Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 785 (2003) 245–251
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Urinary concentrations of morphine after the administration of herbal teas containing *Papaveris fructus* in relation to doping analysis

W. Van Thuyne, P. Van Eenoo, F.T. Delbeke*

Doping Control Unit, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

Received 16 August 2002; received in revised form 21 October 2002; accepted 31 October 2002

Abstract

A quantitative method for the analysis of morphine in human urine in the concentration range between 0.25 and 2 μg/ml is described and validated. Morphine was determined after enzymatic hydrolysis of the urine. After liquid–liquid extraction with dichloromethane–methanol (9:1) at pH 9.5, morphine was derivatized with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and analyzed with GC–MS (full scan). The limit of quantification of the method was 0.25 μg/ml. Two *Papaveris fructus* containing herbal teas were administered to five male volunteers and urine samples were taken quantitatively during the first 12 h after the administration. The morphine concentration in the tea was 10.4 and 31.5 μg/ml, respectively. Morphine was detected in the urine of all volunteers by 1 h after drinking the tea. Maximum morphine concentrations, 4.3 and 7.4 μg/ml, respectively, were obtained 4–6 h after administration. Doping positive urine samples were delivered for 1–9 h.

Keywords: Doping analysis; Morphine

1. Introduction

Morphine, extracted from the plant *Papaver somniferum*, is a narcotic analgesic commonly used for the short-term treatment of post surgery pain and in the long term for the pain relief of cancer patients [1]. Because of this painkilling effect morphine can be misused by athletes during training or in competition to overcome pain associated with strenuous exercises. This was the major reason why morphine was included in the IOC list of banned substances. Before 1991, the presence of morphine in human urine, irrespective of its concentration, resulted in a positive doping test. This changed in 1991 because the International Cycling Federation introduced a 1 μg/ml threshold level for morphine as well as for codeine, one of the precursors of morphine. The rules were later on adapted to the rules that are now in force. At present the use of codeine and ethylmorphine, another morphine precursor, is allowed while morphine is forbidden in concentrations higher than 1 μg/ml.

GC–MS is one of the recommended analytical techniques for the identification, quantification and

*Corresponding author. Tel.: +32-9-264-7347; fax: +32-9-264-7497.
E-mail address: frans.delbeke@rug.ac.be (F.T. Delbeke).

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00910-8

confirmation of opiates including morphine, in urine [2–5]. Hydrolysis is required to obtain unconjugated morphine, as morphine is excreted in urine as its 3- and 6-glucuronides. Enzymatic hydrolysis with β-glucuronidase has proven to give the best result [6]. To accomplish GC separation of morphine, the phenolic hydroxy group of morphine must be derivatized before GC separation and detection. Various derivatization procedures, including silylation [7] have been described in the literature.

In 1991 a study was carried out by Delbeke and Debackere [8] proving that morphine positive urine samples could be delivered 6–9 h after the administration of a therapeutic dose of codeine. A study by Selavka [9] dealt with the possible contribution of poppy seed containing food to positive opiate urinalysis results. This ''poppy seed defense'' was mainly used by job-applicants controlled by the U.S. Department of Defense or by the National Institute on Drug Abuse [10–13]. Several other experiments were also carried out on the detection of opiates, mainly morphine, after the use of various poppy seed containing food products including cakes and bread rolls [14–17]. The major results from these investigations were that morphine could be detected after the use of poppy seed incorporated in food products but that the resulting concentrations of morphine in urine were too low to result in a positive urine sample. Another conclusion was that the morphine concentration of the poppy seeds could substantially differ depending on the origin of the investigated seed. Although several studies dealt with the detection of morphine after the use of poppy seed containing food [9–17], no other related studies were found describing the possible contribution of other parts of the plant *P. somniferum* in positive opiate results. The objective of the present study was to determine whether doping positive urine samples could be delivered after the administration of herbal tea containing parts from the plant *P. somniferum*. The quantitative GC–MS method was validated according to ISO 17025 norms.

2. Experimental

2.1. Reagents

Morphine.HCl was purchased from Lipomed (Arlesheim, Switzerland) and nalorphine.HBr was obtained from Janssen Pharmaceuticals (Beerse, Belgium). The enzyme preparation β-glucuronidase type HP-2 from *Helix pomatia* (127 300 U/ml β-glucuronidase) was obtained from Sigma (St. Louis, MO, USA). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was bought from Chem. Fabrik. Karl Bucher (Waldstetten, Germany). All other chemicals were of analytical grade.

The herbal teas were bought at a local chemist. The labeled content of the first tea (A) (Tisane Ardennaise, Tilman, Bomal, Belgium) was as follows: *Papaveris fructus*: 15%; *Crataegi folium* 12.5%; *Passiflorae mexico herba* 10%; *Viscum album* 7.5%; *Oleae europaea folium* 13.75%; *Cynodon dactylon rhizae* 12.5%; *Triticum rhizae* 1.25%; *Frangulae cortex* 13.75%; *Glycyrrhizae radix* 13.75%. The recommended dosage was one to three cups a day. The content of the second tea (B) (Thé de sapin, Laboratoires Colin, Blegny, Belgium) was: *Rubi fructicosi folium* 31.4%; Carraghene 24%; *P. fructus* 14.7%; *Thymi serpylli herba* 7.8%; *Eucalypti folium* 7.8%; *G. radix* 6.9%; *Anisi stellati fructus* 3%; *Hederae terrestris herba* 1.2%; *Erysimi herba* 1.2%; *Capilli veneri herba* 1%; *Salviae folis* 1%; cum pini essent imbuta. A dosage of one to three cups a day was recommended.

2.2. Gas chromatographic conditions

The GC–MS analysis was conducted in full scan mode on an HP 6890 gas chromatograph directly coupled to an HP 5973 mass selective detector (HP, Palo Alto, USA). The GC column was a HP-Ultra 1 (J&W, Folsom, USA), 100% methylsilicone column with a length of 17 m, an internal diameter of 0.2 mm and a film thickness of 0.11 μm. Helium was used as the carrier gas (linear velocity: 41 cm/s). A total of 0.5 μl was injected splitless. The oven temperature program was as follows: 70 °C (0 min), 70 °C/min→96 °C (1 min), 20 °C/min→320 °C (1 min). The electron energy was set at 70 eV and the ion source temperature was 230 °C.

2.3. Urine analysis

A 1 ml sample of a sodium acetate buffer (pH 5.2, 1 *M*) and 50 μl β-glucuronidase were added to 3 ml of urine. The mixture was hydrolyzed during 2.5 h at

56 °C. After cooling, 50 μl of the internal standard (nalorphine, 20 μg/ml, MeOH) and 0.5 ml of an ammonium buffer (pH 9.5) were added. Extraction was performed with 5 ml of the mixture CH_2Cl_2–MeOH (9:1) by rolling for 20 min. After centrifugation (1200 *g*, 5 min) the organic layer was separated, dried over anhydrous Na_2SO_4 and evaporated under oxygen free nitrogen. The residue was derivatized with 100 μl MSTFA at 80 °C for 10 min and transferred to an autosampler microvial.

2.4. Analytical method validation

An equal weighted linear calibration curve (not forced through zero) was established in the concentration range between 0 and 2 μg/ml (0, 0.25, 0.50, 1.00, 1.50 and 2.00 μg/ml) by plotting the relative abundances of the ions *m*/*z* 429 and 455 for morphine and nalorphine, respectively. Therefore, negative urine, checked for the presence of morphine and nalorphine, spiked with the appropriate amount of morphine standards (100 and 10 μg/ml in MeOH), was analyzed. Each concentration was analyzed in triplicate, and the averages were used to construct the calibration curve. The precision was evaluated by the determination of the repeatability and the reproducibility. To measure the repeatability six samples at three different concentrations (0.25, 1.00 and 2.00 μg/ml) were analyzed. The reproducibility was examined by analyzing three times six samples at the same concentrations as for the repeatability. This was done by different analysts, at different times. Selectivity was tested by the analysis of negative urine spiked with structural related compounds such as codeine and ethylmorphine and some other compounds including methadone, pholedrine, pethidine, prolintane, etc. Specificity was tested by analyzing 20 different negative urines.

2.5. Morphine content of tea

A 0.5 ml sample of an ammonium buffer (pH 9.5) and 50 μl of the internal standard nalorphine (20 μg/ml, MeOH) were added to 3 ml of the prepared tea. Extraction was performed with 5 ml of CH_2Cl_2–MeOH (9:1) by rolling for 20 min. The organic layer was separated after centrifugation (1200 *g*, 5 min), dried over anhydrous Na_2SO_4 and evaporated under oxygen free nitrogen. The residue was derivatized with 100 μl MSTFA at 80 °C for 10 min. The herbal teas were extracted and analyzed in duplicate by this method. Tea A was diluted 20 times with distilled water, tea B 30 times.

2.6. Excretion studies

The study was performed on five healthy male volunteers. The purpose of the study was explained to each volunteer who was asked not to use any morphine, codeine or ethylmorphine containing preparations, nor to eat food containing poppy seeds for 1 week before the start of the experiment. Both teas were made following the instructions on the package. A total of 12 tablespoons of tea A were infused in 12 cups of boiling water for 10 min after which the liquid was sieved. Each volunteer had two cups of this tea, ~120 ml/cup. Urine samples were collected before (0 h) and quantitatively after 1, 2, 4, 6, 9, and 12 h. An additional sample was taken after 24 h. Tea B was prepared by pouring 1.5 l boiling water over a filter containing 22 tablespoons of the tea. Each volunteer drank two cups of tea each 130 ml. Urine sampling was similar as in the previous experiment except that no sample was taken after 24 h. All urine samples were either analyzed directly or stored deep-frozen for later analysis. Urinary pH, volume and density were measured and all samples were analyzed in duplicate. When appropriate, urine samples were diluted with distilled water before extraction in order to obtain a concentration in the range of the calibration curve.

3. Results and discussion

Under the chromatographic conditions described before, morphine as well as the internal standard nalorphine gave sharp peaks with retention times of 9.35 and 9.84 min, respectively (Fig. 1). Quantification of the samples was done by determination of the relative abundance of the molecular ion *m*/*z* 429 of TMS-derivatized morphine to *m*/*z* 455 of the internal standard nalorphine. Unequivocal determination in doping analysis is mostly based on full scan mass spectrometry. The spectrum of TMS-derivatized morphine is shown in Fig. 2. Other diagnostic ions besides the molecular ion are the ions with *m*/*z* 236, 287, 324, 401 and 414. A maximal relative deviation

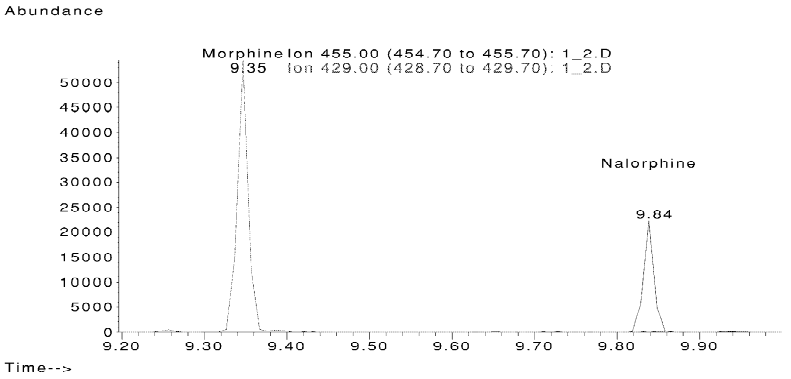

Fig. 1. Chromatogram of morphine (m/z 429) and nalorphine (m/z 455) in urine 1 h after the administration of herbal tea B.

of 20% in the intensities of these diagnostic ions was used as a qualitative criterion for the relative abundances when compared to a quality control sample spiked with morphine at 1 μg/ml. All urine samples from the method validation and the excretion studies in which morphine was detected fulfilled this qualitative criterion.

The correlation coefficient of the calculated calibration curve was 0.9983. The deviation of the mean measured concentration to the theoretical concentration (trueness) was far below the maximal value of 15% [18]. The relative standard deviation, an indicator of the repeatability, for each of the three concentrations (0.25, 1.00 and 2.00 μg/ml) never exceeded the limit values derived from the Horwitz equation [19]. Measured concentrations, relative standard deviations and maximal limits for the repeatability are listed in Table 1. The results for the reproducibility are summarized in Table 2. The original limit values calculated by the Horwitz

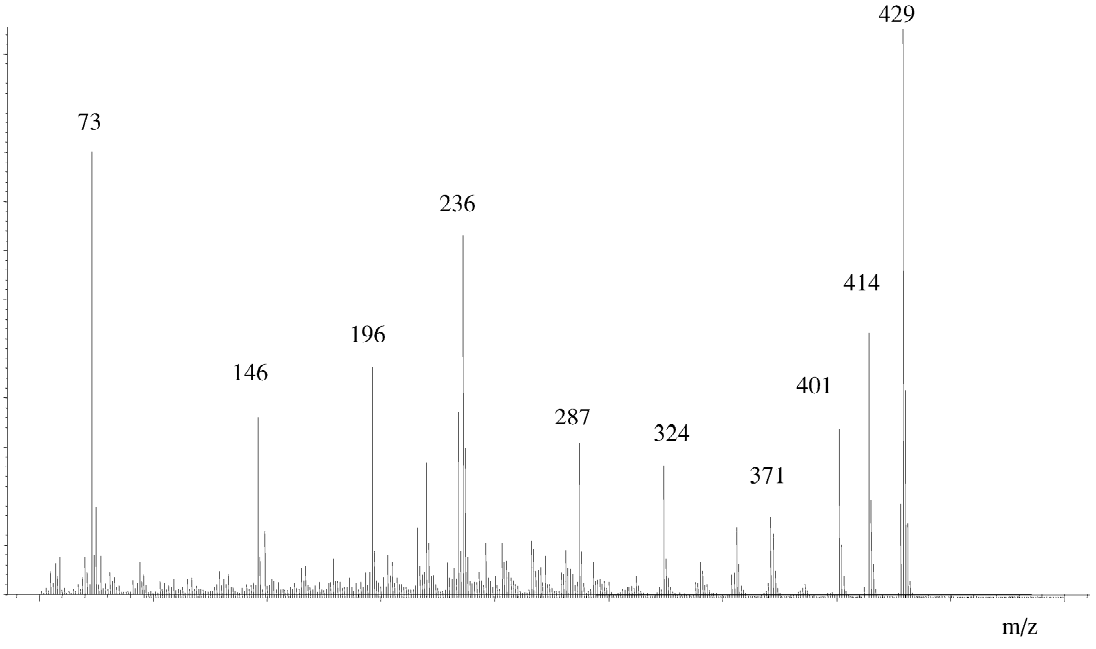

Fig. 2. Mass spectrum of TMS-derivatized morphine.

Table 1
Method repeatability

Theoretical concentration (μg/ml)	0.25	1.00	2.00
Average measured concentration (μg/ml) ($n=6$)	0.25	0.95	2.04
SD (μg/ml)	0.01	0.03	0.06
RSD (%)	3.82	3.09	2.97
2/3 RSD_{max} (%)	13.10	10.67	9.61
Accuracy (%)	0.19	−5.03	1.94

Table 2
Reproducibility of the method

Theoretical concentration (μg/ml)	0.25	1.00	2.00
Average measured concentration (μg/ml) ($n=18$)	0.24	0.91	1.99
SD (μg/ml)	0.01	0.04	0.05
RSD (%)	2.88	4.30	2.59
RSD_{max} (%)	19.65	16.01	14.42
Accuracy (%)	−2.53	−9.49	−0.42

equation are used while for the repeatability the margin was narrowed to two-thirds of the calculated value. For both repeatability and reproducibility the accuracy was measured and is listed in the respective tables. A limit value of 15% is used, similar as for the trueness of the data points of the calibration curve.

No interference by any of the structural related or other compounds spiked in negative urine could be found for morphine or for the internal standard nalorphine. Hence, the method was selective for morphine and nalorphine. Analysis of 20 different negative urines did not result in the detection of matrix interference, proving the specificity of the method.

The limit of quantification of the method was 0.25 μg/ml or the lowest point of the calibration curve where morphine could be detected in a reproducible way. The limit of detection was set at 0.125 μg/ml, half the limit of quantification.

Analysis of tea A resulted in a morphine concentration of 10.4 μg/ml, equivalent to the ingestion of 2.5 mg of morphine when two cups of 120 ml were administered. Tea B contained 31.5 μg/ml morphine, resulting in the ingestion of 8.51 mg after two cups.

The urinary excretion profiles of morphine during the first 12 h after the administration of herbal tea A are shown in Fig. 3. Morphine could already be detected in the urine of all volunteers at 1 h and remained detectable for at least 24 h. Subject 1 delivered a doping positive urine sample after 6 h while morphine in the urine of four volunteers already exceeded the 1 μg/ml doping threshold 1 h after drinking the tea. The maximum morphine concentration that was found after drinking this herbal tea was 4.34 μg/ml. Morphine positive urine samples were detected up to 6 h. All subjects tested negative for morphine after 9 h. Urinary excretion

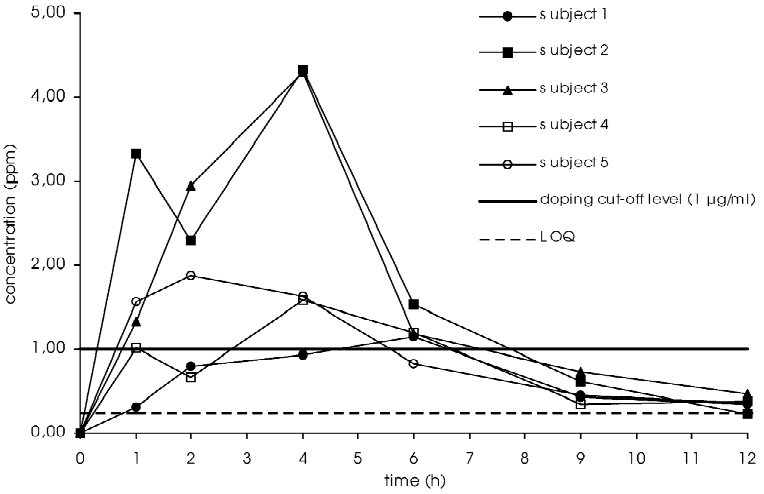

Fig. 3. Urinary morphine concentration profiles after drinking two cups of herbal tea A in five subjects.

Table 3
Amounts (mg) and excretion rates (mg/h) of morphine in the urine of five subjects after drinking herbal tea A

Time interval (h)	Excreted amount (mg)					Excretion rate (mg/h)				
	1	2	3	4	5	1	2	3	4	5
0–1	0.18	0.30	0.17	0.14	0.16	0.18	0.30	0.17	0.14	0.16
1–2	0.26	0.41	0.25	0.21	0.32	0.26	0.41	0.25	0.21	0.32
2–4	0.36	0.43	0.28	0.25	0.40	0.18	0.22	0.14	0.12	0.20
4–6	0.13	0.08	0.07	0.11	0.20	0.07	0.04	0.04	0.06	0.10
6–9	0.13	0.12	0.06	0.03	0.11	0.04	0.04	0.02	0.01	0.04
9–12	0.15	0.21	0.07	0.07	0.09	0.05	0.07	0.02	0.02	0.03
Total	1.20	1.55	0.91	0.80	1.29					

and excretion rates of morphine during the first 12 h after drinking this tea are given in Table 3. Maximum excretion was obtained after 2–4 h. Inter-individual differences in excretion of morphine were observed. An average amount of 1.15 mg morphine was excreted during the first 12 h. Based on the ingested amount of 2.5 mg, 46% of the administered dose was excreted during that period.

Similar to the previous experiment, morphine could already be detected in the urine of all volunteers 1 h after drinking tea B and remained detectable after 12 h (maximum concentration 7.44 μg/ml) (Fig. 4). Three subjects tested doping positive for morphine after 1 h. Similar results were found until 6–9 h after drinking the tea, and doping negative results were found after 12 h. The excreted amounts and excretion rates of morphine are listed in Table 4.

As illustrated in Figs. 3 and 4 doping positive results were obtained after drinking herbal tea. Unlike tea A, the package of tea B did not mention any sedative or calming effect. An athlete should be aware of the presence and the possible detection of morphine after the use of these kinds of teas.

Problems with positive doping cases for morphine are frequently connected with the therapeutic use of its precursors codeine and ethylmorphine, substances no longer considered as doping agents. As codeine and ethylmorphine are allowed, the actual doping rules for morphine need to be revised. Hence, the simultaneous presence of morphine and codeine or morphine and ethylmorphine should not constitute a doping offense. To differentiate between the accidental intake of morphine by poppy seeds [9] or herbal teas and the oral or systemic administration of morphine, the actual doping threshold level for

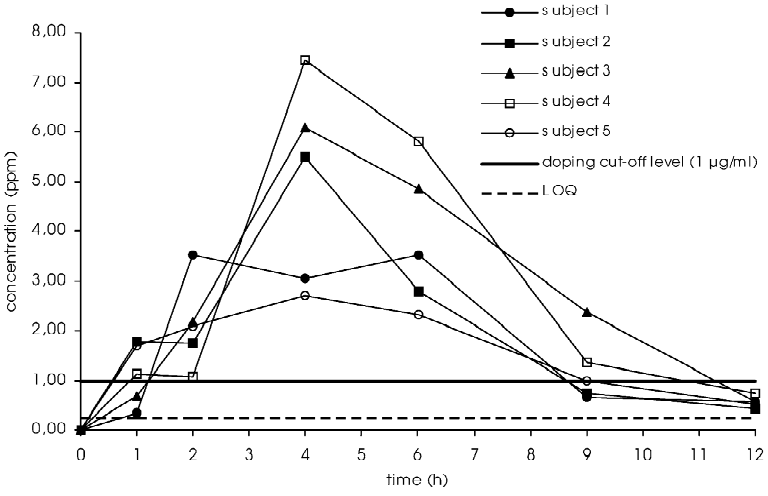

Fig. 4. Urinary concentration profiles of morphine after the administration of two cups of herbal tea B in five subjects.

Table 4
Amounts (mg) and excretion rates of morphine in the urine of five volunteers after drinking herbal tea B

Time interval (h)	Excreted amount (mg)					Excretion rate (mg/h)				
	1	2	3	4	5	1	2	3	4	5
0–1	0.22	0.36	0.44	0.18	0.22	0.22	0.36	0.44	0.18	0.22
1–2	0.48	0.63	0.61	0.31	0.53	0.48	0.63	0.61	0.31	0.53
2–4	0.87	0.88	0.76	0.37	0.82	0.43	0.44	0.38	0.19	0.41
4–6	0.40	0.44	0.36	0.29	0.39	0.20	0.22	0.18	0.15	0.20
6–9	0.29	0.28	0.43	0.16	0.33	0.10	0.09	0.14	0.05	0.11
9–12	0.20	0.13	0.23	0.09	0.10	0.07	0.04	0.08	0.03	0.03
Total	2.45	2.72	2.82	1.41	2.40					

morphine in urine of 1 μg/ml should be increased. Following international doping rules athletes remain responsible for the presence of doping substances in their bio-fluids, irrespective of the origin. Hence, athletes should be warned against the use of food products containing poppy seeds or herbal teas containing parts from the plant *P. somniferum*.

Acknowledgements

The authors wish to thank the Flemish Ministry of Health for financial support (W.V.T.). The technical assistance of D. D'Haenens and N. Desmet is gratefully acknowledged.

References

[1] World Health Organization, Cancer Pain Relief Program, WHO, Geneva, 1986.
[2] A. Solans, R. De La Torre, J. Segura, J. Pharm. Biomed. Anal. 8 (1990) 905.
[3] R. Meatherall, J. Anal. Toxicol. 23 (1999) 177.
[4] C. Meadway, S. George, R. Braitwaite, Forensic Sci. Int. 127 (2002) 136.
[5] G. Ceder, A.W. Jones, J. Forensic Sci. 47 (2002) 366.
[6] F.T. Delbeke, M. Debackere, J. Pharm. Biomed. Anal. 11 (1993) 339.
[7] A. Clarke, R.L. Foltz, Clin. Chem. 20 (1974) 465.
[8] F.T. Delbeke, M. Debackere, J. Pharm. Biomed. Anal. 9 (1991) 959.
[9] C.M. Selavka, J. Forensic Sci. 36 (1991) 685.
[10] M.A. ElSohly, A.B. Jones, Forensic Sci. Rev. 1 (1989) 13.
[11] L.W. Hayes, W.G. Krasselt, P.A. Mueggler, Clin. Chem. 33 (1987) 806.
[12] B.C. Pettitt, S.M. Dyszel, L.V.S. Hood, Clin. Chem. 33 (1987) 1251.
[13] H.N. ElSohly et al., J. Forensic Sci. 33 (1988) 347.
[14] K.D. Meneely, J. Forensic Sci. 37 (1992) 1158.
[15] D.S. Lo, T.H. Chua, Med. Sci. Law 32 (1992) 296.
[16] M.G. Pelders, J.J. Ros, J. Forensic Sci. 41 (1996) 209.
[17] C. Meadway, S. George, R. Braitwaite, Forensic Sci. Int. 96 (1998) 29.
[18] F. Bressole, M. Bromet-Petit, M. Audrau, J. Chromatogr. B 68 (1996) 3.
[19] R.J. Heitzman, in: Veterinary Drug Residues Report EUR 15127-EN, Residues in Food Producing Animals and Their Products: Reference Materials and Methods, Office for Official Publications of the European Communities, Luxembourg, 1994, p. 517.