Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 654–658
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Analysis of exogenous nandrolone metabolite in horse urine by gas chromatography/combustion/carbon isotope ratio mass spectrometry

Masayuki Yamada [a,*], Kenji Kinoshita [a], Masahiko Kurosawa [a], Koichi Saito [b], Hiroyuki Nakazawa [b]

[a] *Laboratory of Racing Chemistry, 1731-2 Tsurutamachi, Utsunomiya, Tochigi 320-0851, Japan*
[b] *Department of Analytical Chemistry, Hoshi University, 2-4-41 Ebara, Tokyo 142-8501, Japan*

Received 12 April 2007; received in revised form 2 July 2007; accepted 5 July 2007
Available online 10 July 2007

Abstract

Nandrolone (17β-hydroxy-4-estren-3-one, NAD) is an endogenous steroid hormone; thus, the detection of its metabolites is not conclusive of NAD doping in racehorses. NAD doping control in male horses is based on the threshold, namely, the concentration ratio of 5α-estran-3β,17α-diol (ETA) to 5(10)-estren-3β,17α-diol (ETE). The ETA/ETE ratio of 1/1 was determined based on statistical data of authentic horses in International Federation of Horseracing Authorities. To individuals with complex metabolic disorders, however, such a threshold might not be applicable. The aim of this study was to establish an analytical method that discriminates endogenous steroids from exogenous ones in horse urine after NAD administration using gas chromatography/combustion/carbon isotope ratio mass spectrometry (GC/C/IRMS). Urine was sampled from NAD-administered and authentic horses. Ten millilitres of urine was hydrolyzed and subjected to liquid-liquid extraction and solid phase extraction. The residue of the extracts purified by HPLC was derivatized by acetylation. As a result of measurement of the $^{13}C/^{12}C$ ratio ($\delta^{13}C$) by GC/C/IRMS, the $\delta^{13}C$ values of ETA for NAD-administered and authentic horses were $-32.20 \pm 0.35‰$ and $-27.85 \pm 0.75‰$ ($n = 60$), respectively. The detection limit of ETA in this GC/C/IRMS analysis was approximately 25 ng/ml. This study indicates that the measurement of $\delta^{13}C$ by GC/C/IRMS enables us to discriminate exogenous ETA derived from NAD administration from endogenous ETA, proving that GC/C/IRMS is a useful technique to complement the ETA/ETE ratio.

Keywords: Nandrolone; Horse; Urine; Carbon isotope mass spectrometry

1. Introduction

Nandrolone (17β-hydroxy-4-estren-3-one, NAD) is an anabolic androgenic steroid that may be abused for the purpose of enhancing athletic performance in racehorses and athletes. The metabolism of NAD in the horse has been investigated in detail by Houghton et al. [1,2], who reported that NAD was mainly excreted as 5α-estran-3β,17α-diol (ETA) and 5α-estran-3β,17β-diol of sulfate and glucuronide in horse urine. It is known that NAD is generated in the biosynthesis of estradiol from testosterone in male horses [3]. Thus, its endogenous metabolites, which are not derived from the administration of NAD, are also excreted in urine. For this reason, NAD doping control in male horses has been conducted on the basis of the threshold, namely, the concentration ratio of ETA to 5(10)-estren-3β,17α-diol (ETE), which is an endogenous substance that is not derived from NAD administration. The threshold, ETA/ETE = 1/1, is based on the statistical data of authentic horses in International Federation of Horseracing Authorities. In human sports, the use of testosterone is prohibited by the World Anti-Doping Code [4] defined by the World Anti-Doping Agency (WADA), and the threshold of the concentration ratio of testosterone to 17-epitestosterone has been adopted for its detection. However, in individuals with complex metabolic disorders, the threshold might not be applicable. In spite of test results showing that the threshold has been exceeded in doping tests conducted after an athletic event, it was proved that this was due to complex metabolic disorders, based on the results of continuous out-of-competition tests [5,6]. To control NAD doping in horseracing, a novel method is preferred over the threshold method to improve

* Corresponding author. Tel.: +81 28 647 4471; fax: +81 28 647 4473.
E-mail address: m-yamada@lrc.or.jp (M. Yamada).

0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2007.07.005

judgment of cases and to reduce false-positive results. Therefore, we used the $\delta^{13}C$ values of NAD metabolites measured by gas chromatography/combustion/carbon isotope ratio mass spectrometry (GC/C/IRMS). According to reports in human sports [5–18], the measurement of $\delta^{13}C$ values by GC/C/IRMS enabled discrimination of exogenous steroids from endogenous ones. It is one of the steroid detection methods recommended by the World Anti-Doping Code. In this study, we used GC/C/IRMS to measure the $\delta^{13}C$ values of urinary ETA obtained from NAD-administered racehorses and authentic racehorses. Based on the $\delta^{13}C$ values of urinary ETA, we investigated the applicability of GC/C/IRMS to the NAD doping test for racehorses, to distinguish exogenous ETA from endogenous ETA.

2. Experimental

2.1. Chemicals and reagents

All reagents and solvents were of analytical or HPLC grade (Wako Pure Chemical Industries, Tokyo, Japan or Kanto Kagaku Reagent Division, Tokyo, Japan). The NAD decanoate used was Deca Duramin 50 Injection (50 mg/ampul, Fuji Pharmaceutical, Tokyo, Japan). ETA and 5α-androstan-3β-ol were purchased from Steraloids Inc. (Newport, USA). CO_2 gas (purity is more than 99.999%) for calibrating $\delta^{13}C$ values was purchased from Showa Tansan (Tokyo, Japan). 5α-Androstane with known $\delta^{13}C$ values was a gift from Dr. Arndt Schimmelmann (Indiana University, Indiana, USA). Sep-Pak Plus C_{18} 360 mg/cartridge from Nihon Waters (Tokyo, Japan) was used as SPE column. β-Glucuronidase (from *Pomacea canaliculata*, Type A–I, 22,000 Fishman units/ml) was purchased from Nihon Biotest (Tokyo, Japan).

2.2. Urine samples

All urine samples were stored at temperatures below −40 °C prior to analysis.

2.2.1. Urine samples collected after NAD administration

We used three male horses (A, B, and C) housed at the Equine Research Institute, Japan Racing Association. NAD decanoate was administered intramuscularly to each horse at 0.8 mg/kg. Urine samples were collected prior to NAD administration and at 6 and 12 h, and days 1, 2, 3, 7, 14, 21, 23, 25, and 28 post NAD administration from each horse.

2.2.2. Authentic urine samples

We used the urine samples of post-race male horses submitted for routine doping tests as authentic urine samples. All the urine samples were sent from 13 race courses in Japan after horse racing.

2.3. Sample preparation for GC/C/IRMS

Ten millilitres of urine was adjusted to pH 5 and hydrolyzed with β-glucuronidase purified from *P. canaliculata* for 30 min at 60 °C (11,000 Fishman units). The enzyme-hydrolyzed urine sample was centrifuged at 1663 × *g* for 10 min, and loaded onto Sep-Pak Plus C_{18} (pretreated with 5 ml of methanol and 5 ml of deionized water). After washing the column with 10 ml of water and 5 ml of *n*-hexane, the fraction that was eluted with 5 ml of ethyl acetate was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 5 ml of diethyl ether and was purified twice by liquid–liquid extraction with 2 ml of 1 M NaOH. After evaporating the solvent, the residue was dissolved in 0.2 ml of *n*-hexane/2-propanol (1:1, v/v), and the entire volume of the sample was injected into HPLC (HPLC-I purification). The mobile phase was *n*-hexane/2-propanol (95:5, v/v), the flow rate was 1 ml/min, the column was an Inertsil NH_2 (5 μm, 150 mm × 4.6 mm, GL Science, Japan), and the column temperature was set at 40 °C. HPLC-I eluates were collected from 7 min to 13 min. The fraction was evaporated to dryness under a stream of nitrogen, the residue was dissolved with 0.1 ml of pyridine and 0.1 ml of acetic anhydride, and the reaction was allowed to proceed at 80 °C for 60 min. After removal of the excess reagent, the derivatized residue was dissolved with 0.2 ml of *n*-hexane/2-propanol (1:1, v/v), and the entire volume of the sample was injected into HPLC (HPLC-II purification). The conditions for HPLC-II were the same as those for HPLC-I except for the mobile phase [*n*-hexane/2-propanol (98:2, v/v)]. HPLC-II eluates were collected from 2 min to 3.5 min. The fraction was evaporated to dryness under a stream of nitrogen, and the residue was dissolved in 20 μl of acetonitrile containing 0.1 mg/ml 5α-androstan-3β-ol acetate as the IS, and this was applied to GC/C/IRMS.

If the concentration of ETA in urine were low, we would not be able to obtain reliable $\delta^{13}C$ values using this volume of urine. In this case, we used 20–50 ml of urine samples that were divided into 10 ml portions. Each 10 ml sample was extracted with Sep-Pak Plus C_{18} and residues from the combined extracts were purified according to the methods for the subsequent preparation procedure. The maximum volume of urine sample used was 50 ml because endogenous substances interfered with the GC/C/IRMS analysis at volumes larger than 50 ml.

2.4. GC/C/IRMS analysis

An Isoprime TM isotope ratio mass spectrometer (Micromass, Manchester, UK) connected to an HP 6890 series GC (Agilent Technologies, Tokyo, Japan) equipped with a DB-17 capillary column (J&W Scientific, CA, USA, 0.25 μm, 30 m × 0.25 mm) was used as the GC/C/IRMS system. Two microlitres of the final extract was injected in the splitless mode. The injection temperature was 260 °C. The oven temperature was 150 °C initially (initial hold 2 min), and was increased at 30 °C/min to 250 °C, and then at 5 °C/min to 300 °C (final hold 2 min). The carrier gas was helium at a flow rate of 1 ml/min. After separation, the substance was passed through the interface at 350 °C, and was converted into CO_2 and H_2O in the combustion furnace maintained at 850 °C. The combustion gases were passed through a cryo-trap (−100 °C with liquid nitrogen) for the removal of water. CO_2 molecules were ionized, and the masses of CO_2 (*m/z* 44, 45, 46) were analyzed by mass spectrometry.

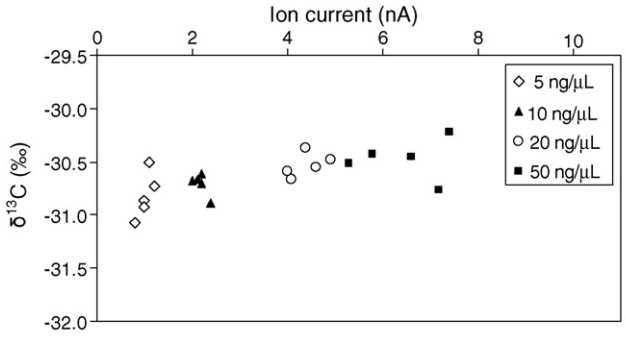

Fig. 1. Relation between the δ^{13}C values and the ion current at *m/z* 44 of ETA.

3. Results

3.1. Validation for GC/C/IRMS analysis

CO_2 calibration gas was analyzed simultaneously with each sample injection. The δ^{13}C value of the used CO_2 gas was measured previously at Taiyo Nippon Sanso (Tokyo, Japan), which had been calibrated against certified reference CO_2 obtained from NBS19. Furthermore, in order to assess the reliability of our GC/C/IRMS system in measuring steroid samples, 5α-androstane ($\delta^{13}C = -31.51‰$, calibrated against certified reference CO_2 obtained from NBS19), which was provided by Dr. Arndt Schimmelmann (Indiana University, Indiana, USA), was measured and its δ^{13}C value was confirmed.

To estimate the linearity and the limit of detection (LOD) of acetylated ETA with the GC/C/IRMS system, within-day analysis of five replicates of ETA (2 μl) at 5, 10, 20, and 50 ng/μl was carried out. The relationship between δ^{13}C value and ion current at *m/z* 44 (I^{44}) is shown in Fig. 1. The results indicate that the I^{44} and δ^{13}C values of 10 and 20 ng/μl samples tended to be stable. The I^{44} values of those two samples ranged from approximately 1.5 to 5 nA, and the δ^{13}C values were $-30.71 \pm 0.12‰$ and $-30.53 \pm 0.11‰$, respectively. Therefore, only the δ^{13}C values of ETA within the above range were adopted. The minimum value of 1.5 nA was equivalent to the I^{44} of ETA that was obtained from the urine sample spiked with 25 ng/ml ETA. Thus, the LOD of ETA in horse urine using our GC/C/IRMS system was 25 ng/ml. Further, to estimate specificity, the δ^{13}C values of ETA were obtained by measuring urine samples spiked with 25 ng/ml and 50 ng/ml ETA, and were $-30.53 \pm 0.23‰$ and $-30.40 \pm 0.16‰$, respectively. These results indicated that the δ^{13}C values of ETA were not influenced by the sample preparation method for GC/C/IRMS.

The recovery rates of ETA using the sample preparation method for GC/C/IRMS shown in Section 2.3 were calculated as follows: standard curves were prepared over the concentration range of 20–1000 ng/ml, and the correlation coefficient was higher than 0.995. The means of the recovery rates were 77.3% (500 ng/ml, $n = 3$) and 63.2% (50 ng/ml, $n = 3$), and their coefficients of variation were 0.25% and 3.3%, respectively.

3.2. δ^{13}C values of exogenous ETA

Prior to GC/C/IRMS analysis of urine samples after NAD administration, the ETA/ETE ratio was measured. We found that urine samples from day 1 or 2 to day 23 after NAD administration had ETA/ETE ≥ 1/1. As shown in Table 1, GC/C/IRMS analysis of urine samples obtained after NAD administration revealed that the δ^{13}C value could be measured not only in all the samples that showed ETA/ETE ≥ 1/1 but also in some samples that showed ETA/ETE < 1/1. The means and S.D.s of the δ^{13}C values of ETA were $-32.13 \pm 0.21‰$ in horse A, $-32.26 \pm 0.48‰$ in horse B, and $-32.05 \pm 0.43‰$ in horse C, respectively. GC/C/IRMS chromatograms are shown in Fig. 2.

3.3. δ^{13}C values of endogenous ETA

The urinary concentration of endogenous ETA in authentic male horses is usually very low and differs with individual horses. For this reason, GC/C/IRMS analysis was performed not in all the authentic urine samples, but only in the urine samples in which endogenous ETA was detected during routine doping tests with GC/MS (LOD 10 ng/ml). We determined the urinary concentrations of endogenous ETA in more than 1000 urine samples submitted for doping tests of racehorses in Japan, and obtained only 60 urine samples for GC/C/IRMS analysis. The mean and S.D. of the endogenous ETA δ^{13}C values of those urine samples were $-27.85 \pm 0.75‰$ and their frequency distribution is shown in Fig. 3.

4. Discussion

The calculation of δ^{13}C values obtained by GC/C/IRMS measurement would not be reliable if peaks of the target substance overlapped with those of endogenous substances on the chromatogram. In order to obtain reliable results in GC/C/IRMS measurement, it is necessary to separate the peaks. Previous studies of doping control in human sports using GC/C/IRMS have shown that HPLC purification was performed twice [18]. However, this method presented some complications and required a long preparation time, because both the HPLC column and the mobile phase had to be changed after each operation. In this study, we carried out the first HPLC purification before acetyl derivatization and the second one after derivatization. As a

Table 1
ETA δ^{13}C values in urine of NAD-administered horses

Horse	pre	6 h	12 h	Day 1	Day 2	Day 3	Day 7	Day 14	Day 21	Day 23	Day 25	Day 28	mean	S.D.
A	–	–	–	−32.02	−31.74	−32.25	−32.47	−32.11	−32.15	−31.99	−32.35	−32.08	−32.13	0.21
B	–	–	−31.14	−32.72	−32.26	−32.53	−32.53	−31.94	−32.57	−32.31	−32.34	–	−32.26	0.48
C	–	–	−31.43	−32.37	−31.65	−31.34	−32.47	−32.23	−32.46	−31.99	−32.2	−32.38	−32.05	0.43

–: calculation was not possible.

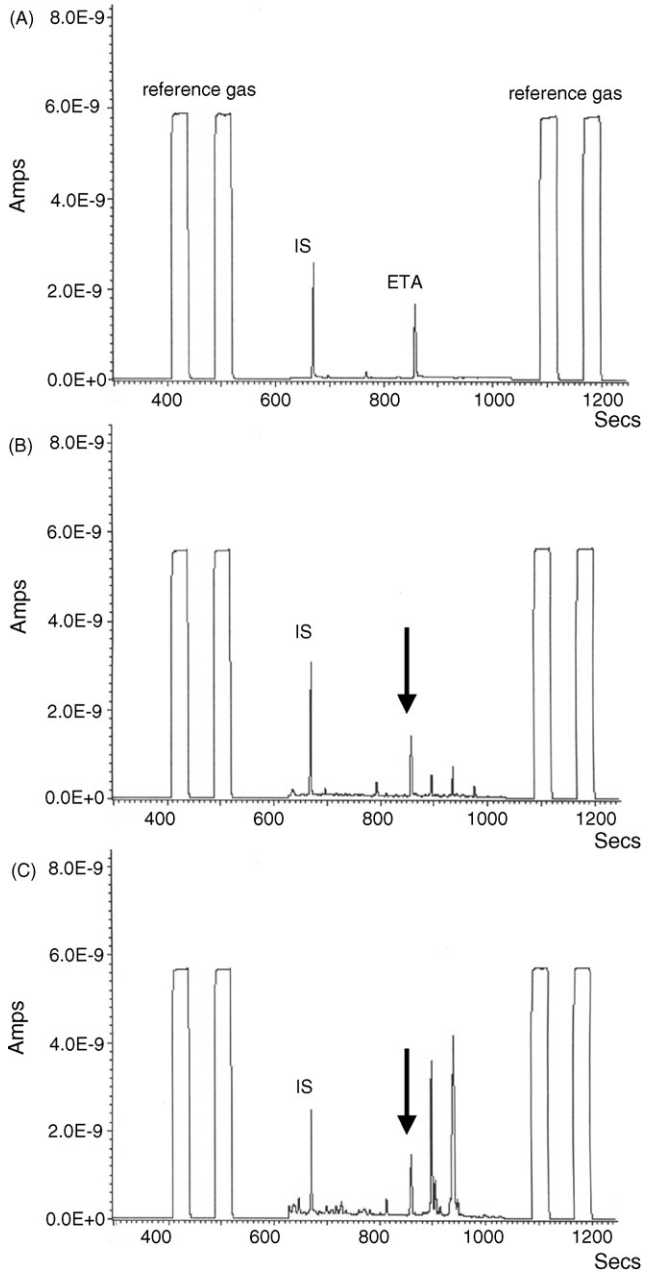

Fig. 2. GC/C/IRMS chromatograms (*m/z* 44), (A) standard ETA, (B) exogenous ETA (horse A, days 23 after NAD administration), and (C) endogenous ETA.

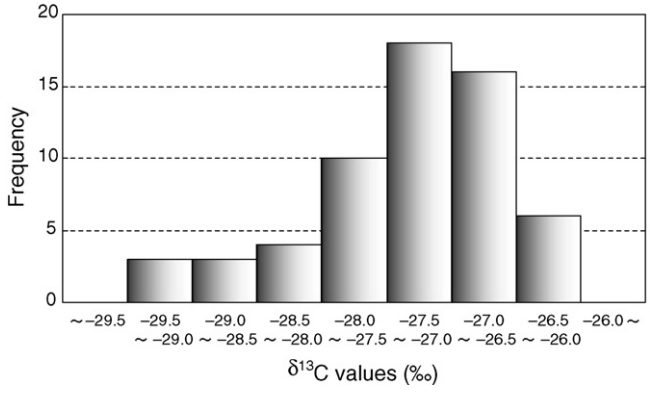

Fig. 3. Frequency distributions of endogenous ETA δ^{13}C values.

result, HPLC purification could be performed twice under almost the same conditions, and efficient purification was achieved.

Previous studies of doping control in human sports using GC/C/IRMS have indicated that comparing the δ^{13}C values between exogenous target substance and endogenous reference substance was useful for positive identification [11,12]. ETE is an endogenous substance that is not derived from NAD administration, and is used as an index of threshold for NAD doping control in male horses. It was present together with ETA in the extracts for GC/C/IRMS. ETE could be used as the endogenous reference substance for NAD doping analysis with GC/C/IRMS in male horses, although the δ^{13}C values of endogenous and exogenous target substances were compared based on statistical analysis in this study.

The minimum δ^{13}C value of the endogenous ETA in this study was −29.30‰, which was slightly different from other δ^{13}C values. This value was considered to be unusual, although it was above the mean − 3.09S.D. of −30.17‰ for endogenous ETA. Balizs et al. reported that the δ^{13}C values of endogenous substance in bovine urine were influenced by food [19]. As racehorses are also herbivores, we speculate that the δ^{13}C values of endogenous substance in horse urine are probably influenced by food as well. Thus, we assumed that an unusual δ^{13}C value could be detected owing to the type of food ingested or to individual differences. Further, in the event that an unusual δ^{13}C value was measured in the doping tests of horse urine, continuous measurement of δ^{13}C value in the same horse should be carried out, and the measured values should be re-evaluated.

5. Conclusion

The δ^{13}C values of endogenous and exogenous ETAs were compared based on statistical analysis, in order to confirm the utility of GC/C/IRMS for the detection of NAD doping. The δ^{13}C value of endogenous ETA is −27.85‰ ± 0.75‰ (mean ± S.D., $n = 60$), and its "mean − 3.09S.D." is −30.17‰. Hence, if the calculated δ^{13}C value of ETA were below −30.17‰, the statistical probability of a false-positive would be below 1/1000. The δ^{13}C values of ETA following NAD administration for all the urine samples in which calculation was possible were below −30.17‰. As far as we know, there are no reports of racehorses with complex metabolic disorders. However, as there are reports of complex metabolic disorders in humans [5,6], the possibility of such disorders occurring in racehorse cannot be completely denied. Our results indicate that the measurement of δ^{13}C values with GC/C/IRMS enables us to discriminate exogenous ETA derived from NAD-administered horses from endogenous ETA. Although the detection of NAD doping in racehorses relies much on the ETA/ETE ratio, GC/C/IRMS is a useful technique to complement the ETA/ETE ratio for the confirmation of NAD doping in racehorses.

References

[1] P. Teale, E. Houghton, Biol. Mass Spectrom. 20 (1991) 109–114.
[2] E. Houghton, in: C.R. Short (Ed.), Proceedings of the 9th International Conference of Racing Analysts and Veterinarians, Dupre's Printing & Copying, Louisiana, 1992, pp. 3–16.

[3] M.C. Dumasia, E. Houghton, M. Jackiw, J. Endocrinol. 120 (1989) 223–229.
[4] WADA, Reporting The 2007 Prohibited List, World Anti-Doping Agency, Montreal, Canada. 2007. http://www.wada-ama.org/rtecontent/document/2007_List_En.pdf.
[5] M. Ueki, Jpn. J. Toxicol. Environ. Health 44 (1998) 75–82.
[6] M. Ueki, M. Okano, Rapid Commun. Mass Spectrom. (1999) 2237–2243.
[7] M. Becchi, R. Aguilera, Y. Farizon, M.M. Flament, H. Casabianca, P. James, Rapid Commun. Mass Spectrom. 8 (1994) 304–308.
[8] R. Aguilera, M. Becchi, C. Grenot, H. Casabianca, C.K. Hatton, J. Chromatogr. B 687 (1996) 43–57.
[9] R. Aguilera, M. Becchi, H. Casabianca, C.K. Hatton, D.H. Catlin, B. Starcevic, H.G. Rope, J. Mass Spectrom. 31 (1996) 169–176.
[10] C.H.L. Shackleton, E. Roitman, A. Phillips, T. Chang, Steroids 62 (1997) 665–673.
[11] R. Aguilera, D.H. Catlin, M. Becchi, A. Phillips, C. Wang, R.S. Swerdloff, H.G. Rope, C.K. Hatton, J. Chromatogr. B 727 (1999) 95–105.
[12] E. Bourgogone, V. Herrou, J.C. Mathurin, M. Becchi, J. de Ceaurriz, Rapid Commun. Mass Spectrom. 14 (2000) 2343–2347.
[13] J.C. Mathurin, V. Herrou, E. Bourgogne, L. Pascaud, J. de Ceaurriz, J. Chromatogr. B 759 (2001) 267–275.
[14] M.C. Desroches, J.C. Mathurin, Y. Richard, P. Delahaut, J. de Ceaurriz, Rapid Commun. Mass Spectrom. 16 (2002) 370–374.
[15] R. Aguilera, C.K. Hatton, D.H. Catlin, Clin. Chem. 48 (2002) 629–636.
[16] A. Maitre, C. Saudan, P. Mangin, M. Saugy, J. Anal. Toxicol. 28 (2004) 426–431.
[17] A.T. Cawley, R. Kazlauskas, G.J. Trout, J.H. Rogerson, A.V. George, J. Chromatogr. Sci. 43 (2005) 32–38.
[18] M. Hebestreit, U. Flenker, G. Fussholler, H. Geyer, U. Guntner, U. Mareck, T. Piper, M. Thevis, C. Ayotte, W. Schanzer, Analyst 131 (2006) 1021–1026.
[19] G. Balizs, A. Jainz, P. Horvatovich, J. Chromatogr. A 1067 (2005) 323–330.