionic acid, formerly referred to as RSR13, Scheme 1, **1**) is a typical representative of the class of synthetic allosteric modifiers of Hb and has demonstrated promising results in clinical trials.[4]

However, in addition to its clinical importance, tissue oxygenation is a crucial parameter also from an athletic performance point of view. The concerns of a potential misuse in amateur or professional sports have been substantiated by reports that efaproxiral has caused increased muscle oxygenation in animal studies.[9,10] Hence, two assays, which enabled the determination of efaproxiral in human urine using gas chromatography–mass spectrometry (GC–MS) for doping control purposes, were published in 2002 and 2004 with a recommendation to ban this substance in sports.[11,12] As a consequence, the World Anti-Doping Agency (WADA) added efaproxiral to the list of prohibited compounds in 2004 (Prohibited methods, M1. Section: Enhancement of oxygen transfer),[13] suspecting that this therapeutic agent or a structurally related compound may be misused.

In the present study we describe the mass spectrometric characterization of efaproxiral using electrospray ionization (ESI) and tandem mass spectrometry (MS/MS) and propose collision-induced dissociation (CID) pathways based on ^{13}C isotope labeling, synthesis of structurally related compounds and high-resolution/high-accuracy mass spectrometry employing an Orbitrap mass analyzer.[14] In

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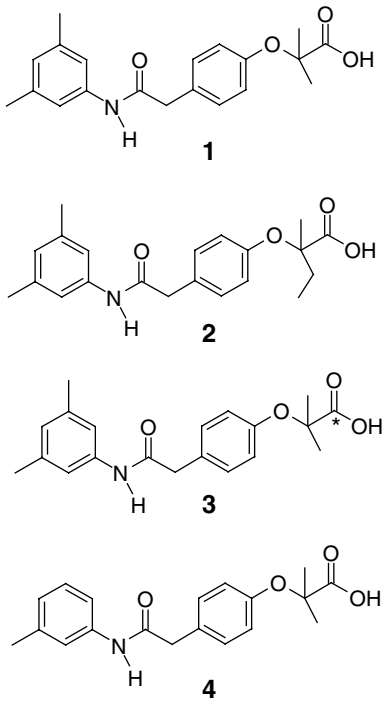

Scheme 1. Chemical structures of synthesized and characterized compounds: efaproxiral (RSR13, **1**, m.w. = 341), 2-{4-[(3,5-dimethylphenylcarbamoyl)methyl]phenoxy}2-methylbutyric acid (**2**, m.w. = 355), ^{13}C-labeled efaproxiral (**3**, m.w. = 342) – the labeled carbon is marked with an asterisk – and 2-methyl-2-[4-(*m*-tolylcarbamoylmethyl)phenoxy]propionic acid (**4**, m.w. = 327).

addition, the implementation of efaproxiral into an existing screening procedure based on liquid chromatography (LC) and MS/MS in doping controls is presented.

EXPERIMENTAL

Chemicals and reagents

3,5-Dimethylaniline (98%), *m*-toluidine (99%), 4-hydroxyphenylacetic acid (98%), ^{13}C-chloroform (99 atom% ^{13}C), thionyl chloride (97%, distilled before use), 2-butanone (99%) and sodium hydroxide (powder, 97%) were obtained from Sigma (Deisendorf, Germany). Acetone, *t*-butyl methyl ether and *n*-hexane were purchased from KMF (St. Augustin, Germany). Deionized water was of MilliQ grade. Hydrochloric acid (32%, p.a.), chloroform (p.a.), silica gel 60 (70–230 mesh) and xylene (p.a.) were obtained from VWR (Darmstadt, Germany), and Serdolit PAD1 was purchased from Serva Electrophoresis GmbH (Heidelberg, Germany).

Synthesis of efaproxiral and related compounds

The synthesis of efaproxiral and structural analogs was accomplished according to established procedures described elsewhere.[6,8] Briefly, 4-hydroxyphenylacetic acid (2.6 g, 17 mmol) was converted into the corresponding acetyl chloride by reflux in 10 ml of thionyl chloride for 30 min. Excess of thionyl chloride was evaporated, 3,5-dimethylaniline (5.7 g, 35 mmol) was added and the mixture was refluxed in 50 ml of xylene for 3 h to give 4-[[(3,5-dimethylanilino)carbonyl]methyl]phenol. The solid was filtered and washed with 20 ml of 0.1 M aqueous HCl, 20 ml of water, extracted into 30 ml of 1 M aqueous NaOH and washed with 10 ml of *t*-butyl methyl ether. The desired product was precipitated by acidification of the aqueous layer using 6 M aqueous HCl, collected by filtration and dried in a desiccator over phosphorus pentoxide *in vacuo*.

To 1.5 g (5 mmol) of 4-[[(3,5-dimethylanilino)carbonyl]methyl]phenol, 25 ml of acetone and 2.75 g of powdered sodium hydroxide were added followed by a dropwise addition of 1.25 ml of chloroform yielding crude efaproxiral. The product was purified by flash chromatography on silica gel and fractions containing the desired compound were evaporated to dryness. The resulting residue was subsequently precipitated from acetone/*n*-hexane yielding an oily semisolid, which was dried *in vacuo* over phosphorus pentoxide to give the pale-yellow crystalline product.

In order to prepare structurally related or isotopically labeled compounds, acetone was substituted by 2-butanone, chloroform by ^{13}C-chloroform and 3,5-dimethylaniline by *m*-toluidine, yielding the compounds **2**, **3** and **4** respectively, as depicted in Scheme 1.

Electrospray ionization-tandem mass spectrometry

ESI-MS(/MS) was performed on a Thermo LTQ Orbitrap mass spectrometer employing positive as well as negative ionization. The instrument was calibrated using the manufacturer's calibration mixture, which allowed for mass accuracies <3 ppm in positive and <6 ppm in negative ionization analyses. Analytes were dissolved in acetonitrile/water (1 : 1, v : v) at concentrations of 2 μg/ml and introduced into the mass spectrometer using a syringe pump at a flow rate of 5 μl/min. The ionization voltages were 4.5 and −3.3 kV for positive and negative ionization, respectively. The capillary temperature was set to 270 °C, and protonated or deprotonated precursor ions were dissociated using normalized collision energies between 25 and 35. Damping gas in the linear ion trap was helium 5.0, and gas supplied to the curved linear ion trap (CLT) was nitrogen obtained from a Peak Scientific NM30L nitrogen generator.

Liquid chromatography–tandem mass spectrometry

Routine LC–MS/MS analyses were performed on an Agilent 1100 Series HPLC interfaced by atmospheric pressure chemical ionization (APCI) to an Applied Biosystems API2000 triple-quadrupole mass spectrometer. The LC was equipped with a Macherey–Nagel Pyramid column (4 × 70 mm, 3-μm particle size), and the eluents used were 5 mM ammonium acetate containing 0.1% acetic acid (mobile phase A) and acetonitrile (mobile phase B). After 3 min of isocratic flow at 100% A, a gradient was employed to 100% B within 7 min, and the column was re-equilibrated at 100% A for 2.2 min. The flow rate was set to 800 μl/min with a postcolumn split of approximately 1 : 5. The ion source was operated in the negative mode at 350 °C, and the analyte efaproxiral was detected by means of characteristic fragment ions generated from the deprotonated molecule by CID utilizing the multiple reaction monitoring (MRM)

mode. Collision gas was nitrogen at 3.99×10^{-3} Pa (obtained from a Whatman K75-72 nitrogen generator), and collision offset voltages (COV) were optimized for each ion transition. Target product ions generated from $(M - H)^-$ at m/z 340 by CID were: m/z 296 (COV = 25 V), m/z 254 (COV = 30 V) and m/z 120 (COV = 40 V).

Urine sample preparation

The extraction of efaproxiral from human urine specimens was accomplished using solid-phase extraction (SPE) as described elsewhere.[15] Briefly, extraction cartridges (30 mg, 2 ml) were prepared from bulk PAD1 material and preconditioned with 2 ml of methanol and deionized water. Then, a volume of 2 ml of urine was added followed by a washing step with 2 ml of deionized water and an elution step with 2 ml of methanol. The methanolic layer was collected and evaporated to dryness, the residue was reconstituted in 200 µl of a mixture of mobile phase A and B (4 : 1, v : v) and 20 µl was injected into the LC–MS/MS system.

Assay validation for efaproxiral

A screening method for diuretics was validated in our laboratory according to ICH[16] and WADA requirements. In order to implement efaproxiral into this assay, the parameters, lower limit of detection (LLOD), recovery, intra- and interday precision as well as specificity, were determined. Blank urine specimens necessary for the assay validation were obtained from ten different healthy volunteers (three females, seven males). As the ionization mode for the detection of diuretics is negative, the ion transitions m/z 340–254 and m/z 354–254 for efaproxiral and the ISTD (compound **2**) were chosen for validation purposes.

Lower limit of detection

The LLOD was defined as the 'lowest content that can be measured with reasonable statistical certainty'[16] at a signal-to-noise ratio $\geq$3. Ten different blank urine samples spiked with the internal standard (ISTD, compound **2**) only and another ten different blank urine specimens fortified with 2.5 ng/ml of efaproxiral were prepared and analyzed according to the established protocol providing the data necessary to estimate the LLOD.

Recovery

The recovery of efaproxiral from 2 ml of human urine by SPE was determined at 100 ng/ml. Ten blank urine samples were fortified with efaproxiral before sample preparation, and another ten blank urine specimens were extracted according to the described protocol followed by addition of 50 ng of efaproxiral to the methanolic eluate. To both sets of samples, 100 ng of compound **2** (ISTD) was spiked into the methanol layer before evaporation. Recovery was calculated by comparison of mean peak area ratios of the analyte and ISTD of the samples fortified prior to and after SPE.

Intraday precision

During a one-day period, ten urine samples of low (10 ng/ml), medium (50 ng/ml) and high (250 ng/ml) concentrations of efaproxiral were prepared and analyzed, and the intraday precision was calculated for each concentration level.

Interday precision

On three consecutive days, ten urine samples of low (10 ng/ml), medium (100 ng/ml) and high (250 ng/ml) concentrations of efaproxiral were prepared and analyzed randomly, and the assay interday precision was calculated for each concentration level.

Specificity

Fifty different urine specimens were prepared as described, in order to probe for interfering peaks in the selected ion chromatograms at the expected retention time of efaproxiral.

Excretion-study urine sample

One urine sample obtained after administration of efaproxiral was kindly provided by the World Association of Anti-Doping Scientists (WAADS). The urine specimen was stabilized with sodium azide (1 mg/ml) and stored at −20 °C until analysis.

RESULTS AND DISCUSSION

Synthesis of efaproxiral and related compounds

The syntheses yielded compounds **1–4**, and accurate mass measurement employing ESI-MS on the LTQ Orbitrap mass analyzer allowed the determination of elemental compositions of protonated molecules as shown in Table 1. Purity was greater 90% as measured by means of LC–MS, and overall yields ranged from 31 to 42% of the theoretical values. Owing to detailed studies on the principal synthesis and series of products resulting from the employed approach,[6,8] structure confirmations using conventional strategies such as NMR or IR were not performed, and the determination of elemental compositions using high-resolution/high-accurate mass analyses was considered sufficient.

Table 1. Measured elemental compositions of synthesized compounds **1–4** in protonated form

Compounds	Elemental composition (protonated species)	Molecular weight (theoretical)	Molecular weight (experimental)	Error (ppm)
1	$C_{20}H_{24}O_4N$	342.1700	342.1702	0.7
2	$C_{21}H_{26}O_4N$	356.1856	356.1861	1.4
3	$^{12}C_{19}{}^{13}C_1H_{24}O_4N$	343.1734	343.1740	1.8
4	$C_{19}H_{22}O_4N$	328.1543	328.1549	1.8

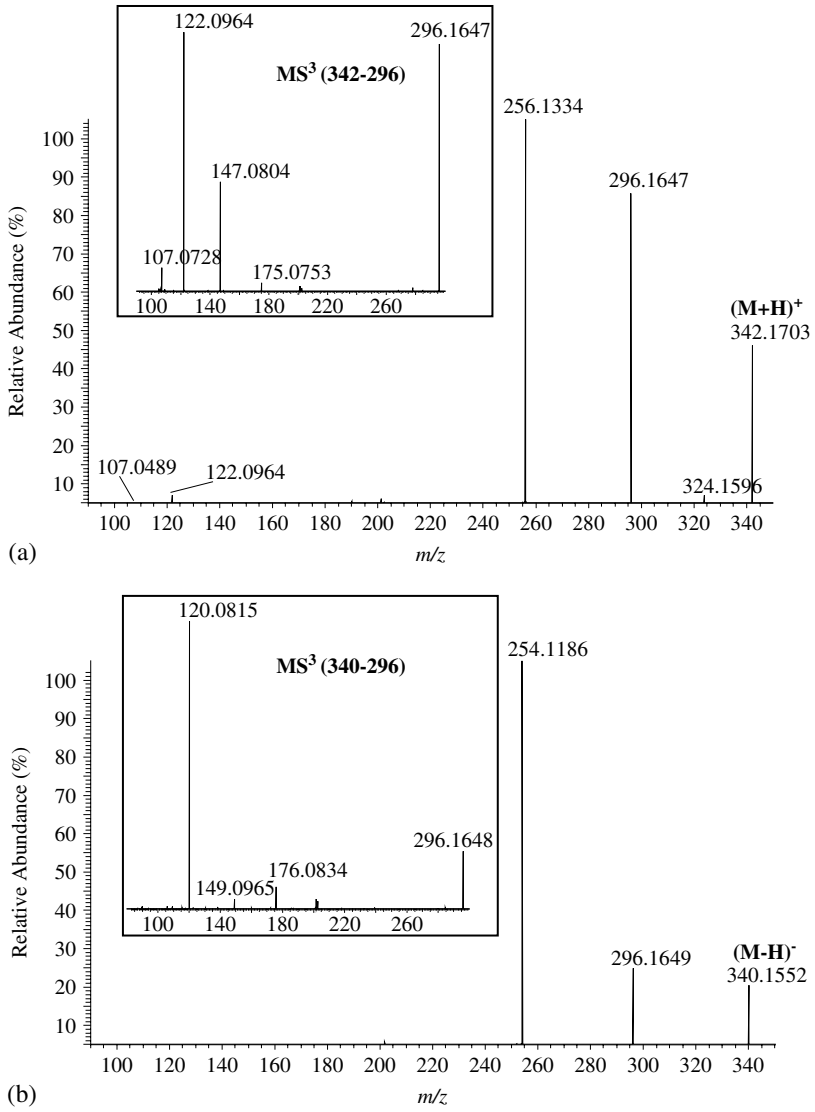

Figure 1. ESI product ion spectra of efaproxiral. (a) Positively charged precursor ions at *m/z* 342 (MS^2) and *m/z* 342–296 (MS^3, inset) were dissociated at normalized collision energies of 25 and 35, respectively. (b) Negatively charged precursor ions at *m/z* 340 (MS^2) and *m/z* 340–296 (MS^3, inset) were dissociated at normalized collision energies of 25 and 35, respectively.

Electrospray ionization-tandem mass spectrometry

Product ion mass spectra of efaproxiral and its analogs in the positive and negative ionization modes were recorded on the LTQ Orbitrap mass spectrometer using a normalized collision energy of 25 for CID of protonated and 35 for CID of deprotonated molecules. The resulting product ion spectra of efaproxiral are shown in Fig. 1, while accurate masses, elemental compositions and relative abundances of dominant fragment ions of all four synthesized compounds are listed in Tables 2–5.

Positive ESI

Using positive ESI, the protonated precursor of efaproxiral was generated at *m/z* 342 (Fig. 1(a)), which gave rise to a number of characteristic product ions upon CID, the proposed structures of the most abundant ones being depicted in Scheme 2. The suggested origin of the fragment ion at *m/z* 296 is a loss of formic acid, as substantiated by the elemental composition of *m/z* 296 (Table 2) and ^{13}C labeling, which proved the release of HCOOH including the carboxyl residue (Table 3). Further dissociation of *m/z* 296 (Fig. 1(a), inset) gave rise to abundant fragment ions at *m/z* 175, 147, 122 and 107, the suggested structures of which are shown

Table 2. Characteristic product ions of $(M + H)^+$ *m/z* 342 of efaproxiral generated by CID. Determined masses represent average values ($n = 30$)

Production (*m/z*)	Elemental composition	Molecular weight (theoretical)	Molecular weight (experimental)	Error (ppm)
296	$C_{19}H_{22}O_2N$	296.1645	296.1647	0.5
256	$C_{16}H_{18}O_2N$	256.1332	256.1333	0.6
193	$C_{11}H_{13}O_3$	193.0859	193.0860	0.1
175	$C_{11}H_{11}O_2$	175.0754	175.0753	−0.3
147	$C_{10}H_{11}O$	147.0805	147.0804	−0.5
122	$C_8H_{12}N$	122.0965	122.0964	−0.5
107	C_7H_7O	107.0491	107.0490	−1.3
107	C_7H_9N	107.0730	107.0728	−1.2

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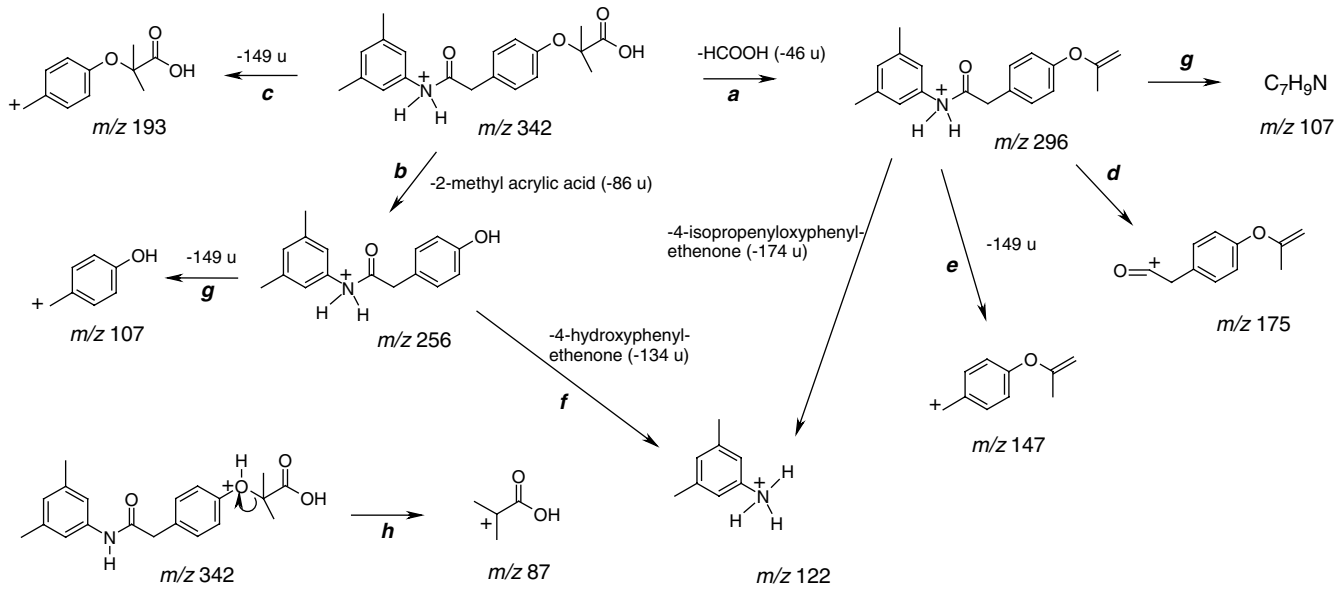


Scheme 2. Proposed fragmentation pathway of efaproxiral using positive ionization and CID.

Table 3. Comparison of product ions generated by CID from compounds **1–4** using positive ESI. Suggested fragmentation routes corresponding to Scheme 2 are indicated as a–h

		Product ions (m/z)							
Compounds	Protonated molecule (m/z)	a	b	c	d	e	f	g	h
1	342	296	256	193	175	147	122	107	87
2	356	310	256	207	189	161	122	107	101
3	343	296	256	194	175	147	122	107	88
4	328	282	242	193	175	147	108	107/93	87

in Scheme 2. The product ions at m/z 175 and 147 are generated only from m/z 296 by eliminations of dimethylaniline (−121 u) and dimethylaniline plus carbon monoxide (−149 u), respectively. No alternative precursor ions were observed in MS^3 experiments for these particular ions. Both fragment ions were increased by 14 u in case of compound **2,** but were not influenced in case of **4** or by ^{13}C labeling in case of **3** (Table 3). In combination with accurate masses of these product ions and calculated elemental compositions, the structures depicted in Scheme 2 were proposed. The product ion at m/z 122 is suggested to originate from a loss of 4-isopropenyloxyphenyl ethenone (−174 u) and consists of protonated dimethylaniline. Support for this proposal was obtained from accurate mass measurement as well as from the CID spectra of compound **4**, which contained a corresponding fragment ion at m/z 108 owing to the lack of a methyl residue (Table 3).

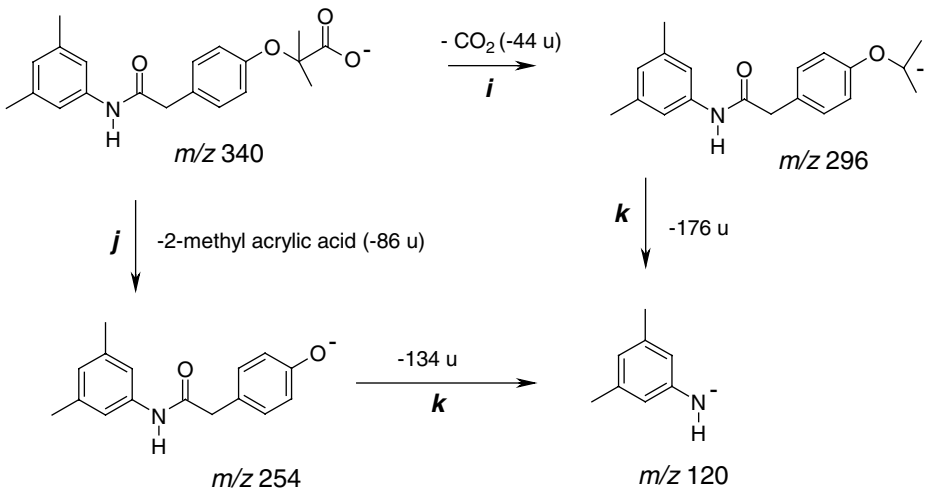
The protonated efaproxiral (Scheme 1, **1**) also generated an abundant fragment ion at m/z 256 upon CID, the proposed structure of which is shown in Scheme 2. It is suggested to originate from the elimination of 2-methyl acrylic acid (−86 u) as substantiated by determination of the elemental composition of m/z 256 (Table 2). Additionally, all synthesized analogs (**2–4**) support the postulated fragmentation pathway, as compound **2** gave rise to a corresponding loss of 100 u accounting for 2-methylene butyric acid, compound **3** eliminated 87 u due to the ^{13}C atom and compound **4** released the same 86 u as efaproxiral (Table 3). Further dissociation of m/z 256 in MS^3 experiments caused a loss of 4-hydroxyphenyl ethenone (−134 u) giving rise to the same fragment ion at m/z 122 (dimethylaniline) as observed in the MS^3 experiments on m/z 296.

Both precursor ions at m/z 296 and 256 generated fragment ions at m/z 107, the high-resolution/high-accuracy mass measurements of which demonstrated two different compositions. While m/z 256 preferably produced a fragment ion with the elemental composition of C_8H_6O, the ion at m/z 296 predominantly gave rise to an ion with the composition C_7H_9N (Scheme 2, Table 2).

Additionally, characteristic but very low abundant fragment ions of **1** obtained by CID were observed at m/z 193 and 87, the proposed structures of which are shown in Scheme 2. The generation of m/z 193 is according to the fragmentation of m/z 296 to m/z 147 based on the release of dimethylaniline plus carbon monoxide (−149 u), while m/z 87 is suggested to consist of isobutyric acid.

Negative ESI

Using negative ESI, efaproxiral (Scheme 1, **1**) generated a deprotonated precursor ion at m/z 340. CID gave rise to a few abundant fragment ions, the proposed structures of which are summarized in Scheme 3. An initial loss of carbon dioxide (−44 u) is observed, producing the product ion at m/z 296, the composition of which was determined by



Scheme 3. Proposed fragmentation pathway of efaproxiral using negative ionization and CID.

Table 4. Characteristic product ions of $(M-H)^-$ *m/z* 340 of efaproxiral generated by CID. Determined masses represent average values ($n = 30$)

Product ions (*m/z*)	Elemental composition	Molecular weight (theoretical)	Molecular weight (experimental)	Error (ppm)
296	$C_{19}H_{22}O_2N$	296.1645	296.1648	1.0
254	$C_{16}H_{16}O_2N$	254.1176	254.1186	3.9
120	$C_8H_{10}N$	120.0808	120.0815	5.9

accurate mass measurement (Table 4). Moreover, ^{13}C labeling of the carboxyl residue (compound **3**) proved its absence from the fragment ion at *m/z* 296 (Table 5). Further dissociation of the anion at *m/z* 296 (Fig. 1(b), inset) gave rise to the product ion at *m/z* 120, the suggested structure of which is deprotonated dimethylaniline, as depicted in Scheme 3.

Corresponding to its positively charged counterpart at *m/z* 342, the anion of efaproxiral at *m/z* 340 also eliminates 2-methyl acrylic acid (−86 u) generating a characteristic fragment ion at *m/z* 254, which subsequently loses 134 u giving rise to *m/z* 120, as proved by MS^3 experiments. Also, here the derivative **4** substantiated the composition of *m/z* 120 by producing a corresponding ion at *m/z* 106, as well as by accurate mass measurement and determination of its elemental composition (Tables 4, 5).

Table 5. Comparison of product ions generated by CID from compounds **1–4** using negative ESI. Suggested fragmentation routes corresponding to Scheme 3 are indicated as i–k

Compounds	Deprotonated molecule (*m/z*)	Productions (*m/z*)		
		i	j	k
1	340	296	254	120
2	354	310	254	120
3	341	296	254	120
4	326	282	240	106

Assay validation for efaproxiral

The drug efaproxiral was incorporated into an existing screening procedure, and the parameters LLOD, recovery, intra- and interday precision as well as specificity were validated. The results are summarized in Table 6.

Lower limit of detection

The LLOD of efaproxiral in the screening procedure was determined at 2.5 ng/ml with a S/N ratio of >3.

Recovery

The recovery of efaproxiral from human urine by SPE was 99% as determined at 100 ng/ml.

Intraday precision

The intraday precision was determined from 30 urine samples at low (10 ng/ml), medium (50 ng/ml) and high (250 ng/ml) concentrations, and it ranged between 3.8 and 8.8% (Table 6).

Interday precision

The interday precision was determined on three days at low, medium and high concentration levels from a total of 90 urine samples yielding precisions of 13.0, 11.6 and 7.9%, respectively.

Specificity

In order to prove specificity of the screening procedure, 50 blank urine specimens were analyzed for efaproxiral, and they did not generate signals in extracted ion chromatograms using the ion transition *m/z* 340–254 at expected retention times.

CONCLUSIONS

Efaproxiral is banned for professional and amateur athletes according to the regulations of the WADA. In order to circumvent the need for new screening procedures, doping control laboratories try to include newly prohibited compounds into existing assays, and efaproxiral has demonstrated suitable physicochemical properties for SPE from

Table 6. Summary of assay validation results

		Intraday precision ($n = 30$)		Interday precision ($n = 90$)	
LLOD (ng/ml)	Recovery (%) at 100 ng/ml	Concentration (ng/ml)	CV (%)	Concentration (ng/ml)	CV (%)
2.5	99	10	3.6	10	13.0
–	–	50	8.8	50	11.6
–	–	250	7.3	250	7.9

urine and LC–ESI-MS/MS analysis using either positive or negative ionization. The elucidation of the fragmentation pathway in both polarities was accomplished by the syntheses of structurally related compounds, stable isotope labeling and high-resolution/high-accuracy mass spectrometry. The determination of efaproxiral in human urine can accurately and reproducibly be accomplished with detection limits as low as 2.5 ng/ml. Considering amounts of administration of 10 mg/kg bodyweight, as used in clinical trials,[11] the LLOD should be sufficient for doping controls.

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