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ORIGINAL PAPER

Screening for benfluorex and its major urinary metabolites in routine doping controls

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Abstract Benfluorex [1-(*m*-trifluoromethylphenyl)-2-(β-benzoyloxyethyl)aminopropane] has been widely used for the treatment of atherogenic metabolic disorders and impaired carbohydrate metabolism (particularly in obese type-II diabetic patients) as well as an anorectic drug. Due to its potentially performance-enhancing properties, benfluorex has been added to the list of prohibited compounds and methods of doping by the World Anti-Doping Agency (WADA) in 2010, necessitating the implementation of the drug as well as its major metabolites into routine doping control procedures. In the present study, human urinary metabolites of benfluorex were characterized by gas chromatography–electron ionization–mass spectrometry (GC-EI-MS) as well as liquid chromatography–electrospray ionization–high resolution/high accuracy tandem mass spectrometry (LC-ESI-MS/MS). Commonly employed sports drug testing approaches consisting of liquid–liquid extraction followed by GC-MS or urine dilution and immediate LC-MS/MS analysis were expanded and validated with regard to specificity, recovery (48–54%, GC-MS only), intra- and interday precision (<25%), limits of detection (5–8 ng/mL for LC-MS/MS and 80 ng/mL for GC-MS), and ion suppression (for LC-ESI-MS/MS only) to allow the detection of benfluorex metabolites 1-(*m*-trifluoromethylphenyl)-2-(2-hydroxyethyl)aminopropane (M1), 1-(*m*-trifluoromethylphenyl)-2-(2-carboxymethyl)aminopropane (M2), and 1-(*m*-trifluoromethylphenyl)-2-aminopropane (M3) as well as the glucuronic acid conjugate of M1.

Keywords Sport · Doping · Mass spectrometry · Benfluorex · Orbitrap · Stimulants

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Introduction

The synthesis of benfluorex [1-(*m*-trifluoromethylphenyl)-2-(β-benzoyloxyethyl)aminopropane, Fig. 1, 1] was first reported approximately 40 years ago [1], followed by the introduction of a drug termed Mediator (Servier, France) in 1976 containing the hydrochloride of benfluorex [2]. Comprehensive studies demonstrated its considerable utility and versatility in the treatment of various medical conditions by supporting the glycemic control in obese patients suffering from non-insulin-dependent diabetes mellitus and improving the obese–diabetic–dyslipidemic syndrome [3]. The beneficial effects of the therapeutic agent were, however, recently eclipsed by various undesirable effects particularly concerning heart valve damages [4, 5], which led to a recommendation of the European Medicines Agency to withdraw benfluorex from the market within the European Union in December 2009 [6]. Despite the apparent and considerable health risks associated even with the controlled use of such compounds, concerns about the

misuse of benfluorex in sports supported by the confession of its administration in 2008 [7] have led to its inclusion into the Prohibited List as established by the World Anti-Doping Agency (WADA) in 2010 [8]. Consequently, current doping control testing procedures [9, 10] have to be extended to cover the drug and/or its major metabolites and allow the unambiguous identification. In the present study, four major urinary metabolites of benfluorex (Fig. 1, 1) namely hydroxyethylnorfenfluramine (M1), carboxymethylnorfenfluramine (M2), norfenfluramine (M3), and the glucuronic acid conjugate of M1 (M4), which were described in early reports on the metabolism and disposition of benfluorex [11–13], were characterized from an administration study urine sample using state-of-the-art mass spectrometric approaches. Subsequently, two procedures based on either gas chromatography–mass spectrometry (GC-MS) or liquid chromatography–tandem mass spectrometry (LC-MS/MS) were expanded and validated concerning the identified target analytes.

Experimental

Chemicals and reagents

Benfluorex hydrochloride (Mediator®) was purchased from Servier (Gidy, France), norfenfluramine hydrochloride from Tropon Werke (Cologne, Germany), *tert*-butyl methyl ether (TBME, distilled before use) from Appli Chem (Darmstadt, Germany), and potassium hydroxide as well as disodium sulfate were from Merck (Darmstadt, Germany).

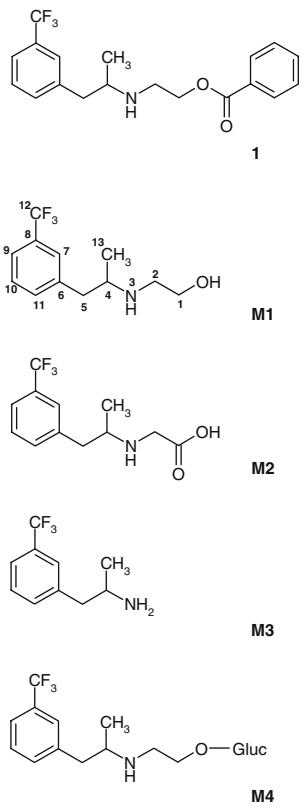


Fig. 1 Structures of benfluorex (1, mol wt.=351), hydroxyethylnorfenfluramine (M1, mol wt.=247), carboxymethylnorfenfluramine (M2, mol wt.=261), norfenfluramine (M3, mol wt.=203), and the glucuronide of M1 (M4, mol wt.=424)

Synthesis of internal standard and hydroxyethylnorfenfluramine (M1)

The internal standard (ISTD, *N*-dodecyl-di-isopropylamine, DIPA-12) was synthesized as described by Nolteernsting et al. [14]. Hydroxyethylnorfenfluramine (M1) was prepared from 1 by hydrolysis using 3 M NaOH in 80% ethanol. The solution was incubated for 1 h at 50°C and the ethanol was subsequently removed under reduced pressure. The aqueous layer was extracted with ethyl acetate, the organic layer was evaporated to dryness, and the oily residue was purified by silica gel chromatography using *tert*-butyl methyl ether (TBME) and methanol (2:1, *v:v*) containing 1% of triethylamine. The final product was characterized by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) with ^{1}H (600 MHz), H,H-COSY, H,C-HMQC, H,C–HMBC, ^{13}C and ^{13}C APT (150 MHz) experiments employing a Bruker AV 600 instrument (Bruker, Karlsruhe, Germany) equipped with a 5 mm inverse probe head (*z*-gradient coil).

GC-MS/NPD analysis

All samples prepared for GC-MS analysis were measured on an Agilent 6890/5973 GC-MS system (Waldbronn, Germany) equipped with an additional nitrogen–phosphorus detector (NPD) and two separate Agilent HP-5MS columns (inner diameter, 0.25 mm; film thickness, 0.25 μm). In order to obtain matching retention times, the column directed to the mass selective detector (MSD) was 26 m long while the other column connected to the NPD was 21 m long (due to different pressure conditions in MSD and NPD). The injection volume was 5 μL (split ratio 1:10), the GC carrier gas was helium (constant pressure at 12 psi), and a temperature gradient was employed starting at 83°C for 0.3 min increasing to 330°C with 28°C/min. The final temperature was kept for 3.8 min. The mass spectrometer was operated with EI and full scan analysis (*m/z* 40–400, four scans/s). The NPD was operated at 290°C using 60 mL/min of dried air, 3 mL/min of hydrogen, and 18 mL/min of helium (as make-up gas).

LC-MS/MS analysis

Two different analytical approaches were pursued in order to characterize the target metabolites in urine specimens or to establish initial testing and confirmation methods.

Accurate masses of intact analytes and product ions derived from collision-induced dissociation (CID) were obtained using a Thermo Accela liquid chromatograph

(LC) interfaced to a Thermo Exactive mass spectrometer (Bremen, Germany). The LC was equipped with a Phenomenex Gemini C_6-Phenyl column (2.0×100 mm, particle size 3 μm), and solvents were 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The flow rate was set to 200 μL/min, and linear gradient elution was conducted starting at 100% A, decreasing to 0% A in 5 min followed by re-equilibration at 100% A. The mass spectrometer (MS) was operated in positive ionization mode and calibrated using the manufacturer's calibration mixture (yielding a total of seven reference masses). Mass accuracies <3 ppm were accomplished for the period of analysis. The ionization voltage was 3.0 kV, the capillary temperature was set to 290°C, and three MS settings were used throughout the analytical runs: (a) full scan MS from *m*/*z* 50–2,000 at a resolution of 25,000 (FWHM), (b) full scan MS (*m*/*z* 50–2,000, resolution set to 10,000) with higher energy collision-induced dissociation (HCD) set to 15 V, and (c) full scan MS (*m*/*z* 50–2,000, resolution set to 10,000) with HCD set to 30 V. Gas supplied to the curved linear ion trap was nitrogen obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany).

Routine doping control procedures were established using the identical LC setup as described above connected to a Thermo Vantage (Dreieich, Germany) mass spectrometer via ESI source. The ion source was operated in the positive mode at 350°C, and the target compounds 1–4 were detected by means of characteristic product ions obtained from the protonated molecules (Table 1, bold text) by CID utilizing the selected reaction monitoring mode (SRM). Collision gas was nitrogen at a nominal pressure of 1.5 mTorr obtained from a CMC nitrogen generator (CMC Instruments, Eschborn, Germany), and collision offset voltages were optimized for each product ion.

Sample preparation for GC-MS

The sample pretreatment for GC-MS analyses was adapted from commonly accepted alkaline extraction procedures for doping control purposes [15, 16]. In brief, 5 mL of urine are basified by means of 0.5 mL of 5 M aqueous potassium hydroxide and 25 μg of the ISTD are added. The aqueous layer is extracted against 2 mL of TBME, and after centrifugation the organic layer is transferred to an injection vial for GC-MS analysis.

Sample preparation for LC-MS/MS

The sample preparation for LC-MS/MS analyses consisted of the addition of 500 ng of the ISTD (*N*-dodecyl-diisopropylamine, DIPA-12) to a urine aliquot of 0.1 mL, addition of 0.9 mL of deionized water, vortexing for 15 s and subsequent centrifugation at 10,000×*g* for 1 min. One hundred microliters were then transferred to autosampler vials, and 2 μL were injected into the LC-MS/MS system.

Assay validation

After oral administration, benfluorex undergoes extensive metabolism, primarily to the metabolites 1-(*m*-trifluoromethylphenyl)-2-(2-hydroxyethyl)aminopropane (M1), 1-(*m*-trifluoromethylphenyl)-2-(2-carboxymethyl)aminopropane (M2), and 1-(*m*-trifluoromethylphenyl)-2-aminopropane (M3). Hence, assay validations with respect to lower limit of detection (LLOD), recovery (GC-MS only), intra- and interday precision, was performed with the synthesized M1 and purchased M3 reference materials.

Lower limit of detection

The lower limit of detection (LLOD) was defined as the 'lowest content that can be measured with reasonable statistical certainty' [17] at a signal-to-noise ratio ≥3. Ten blank urine samples spiked with the internal standard (ISTD) only, and ten blank urine specimens fortified with 80 ng mL^{-1} (GC-MS) or 8 ng mL^{-1} (LC-MS/MS) of compounds M1 and M3 were prepared and analyzed according to the established protocol providing the data necessary to estimate the LLOD.

Recovery (GC-MS only)

The recovery of M1 and M3 from human urine by liquid–liquid extraction (LLE) was determined at 500 ng mL^{-1}. Ten blank urine samples were fortified with target analytes before sample preparation, and another ten blank urine specimens were extracted according to the described protocol followed by addition of 2,500 ng of each metabolite to the ether layer. To both sets of samples, 2,500 ng of the ISTD were spiked into the ether extract before evaporation. Recovery was calculated by comparison of mean peak area ratios of analyte and ISTD of samples fortified prior to and after LLE.

Intra- and interday precision

For GC-MS analyses, ten urine samples of low (500 ng mL^{-1}), medium (2,500 ng mL^{-1}), and high (5,000 ng mL^{-1}) concentrations of the synthesized metabolites M1 and M3 were prepared and measured within 1 day, and the corresponding intraday precision was calculated for each concentration level. For LC-MS/MS analyses, the same approach was pursued but using concentrations of 50, 250, and 500 ng mL^{-1} for low, medium, and high levels, respectively.

Table 1 Elemental compositions of protonated molecules of 1 and M1-M4 and resulting product ions (>5% of relative abundance) using high resolution/high accuracy MS/MS experiments

Compound	Precursor ion (m/z) MS^2	Elemental comp. (exp.)	Error (ppm)	Collision energy (eV)	Product ion (m/z)	Elemental comp. (exp.)	Error (ppm)	Cleaved species
1	352.1517	$C_{19}H_{21}F_3O_2N$	−0.7	35	230.1150	$C_{12}H_{15}F_3N$	−0.3	$C_7H_6O_2$
					187.0727	$C_{10}H_{10}F_3$	−1.2	$C_9H_{11}O_2N$
					159.0417	$C_8H_6F_3$	0.7	$C_{11}H_{15}O_2N$
					149.0598	$C_9H_9O_2$	0.8	$C_{10}H_{12}F_3N$
					105.0340	C_7H_5O	4.7	$C_{12}H_{16}F_3ON$
M1	248.1252	$C_{12}H_{17}F_3ON$	−3.5	25	230.1146	$C_{12}H_{15}F_3N$	−2.0	H_2O
					187.0727	$C_{10}H_{10}F_3$	−1.2	C_2H_7ON
					159.0414	$C_8H_6F_3$	−1.3	$C_4H_{11}ON$
					109.0452	C_7H_6F	3.3	$C_5H_{11}F_2ON$
M2	262.1046	$C_{12}H_{15}F_3O_2N$	−1.2	25	216.0993	$C_{11}H_{13}F_3N$	−1.0	HCOOH
					187.0728	$C_{10}H_{10}F_3$	−0.6	$C_2H_5O_2N$
					159.0415	$C_8H_6F_3$	−0.7	$C_4H_9O_2N$
					109.0453	C_7H_6F	4.5	$C_5H_9F_2O_2N$
M3	204.0993	$C_{10}H_{13}F_3N$	−0.8	25	**187.0728**	$C_{10}H_{10}F_3$	−0.6	NH_3
					159.0415	$C_8H_6F_3$	−0.7	C_2H_7N
					109.0452	C_7H_6F	3.3	$C_3H_7F_2N$
M4	424.1580	$C_{18}H_{25}F_3O_7N$	0.6	25	406.1471	$C_{18}H_{23}F_3O_6N$	−0.2	H_2O
					388.1371	$C_{18}H_{21}F_3O_5N$	1.3	$2 \times H_2O$
					290.1362	$C_{14}H_{19}F_3O_2N$	−0.1	$C_4H_6O_5$
					248.1257	$C_{12}H_{17}F_3ON$	0.0	$C_6H_8O_6$
					230.1152	$C_{12}H_{15}F_3N$	0.5	H_2O, $C_6H_8O_6$
					187.0731	$C_{10}H_{10}F_3$	1.2	C_2H_7ON, $C_6H_8O_6$
					159.0417	$C_8H_6F_3$	0.7	$C_4H_{11}ON$, $C_6H_8O_6$
					85.0288	$C_4H_5O_2$	4.3	$C_{14}H_{20}F_3O_5N$

Product ions written in bold numbers were used in SRM experiments

The determination of the interday precision was accomplished by repetitive sample preparation and analysis of the same ten spiked urine samples of each concentration level on three consecutive days.

Specificity and ion suppression/enhancement effects

Ten different blank urine specimens obtained from five female and five male healthy volunteers were prepared as described above in order to probe for interfering peaks in the selected ion chromatograms at expected retention times of all target analytes, particularly M1 and M3. Tests for ion suppression/enhancement effects were conducted according to established protocols employing post-column infusion of the target compound (1 ng μL^{-1} at 2 μL min^{-1}) with the analysis of four different blank urine samples [18, 19].

Excretion study urine sample

Urine specimens of a male patient (57 years, 69 kg, 180 cm) treated with Mediator® (300 mg, single oral application) with informed written consent were obtained and served as proof-of-principle samples. Urine collection was done before (0 h) and 1.5, 3, 4.75, 6.5, 7.75, 10.5, 13.5, 22, 24, 27, 29, 31, 33, 36.5, 44.5, 59, 52, 60.5, 69.5, 94, 116.5, and 172 h post administration and aliquots were stabilized with sodium azide (1 mg mL^{-1}) and stored at +4°C until analysis.

Results and discussion

Preparation and characterization of M1

The hydrolysis of benfluorex yielded pure M1 (yield: 45% of theory) as demonstrated by ^1H and ^{13}C NMR analysis: ^1H NMR ($CDCl_3$), δ (ppm): 1.08 (d, 3H, H-13, J=6.3 Hz), 2.67 (m, ^1H, H-5a), 2.77 (m, ^1H, H-2a), 2.86 (m, ^1H, H-2b), 2.89 (m, ^1H, H-5b), 2.95 (m, ^1H, H-4), 3.63 (t, 2H, H-1, J= 5.^{1}Hz), 7.36 (d, ^1H, H-11, J=7.5 Hz), 7.40 (t, ^1H, H-10, J= 7.5 Hz), 7.43 (s, ^1H, H-7), 7.47 (d, ^1H, H-9, J=7.5 Hz); ^{13}C NMR ($CDCl_3$), δ (ppm): 19.93 (C-13), 43.01 (C-5), 48.32 (C-2), 54.41 (C-4), 60.91 (C-1), 123.17 (C-9), 125.04 (C-6),

125.86 (C-7), 130.69 (C-12), 132.67 (C-11), 140.03 (C-8). Moreover, accurate mass measurement using ESI-MS proved the desired elemental composition of the protonated target compound with $C_{12}H_{17}F_3ON$ (error=−3.5 ppm, Table 1).

EI-MS characterization of target analytes

GC-MS has been commonly used for initial testing procedures as well as confirmatory analyses of stimulants for decades [20, 21]. Although several LC-MS(/MS)-based methods have been introduced and complemented the array of doping control analytical assays [9, 10], GC-MS offers sufficient sensitivity to meet the minimum required performance levels for the detection of stimulants [22] and further provides a comprehensive data set due to (combined) full scan and nitrogen–phosphorus specific analyses. Hence, initial testing for stimulants can be accomplished by conventional GC-MS approaches, followed by confirmatory analyses based on LC-MS(/MS) methods.

The analytes detected in GC-MS measurements indicating the administration of benfluorex are predominantly M1 and M3. Since the latter represents a common metabolite of benfluorex and fenfluramine [23–25], M1 possesses the higher significance for doping control purposes regarding benfluorex. The EI mass spectra of both compounds (M1 and M3) are depicted in Fig. 2, and comparable dissociation

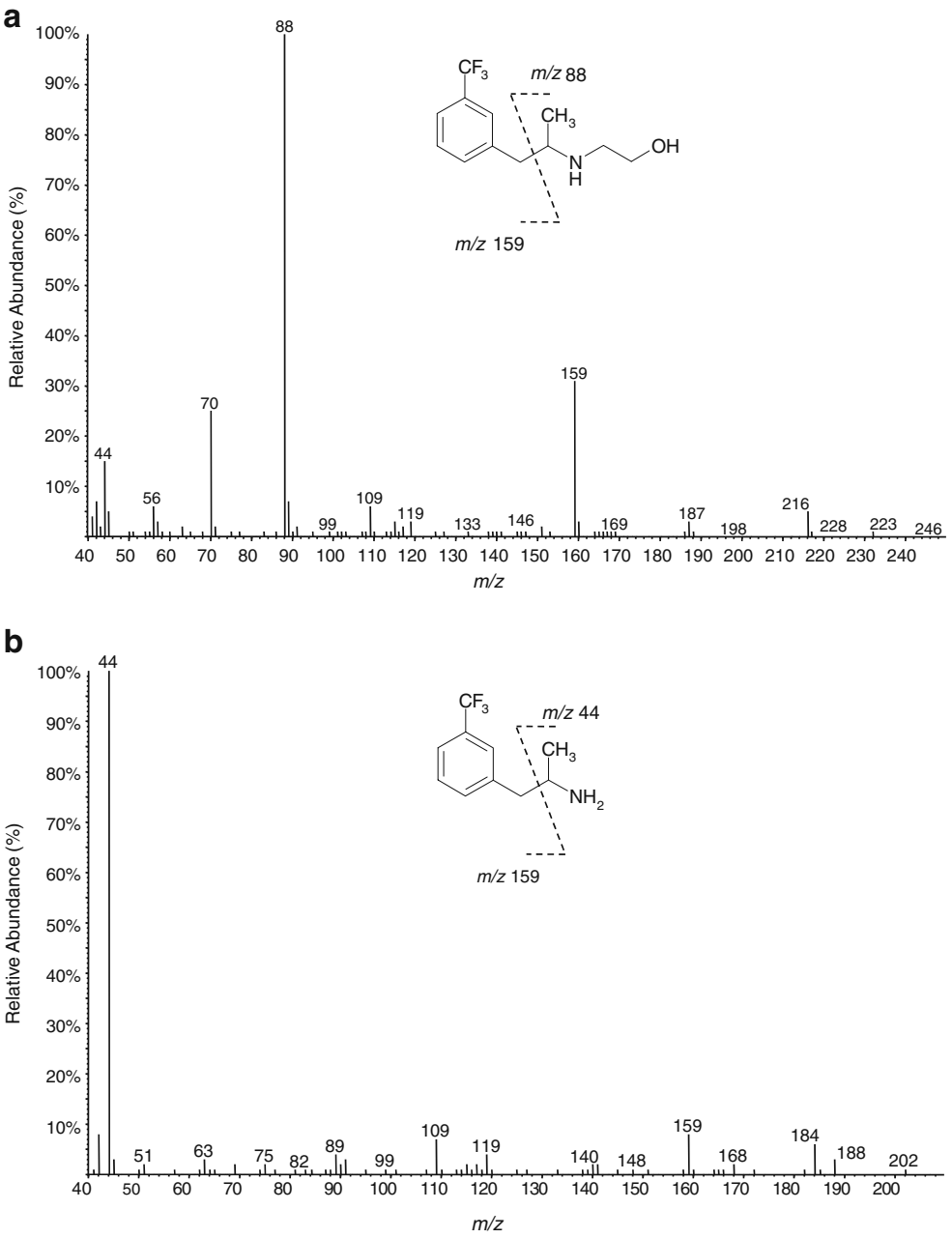


Fig. 2 EI mass spectra of M1 (**a**) and M3 (**b**)

behaviors are observed. While the molecular ions are not visible, M1 and M3 eliminate a hydrogen atom or a methyl radical to yield the low abundant fragments at *m/z* 246 and 202 or *m/z* 232 and 188, respectively. The loss of a fluorine atom (−19 Da) was reported for M3 yielding the ion at *m/z* 184 [24], and the corresponding fragment was observed at low intensity for M1 accordingly at *m/z* 228. The elimination of a hydroxyethyl radical (−45 Da) from M1 is suggested to result in the fragment ion at *m/z* 216, and the complementary fragment ions at *m/z* 88 and 159 the 2-ethylamino-ethanol moiety and the 1-methyl-3-trifluoromethylphenyl residue with the first mentioned fragment ion corresponding to *m/z* 44 of M3. The formations of *m/z* 88 and 44, respectively, are attributed to so-called α-cleavages, following mechanisms studied in detail for aliphatic amines [26–29].

ESI-MS(/MS) characterization of target analytes

In order to unambiguously identify benfluorex and its metabolites in biological fluids by LC-MS(/MS), the detailed mass spectrometric characterization of the analytes under ESI-CID conditions is of particular importance. The product ion mass spectra of all target compounds (i.e., benfluorex, M1–M4) are depicted in Fig. 3, and accurate

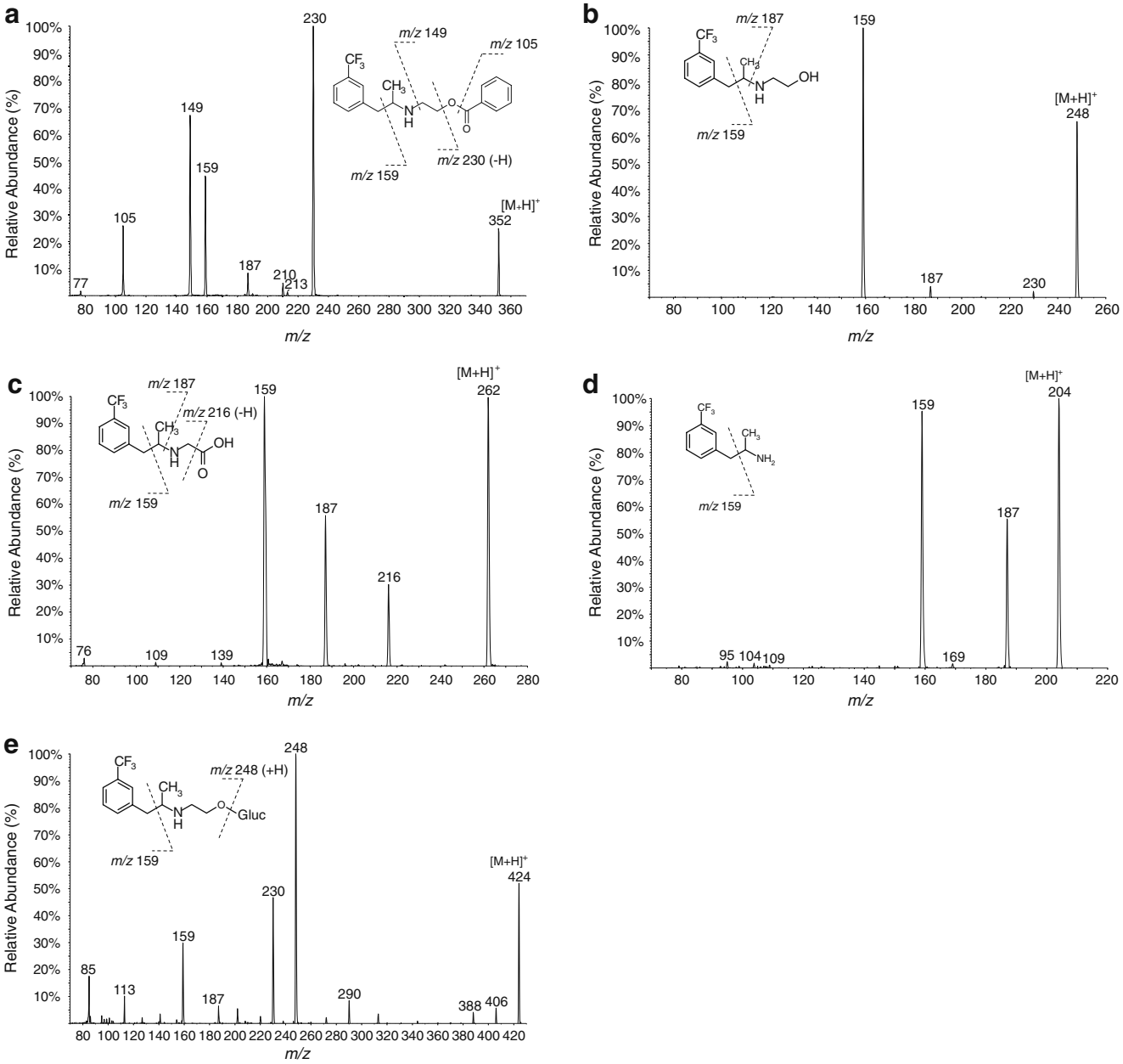
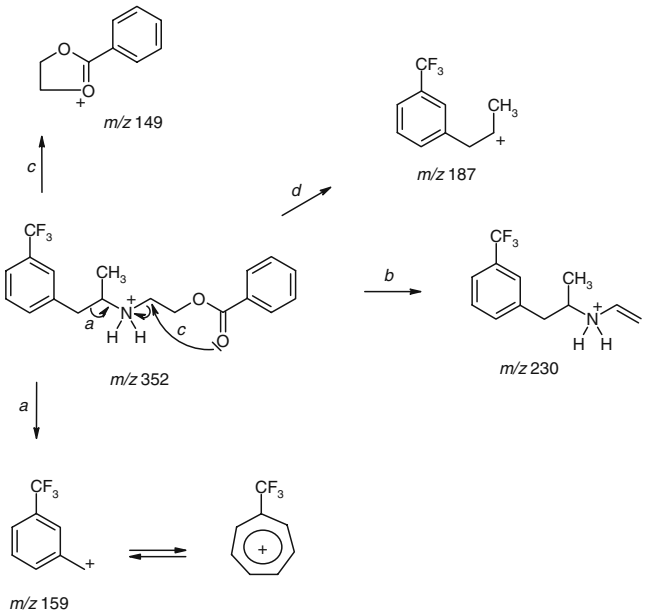


Fig. 3 ESI-product ion mass spectra of benfluorex (**a**), M1 (**b**), M2 (**c**), M3 (**d**), and M4 (**e**)



Scheme 1 Proposed dissociation pathway of benfluorex under ESI-CID conditions

masses of protonated molecules as well as product ions resulting from collisional activation are listed in Table 1.

The most abundant product ions of benfluorex are observed at *m/z* 230, 187, 159, 149, and 105 (Fig. 3a). The release of benzoic acid (−122 Da) is suggested to yield the base peak at *m/z* 230 resulting from a neutral loss forming the cation of [1-methyl-2-(3-trifluoromethyl-phenyl)-ethyl]-vinylamine (Scheme 1b). The low abundant ion at *m/z* 187 as well as the intense product ion at *m/z* 159 are proposed to characterize the trifluoromethylphenyl moiety with the first mentioned comprising the cation of 1-propyl-3-trifluoromethyl-benzene (Scheme 1d) and the latter representing the 1-methyl-3-trifluoromethylphenyl residue. The ion at *m/z* 159 presumably originates from the protonation of the amino residue followed by the elimination of benzoic acid 2-ethylideneamino-ethyl ester (−193 Da, Scheme 1a). The product ions at *m/z* 149 and 105 are suggested to provide structural information about the benzoic acid residue. While *m/z* 105 is attributed to the phenylmethylidyne-oxonium ion, *m/z* 149 is proposed to result from the release of norfenfluramine (M3) from benfluorex, potentially bearing a 2-phenyl-4,5-dihydro-[1, 3]dioxol-1-ylium structure (Scheme 1c).

The diagnostic ions at *m/z* 159 and 187 were detected in all metabolites (Table 1) due to the common structural core consisting of M3. Additional structural information for M1 and M2 was obtained from *m/z* 230 and 216, respectively, indicating the loss of water or formic acid depending on the composition of the N-linked residue of the metabolites (Fig. 3).

GC-MS/NPD analysis

Using the above described data on M1 and M3, an established screening procedure for stimulants [16] was extended to include M1 as indicator for the misuse of benfluorex in addition to the common metabolite M3 of fenfluramine and benfluorex. Due to the alkaline extraction protocol (vide supra), the omission of hydrolyzing phase-II metabolites, and the limited GC properties of the underivatized carboxylic acid moiety of M2, neither M2 nor M4 was detected in routine GC-MS measurements. For initial testing purposes, however, the GC-MS/NPD-based screening procedure proved adequate as demonstrated by method validation (Table 2). The LLODs of M1 and M3 were estimated at 80 ng mL^{-1}, the recoveries (as determined at 500 ng mL^{-1}) were 48% and 54%, respectively, and intra- and interday precisions were better 20% at all concentration levels. In case of adverse analytical findings of M1 and M3 in routine sports drug testing samples, follow-up analyses using an LC-MS/MS approach are recommended as it

Table 2 Assay validation results

Compound	GC-MS						LC-MS/MS				
	LLOD (ng mL^{-1})	Recovery (%) at 500 ng mL^{-1}	Intraday precision (n=30)		Interday precision (n=90)		LLOD (ng mL^{-1})	Intraday precision (n=30)		Interday precision (n=90)	
			conc. (ng mL^{-1})	CV (%)	conc. (ng mL^{-1})	CV (%)		conc. (ng mL^{-1})	CV (%)	conc. (ng mL^{-1})	CV (%)
M1	80	48	500	11.9	500	18.3	3	50	20.6	50	20.7
			2,500	6.0	2,500	17.5		250	15.8	250	17.4
			5,000	5.1	5,000	12.6		500	9.3	500	15.4
M3	80	54	500	8.3	500	13.1	5	50	17.7	50	15.2
			2,500	7.7	2,500	8.8		250	15.0	250	16.0
			5,000	5.1	5,000	6.9		500	17.0	500	18.5

CV coefficient of variation

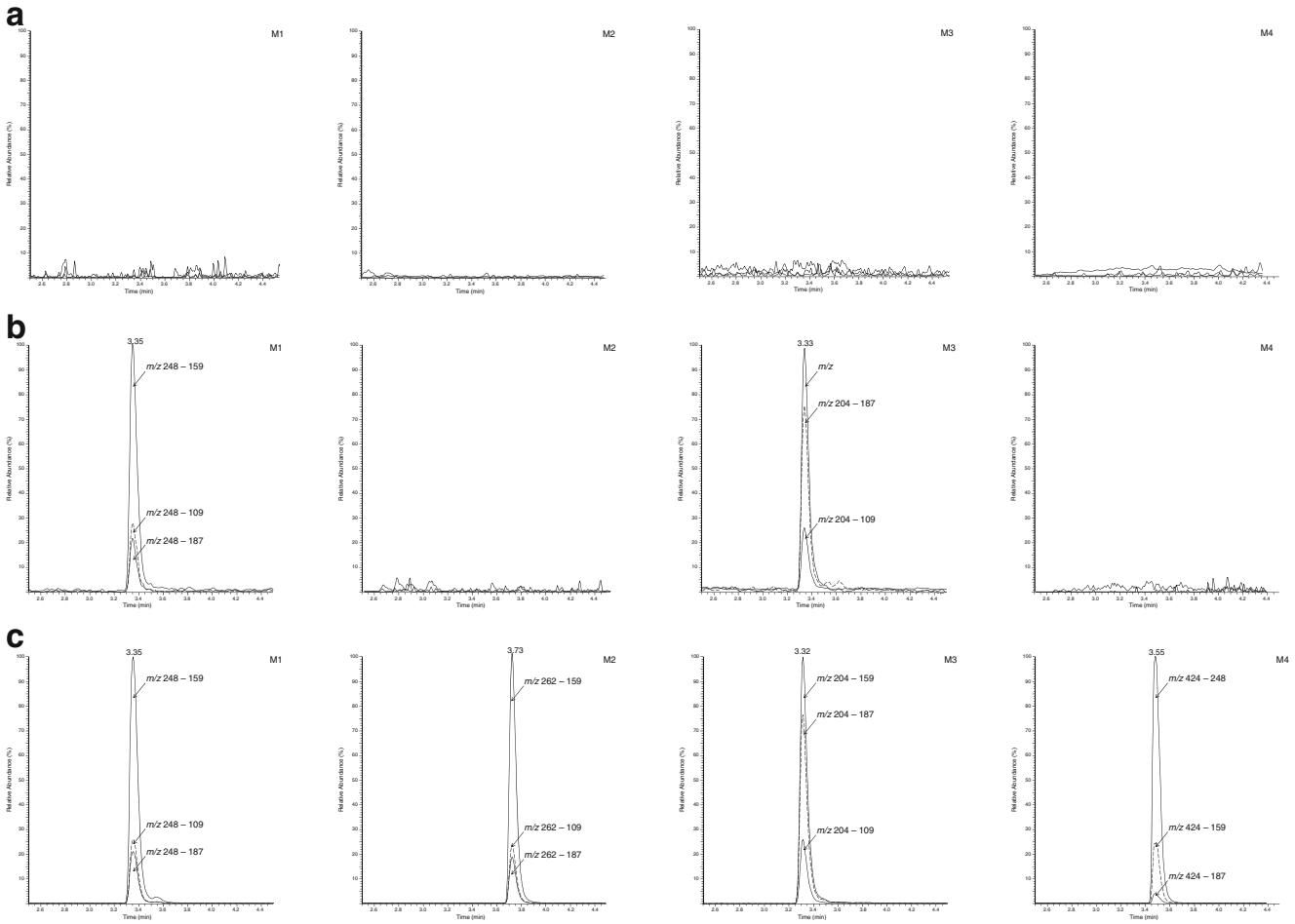


Fig. 4 Extracted ion chromatograms of LC-MS/MS analyses of **a** a blank urine specimen, **b** a urine sample spiked to 50 ng mL^{-1} of M1 and M3, and **c** an excretion study urine sample collected 13 h post administration

allows the simultaneous detection of M1-M4 to confirm the administration of the prohibited stimulant.

LC-MS/MS analysis

By means of urine dilution followed by immediate LC-MS/MS analysis, four major metabolites of benfluorex (M1–M4) are sensitively detected using specific ion transitions (Table 1). As illustrated in Fig. 4, the blank urine specimen (a) does not yield signals at the expected retention times of M1–M4, while the spiked specimen (50 ng mL^{-1} of M1 and M3, (b) as well as the administration study urine sample (13 h post administration, (c) give rise to abundant peaks corresponding to the selected ion transitions. While the active drug was not detected intact in the collected urine specimens, M1 and M3 were observed up to 96 h, M2 for 116 h, and M4 for 69 h after oral application of 300 mg of benfluorex using the presented approach (data not shown). The LLODs of M1 and M3 were estimated at 3 and 5 ng mL^{-1}, respectively, and intra- and interday precisions were better than 21% at all concentration levels (Table 2). Tests for ion suppression/enhancement effects did not show any considerable influence on signal intensities at expected retention times of M1–M4.

Conclusion

The revelation of benfluorex administration in doping controls can be accomplished by means of GC-MS and LC-MS/MS approaches targeting diagnostic metabolites such as hydroxyethylnorfenfluramine (M1, GC-MS and LC-MS/MS), carboxymethylnorfenfluramine (M2, LC-MS/MS only), as well as the glucuronic acid conjugate of M1 (LC-MS/MS only). Initial testing procedures based on GC-MS allow the analysis of M1 and M3, indicating the illicit use of benfluorex by the athlete. Complementary measurements by LC-MS/MS allow further corroboration of the finding by the option to analyze M2 (and M4) that provide substantiating evidence for the application of benfluorex. Moreover, M2 has shown to provide the broadest window of opportunity to detect the administration of benfluorex, although retrospectivity is not of

utmost importance for stimulating agents, which are banned in-competition only. While the presence of M3 can result from applications of other drugs such as fenfluramine, M1, M2, and M4 are unique metabolites of benfluorex.

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