Forensic Science International xxx (2010) xxx–xxx

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Segmental hair analysis using liquid chromatography–tandem mass spectrometry after a single dose of benzodiazepines

Ping Xiang [a,b,*], Qiran Sun [a], Baohua Shen [a], Peng Chen [c], Wei Liu [a], Min Shen [a]

[a] Department of Forensic Toxicology, Institute of Forensic Sciences, Ministry of Justice, Shanghai Key laboratory of Forensic Medicine, China
[b] Department of Forensic & Investigative Science, University of Central Lancashire, UK
[c] Zhejiang Province Wenzhou Public Security Bureau, China

ARTICLE INFO

Article history:
Received 16 January 2010
Received in revised form 26 April 2010
Accepted 28 April 2010
Available online xxx

Keywords:
Segmental hair analysis
Benzodiazepines
A single dose
Liquid chromatography–tandem mass spectrometry
DFC

ABSTRACT

In China, benzodiazepines are the most frequently observed compounds in cases of drug-facilitated crime. Sensitive, specific, and reproducible methods for the quantitative determination of 18 benzodiazepines in hair have been developed using LC–MS/MS. Fourteen volunteers had ingested a single 1–6 mg estazolam tablet. Hair was collected 1 month after administration. All the proximal segments were positive for estazolam. With increased dosage, estazolam can be detected in the 2–4 cm segments in some subject's hair. Even some of 4–6 cm segments were positive. Hair analysis was applied to two authentic criminal cases. Full-length hair samples collected 5 weeks after the offense were cut into segments of 2 cm from the root, analyzed and quantified. The clonazepam concentrations measured in the first two segments for V#1 and V#2 were 15.47 and 11.93 pg/mg, respectively. However, both the 4–6 cm and the 6–8 cm segment of V1# remained positive, while those of V#2 were negative. It needs more substantial guidelines to use segmental hair analysis in drug-facilitated crime.

1. Introduction

Drug-facilitated crime (DFC) is a big problem in China. The majority of the cases are robberies. In addition, drug-facilitated sexual assaults (DFSA) have been increasingly reported. In China, benzodiazepines are the most frequently observed compounds in cases of drug-facilitated crime [1–4].

Benzodiazepines are the most widely and frequently prescribed sedative and hypnotic drugs worldwide and are, therefore, readily available. Their main pharmacological actions are hypnotic, antianxiety, muscle relaxant, and anticonvulsant effects. Benzodiazepines may also induce anterograde amnesia at therapeutic doses, with the risk increasing at higher dosages [5].

Blood and urine are the conventional specimens for documenting drug exposures [6–8]. In most cases, because of amnesia caused by drugs, there will be a 24–72 h or longer delay between a victim's report and the drug's ingestion. In addition, drugs used can be difficult to detect because low does were administered, or the active metabolite is chemically unstable. Some drugs are quickly cleared from the body fluids [9]. Therefore, in such circumstances, blood, and even urine samples are often of limited usefulness in detecting drugs' presence. To prolong the window of detection, hair analysis has been proven to be a solution. Actually, many publications on DFSA cases have demonstrated the usefulness of hair analysis in documenting the involvement of drug(s)/poison(s) [9–16].

Major progress achieved in the detection of benzodiazepines or hypnotics in hair following a single dose is a result of applying liquid chromatography (LC) coupled to MS/MS in forensic laboratories [17]. Kintz and coworkers have published a series of papers, including a general screening procedure [18] and specific methods for bromazepam [13,19], zolpidem [9], zopiclone [20] and alprazolam [15]. They recommended to cut the strand into three segments of 2 cm in order to document any drug-facilitated sexual assault case. Administration of a single dose would be confirmed by the presence of the drug in the proximal segment (root), with no detection in the other segments [17].

In the framework of the setup of a "segmental analysis" procedure based on the recommendations, our purpose was to devise a validated LC–MS/MS method for confirmation and quantification of 18 benzodiazepines in hair, and to apply the developed method to determine the levels of benzodiazepine in hair segments of (i) healthy volunteers after a single intake of estazolam and (ii) victims of two actual crimes.

* Corresponding author at: Department of Forensic Toxicology, Institute of Forensic Sciences, Ministry of Justice, Guangfu Xi Road 1347, Shanghai 200063, PR China. Tel.: +86 021 52352955; fax: +86 021 52352955.
E-mail address: xiangping2630@163.com (P. Xiang).

0379-0738/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.forsciint.2010.04.046

2. Materials and methods

2.1. Chemicals and reagents

Alprazolam, α-hydroxyalprazolam, midazolam, α-hydroxymidazolam, triazolam, α-hydroxytriazolam, estazolam, diazepam, nordiazepam, temazepam, oxazepam, clonazepam, 7-aminoclonazepam, flunitrazepam,7-aminoflunitrazepam, nitrazepam, 7-amnionitrazepam, flurazepam and diazepam-d5 were purchased from Cerilliant (Round Rock, TX, USA) and the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile, methanol, ammonium acetate, and formic acid were obtained from Fluka Chemical Co. (Buchs, Switzerland). Other reagents were all of analytical-reagent grade and no further purification was undertaken. Deionized water was purified using a Milli-Q system (Millipore, MA, USA).

2.2. Sample collection

2.2.1. Healthy volunteers

Fourteen healthy volunteers (ages 23–27 years) were recruited into the study. Subjects agreed to participate in the experimental part of the study through oral informed consent. All protocols were approved by an institutional review committee. Some background data were collected from the subjects such as the frequency of washing hair and the type(s) of hair cosmetic products used. Most subjects have straight black hair except for one female who has curled hair.

Blood samples were obtained before and at 1–8 h after a single oral dose of estazolam tablets (1 mg/tablet) with doses ranged from 1 to 6 mg were used in the study.

Hair was collected 1 month after administration. Strands of about 100 hairs were cut from the posterior vertex as close as possible to the scalp, oriented, and stored in clean paper bag at room temperature.

2.2.2. DFSA case

Two girls (V#1 and V#2) were invited to have a night time snack. After drinking a soft drink, they were unconscious. When they woke up, they realized that they had been raped and went to the police 18 h later. At the medicolegal unit of the hospital, blood samples were collected that revealed the presence of clonazepam and its metabolite 7-aminoclonazepam, confirming their previous declarations. When confronted with evidence of positive blood results, the suspects confessed. This was one criminal gangs case. Hair samples were collected 5 weeks after the offense.

Table 1
LC mobile phase gradient composition.

LC run time (min)	Acetonitrile (%)	Ammonium acetate buffer (%)[a]
0	5	95
3	60	40
7	80	20
20	80	20
20.5	5	95
30	5	95

[a] 20 mM ammonium acetate buffer with 0.1% formic acid, pH 4.0.

2.3. Sample preparation

2.3.1. Hair samples

Hair was segmented and rinsed twice with 5 ml dichloromethane. The last wash was stored for further analysis. After being air-dried, the segments were cut into small pieces of less than 3 mm, and pulverized in a freeze mill (Freezer/Mill, SPEX CertiPrep). 20 mg of powered hair was sonicated in an ultrasonic bath in 1 ml of phosphate buffer, pH 8.4, for 1 h at room temperature, in the presence of 0.4 ng diazepam-d5 as internal standard (IS). Liquid–liquid extraction was performed with 3 ml of dichloromethane. After solvent evaporation at 60 °C, the residue was reconstituted with 100 μl of acetonitrile–20 mM ammonium acetate (70:30, v/v), and 5 μl was injected into the LC–MS/MS system.

2.3.2. Blood samples

The extraction procedure was the same as that previously reported [21]. To 1 ml of blood sample, 10 ng diazepam-d5 (IS) and 2 ml of sodium borate buffer, pH 9.2, were added. Liquid–liquid extraction was performed with 3 ml of diethyl ether.

Table 2
MRM transitions, condition and retention time for benzodiazepines.

Compound	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)	Dwell time (ms)	DP (V)	CE (eV)	Rt (min)
Diazepam	285.1	193.3	30	60	45	9.91
		154.1	30		36	
Oxazepam	287.2	241.2	30	50	31	8.55
		269.3	30		36	
Nordiazepam	271.2	140.2	30	60	36	9.14
		208.1	30		36	
Temazepam	301.2	255.2	30	70	36	9.25
		283.1	30		19	
Clonazepam	316.2	270.1	30	65	36	9.11
		214.1	30		49	
7-Aminoclonazepam	286.1	222.2	30	60	34	7.73
		250.1	30		42	
Nitrazepam	282.2	236.2	30	60	32	8.95
		180.2	30		52	
7-Aminonitrazepam	252.2	121.1	30	80	37	7.69
		146.2	30		38	
Flunitrazepam	314.2	268.3	30	65	35	9.62
		239.3	30		45	
7-Aminoflunitrazepam	284.2	135.2	30	80	39	8.08
		226.2	30		41	
Triazolam	343.2	308.2	30	70	36	8.93
		315.2	30		35	
α-Hydroxytriazolam	359.2	331.2	30	80	38	8.23
		176.1	30		37	
Alprazolam	309.2	281.1	30	70	32	8.94
		274.2	30		33	
α-Hydroxyalprazolam	325.2	297.2	30	70	35	8.23
		279.2	30		33	
Midazolam	326.2	291.4	30	70	37	11.65
		244.2	30		35	
α-Hydroxymidazolam	342.0	324.2	30	60	29	9.26
		203	30		38	
Estazolam	295.2	267.3	30	70	34	8.67
		205.2	30		53	
Flurazepam	388.2	315.2	30	55	32	19.66
		288.1	30		33	
Diazepam-d5 (IS)	290.2	198.2	30	60	45	9.91
		159.2	30		36	

Please cite this article in press as: P. Xiang, et al., Segmental hair analysis using liquid chromatography–tandem mass spectrometry after a single dose of benzodiazepines, Forensic Sci. Int. (2010), doi:10.1016/j.forsciint.2010.04.046

After solvent evaporation at 60 °C, the residue was reconstituted with 100 μl of acetonitrile–20 mM ammonium acetate (70:30, v/v) solvent and 5 μl was injected into the LC–MS/MS system.

2.4. Instrumentation

The liquid chromatography–tandem mass spectrometry (LC–MS/MS) system consisted of an Agilent HPLC (Palo Alto, CA, USA) including a quaternary pump, an on-line degasser and an autosampler, equipped with an MDS Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). The Analyst 1.4.2 software package was used for instrument control and data acquisition.

The analytical column was an Resteck Allure PFP Propyl column (100 mm × 2.1 mm i.d., 5 μm) fitted with an end-capped C18 guard column (12.5 mm × 2.1 mm i.d., 5 μm), at room temperature. According to Villain's method [20], an LC mobile phase gradient (see Table 1 for composition) was used for resolution of the analytes. Total flow rate through the column was 200 μl/min.

The mass instrument was operated in the positive ionization mode. Best results were obtained with an ionspray voltage of 5 kV and source temperature of 500 °C. CAD gas (nitrogen) was 7 psi, CUR gas was 25 psi, and GS1 and GS2 were both 35 psi. Data were recorded in the multiple reaction monitoring (MRM) mode. Parent ions, the corresponding daughter ions, retention time, decluster potential (DP) and

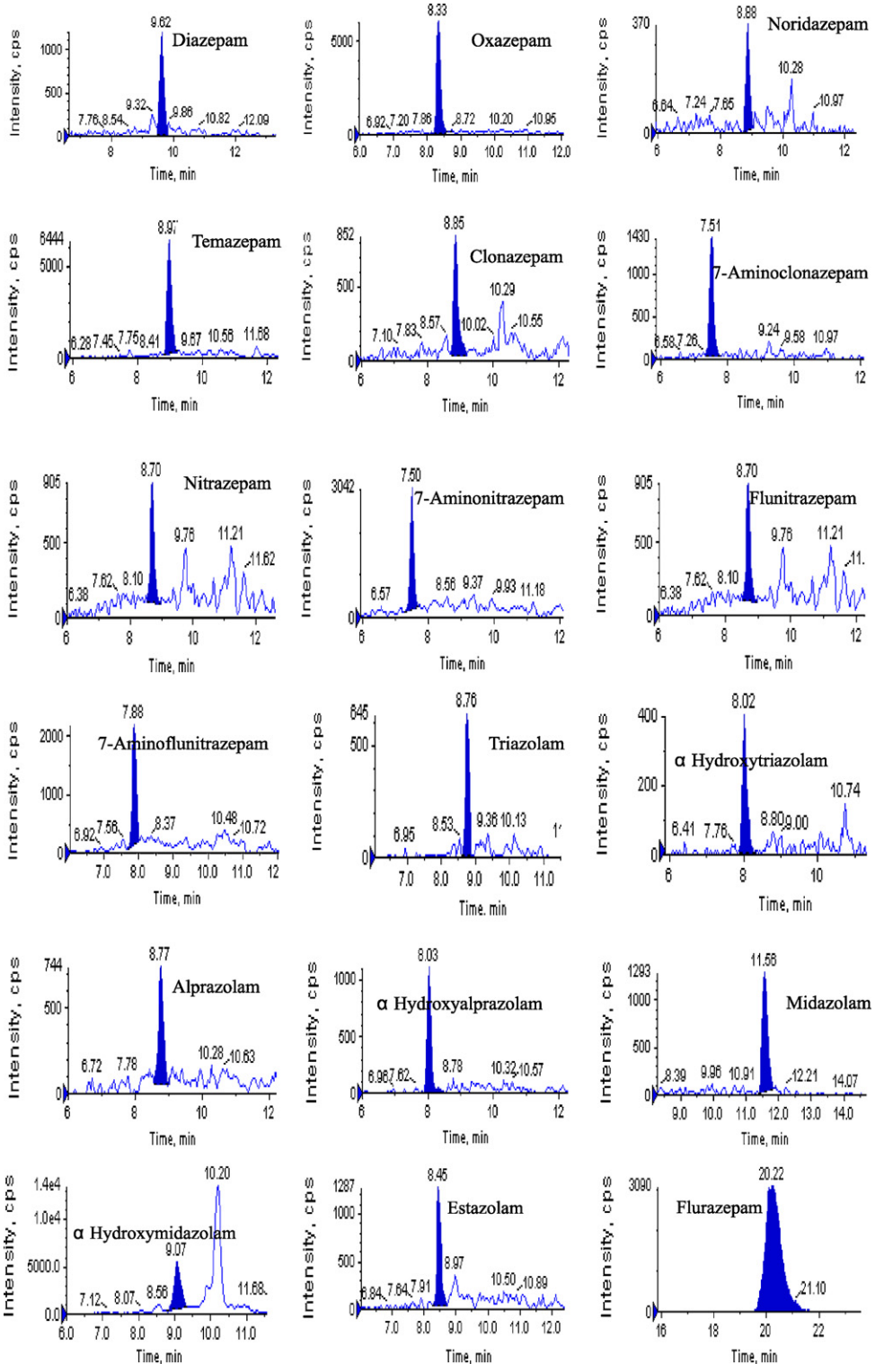

Fig. 1. The ion chromatograms of blank hair spiked with analytes at the Level of LOQ.

Please cite this article in press as: P. Xiang, et al., Segmental hair analysis using liquid chromatography–tandem mass spectrometry after a single dose of benzodiazepines, Forensic Sci. Int. (2010), doi:10.1016/j.forsciint.2010.04.046

collision energy (CE) optimized for analytes and IS are presented in Table 2. The first transition was used for quantification.

2.5. Method validation

The method was validated according to suggestions by Peters et al. [22]. Standard calibration curves were obtained by preparing authentic spiked blank hair powder (20 mg) containing 0.5, 1, 2, 5, 10, 20, 50, 100, 200 and 500 pg/mg final of the 18 benzodiazepines.

Accuracy and precision were determined using blank hair powder spiked with the 18 compounds at low, medium and high concentrations relative to the calibration range. The intra-day precision was determined by assaying six spiked hair samples at each concentration level on the same day and inter-day precision was assayed for 6 replicates on each of 4 days.

Recovery was established, at low, medium and high concentrations, by comparing the analyte peak areas of extracted spiked samples ($n = 6$) with those of blank samples spiked with the same amounts of the analyte after extraction ($n = 6$).

To investigate the sensitivity and potential background interference of the method, 10 sources of blank hair powder were analyzed.

The limit of detection (LOD) was evaluated with decreasing concentrations of the 18 compounds in blank hair powder until a response equivalent to three times the background noise was observed for two monitoring transitions. The limit of quantification (LOQ) was defined as the lowest concentration in the calibration curves, and a response superior to 10 times the background noise was required.

Matrix effects (ME) were evaluated by the method proposed by Matuszewski et al. [23]. The analyte signal in the spiked mobile phase ($n = 6$) was compared with the analyte signal in the matrix fortified after extraction ($n = 6$), and the ME was defined as ME% = (extracted matrix area/mobile phase area) × 100.

3. Results

3.1. Method validation

Assay selectivity was confirmed by the absence of interfering peaks at the retention times for 18 benzodiazepines in blank hair powders. The ion chromatograms of blank hair spiked with analytes and internal standards at the level of the lowest calibrator are shown in Fig. 1.

Calibration curves were made for each compound for the concentration range listed in Table 3. The LOD ranged from 0.2 to 5 pg/mg (Table 3). This sensitivity was sufficient for the determination of benzodiazepines in hair after a single dose.

Data for accuracy and precision (Table 4) were within the required limits. Inter-day and intra-day precisions were less than 20%. Recoveries obtained from spiked samples ranged from 38% to 104%. Although recoveries of 7-aminonitrazepam and 7-amnioclonazepam were lower than 50%, the method showed acceptable accuracy and precision for these 2 compounds. The results listed in Table 4 demonstrate that the hair matrix had a significant ion-suppression influence on benzodiazepines. Nevertheless, since we used diazepam-d5 as the internal standard, the ion-suppression effect does not significantly affect the accuracy of the results.

3.2. Volunteers experiment

A single 1–2 mg dose of estazolam did not influence the behavior of volunteers. Those who took 4 mg or more than 4 mg of estazolam appeared obvious impaired behavior half an hour later. They reacted slowly, claimed limb weakness and became sleepy. Their blood test revealed estazolam concentration was above 20 ng/ml 1 h after dose. The concentration–time curve in blood samples of volunteers was presented as Fig. 2.

The hairs of 14 volunteers that had been administrated a single dose of estazolam were decontaminated, segmented, analyzed and quantified. The results are shown in Table 5. A single 1 mg dose of estazolam can be detected in hair after a month's time. As Fig. 3 shows, there was a good correlation between the dosage and the 0–2 cm segment concentration ($r = 0.766$), with narrow variations for subjects at the same dosage (less than 40%). All the proximal segments were positive for estazolam. With increased dosage, estazolam can be detected in the 2–4 cm segments in some subject's hair. Even some of 4–6 cm segments were positive.

3.3. DFSA case

Clonazepam and its main metabolite 7-aminoclonazepam were detected in both of the blood samples from the two victims. The blood concentrations of clonazepam and 7-aminoclonazepam for V#1 were 1.05 and 20.60 ng/ml, respectively. The concentrations for V#2 were 0.22 and 15.34 ng/ml, respectively.

Full-length hair samples collected 5 weeks after the offense were cut into segments of 2 cm from the root, analyzed and quantified. Figs. 4 and 5 represent the chromatograms of proximal segments (0–2 cm) of V#1 and V#2. The results are shown in Table 6.

The first two segments for V#1 and V#2 were both positive for clonazepam (15.47 pg/mg for first segment and 5.31 pg/mg for the second segment of V#1; 11.93 pg/mg for first segment and 1.31 pg/mg for second segment of V#2). In addition, 7-aminoclonazepam could be detected in the first segments (i.e. 0–2 cm) for both subjects with concentrations of 45.30 pg/mg (V#1) and 33.47 pg/mg (V#2) which were higher in concentrations comparing with the levels of clonazepam found. Both the 4–6 cm and the 6–8 cm segment of V1# remained positive, while those of V#2 were negative.

Table 3
Calibration curves and LOD for 18 benzodiazepines.

Compounds	LOD (pg/mg)	Calibration ranges (pg/mg)	Calibration curves	r
Diazepam	0.5	1–200	$y = 0.0216x + 0.0356$	0.9997
oxazepam	2	5–200	$y = 0.019x + 0.2182$	0.9971
Nordiazepam	0.5	1–200	$y = 0.0109x + 0.0056$	0.9993
Temazepam	2	5–200	$y = 0.0466x + 0.0829$	0.9978
Clonazepam	0.5	1–200	$y = 0.0162x + 0.0133$	0.9997
7-Aminoclonazepam	5	10–500	$y = 0.0041x + 0.0075$	0.9989
Nitrazepam	0.5	1–200	$y = 0.0297x + 0.0389$	0.9996
7-Aminonitrazepam	5	10–500	$y = 0.007x + 0.0304$	0.9989
Flunitrazepam	0.5	1–200	$y = 0.0299x + 0.0356$	0.9997
7-Aminoflunitrazepam	2	10–500	$y = 0.0174x + 0.0233$	0.9995
Triazolam	1	2–200	$y = 0.2264x + 0.6017$	0.9993
α-Hydroxytriazolam	5	10–500	$y = 0.0011x + 0.0061$	0.9991
Alprazolam	0.5	1–200	$y = 0.0493x + 0.0491$	0.9999
α-Hydroxyalprazolam	1	10–500	$y = 0.0165x - 0.0015$	0.9995
Midazolam	1	2–200	$y = 0.0596x + 0.0024$	0.9982
α-Hydroxymidazolam	5	10–500	$y = 0.0226x - 0.1183$	0.9994
Estazolam	0.2	0.5–200	$y = 0.1414x + 0.0272$	0.9998
Flurazepam	0.5	1–200	$y = 0.1562x + 0.1377$	0.9998

Please cite this article in press as: P. Xiang, et al., Segmental hair analysis using liquid chromatography–tandem mass spectrometry after a single dose of benzodiazepines, Forensic Sci. Int. (2010), doi:10.1016/j.forsciint.2010.04.046

Table 4
Validation parameters for 18 benzodiazepines.

Compound	Spiked concentration (pg/mg)	Accuracy (%bias) ($n=6$)	Recovery (%) ($n=6$)	ME (%) ($n=6$)	Precision (%RSD)	
					Intra-day ($n=6$)	Inter-day ($n=24$)
Diazepam	1	12.8	103.0	59.4	11.4	7.6
	10	14	103.0	72.4	11.0	12.4
	150	0.8	102.0	62.4	5.1	8.8
Oxazepam	5	15	82.6	90.3	9.7	7.2
	10	6.8	103.0	86.5	10.6	6.6
	150	3.2	88.0	89.2	3.1	12.7
Nordiazepam	1	4.4	87.0	73.8	6.6	14.5
	10	5.0	91.0	84.5	13.5	7.6
	150	0.3	89.0	81.4	6.6	11.5
Temazepam	5	12.5	89.0	67.0	8.3	11.2
	10	1.2	90.0	73.9	7.7	5.7
	150	1.6	99.0	60.6	4.1	10.2
Clonazepam	1	5.8	102.0	95.4	13.3	13.3
	10	6.0	93.0	94.8	11.5	7.3
	150	0.4	97.0	95.7	6.6	12.0
7-Aminoclonazepam	10	11.5	58.1	35.0	9.3	15.4
	100	6.8	47.3	57.7	5.6	10.9
	400	2.6	49.3	56.7	5.9	11.9
Nitrazepam	1	4.9	75.0	81.3	14.8	18.3
	10	5.0	94.0	86.9	9.3	6.4
	150	0.3	86.0	84.5	8.3	13.3
7-Aminonitrazepam	10	14.8	40.8	53.2	5.5	12.5
	100	2.0	38.0	52.8	7.2	10.1
	400	3.0	39.4	71.4	6.2	14.8
Flunitrazepam	1	2.0	80.0	68.0	10.3	12.6
	10	1.7	97.0	73.4	8.6	12.3
	150	0	104.0	61.7	4.7	12.9
7-Aminoflunitrazepam	10	13.8	50.8	39.1	8.2	17.8
	100	5.8	67.5	45.6	4.7	12.0
	400	3.4	61.5	48.7	8.5	18.4
Triazolam	2	13.3	75.9	61.1	8.1	7.6
	10	14	71.4	68.2	8.2	8.5
	150	2.9	65.1	60.9	5.1	10.0
α-Hydroxytriazolam	10	8.8	50.3	92.6	6.8	11.9
	100	2.0	59.7	96.3	5.5	9.0
	400	2.4	61.0	94.5	3.0	7.9
Alprazolam	2	9.7	54.9	61.5	4.9	10.0
	10	3.3	69.2	74.7	6.5	8.3
	150	0.2	63.9	68.5	5.5	7.1
α-Hydroxyalprazolam	10	12.5	56.1	88.4	10.2	8.9
	100	2.8	77.0	90.7	6.5	8.3
	400	5.2	60.7	92.7	6.0	11.2
Midazolam	2	12.8	91.7	25.7	8.2	14.7
	10	9.5	87.2	30.9	6.5	8.6
	150	0.5	93.7	32.4	4.5	7.8
α-Hydroxymidazolam	10	16.5	65.6	64.0	7.5	14.1
	100	5.4	80.4	67.4	2.8	12.0
	400	0.8	82.6	60.0	8.2	13.9
Estazolam	2	6.4	59.6	69.2	4.0	14.4
	10	2.4	59.1	71.5	3.5	7.2
	150	1.2	62.1	65.4	3.9	7.4
Flurazepam	1	10.9	80.7	109.7	13.5	11.4
	10	0.7	76.5	103.5	8.7	9.7
	150	3.5	85.9	108.2	6.3	8.9

In order to fine tune the clonazepam profiles in hair, V#1 hair sample was sectioned into sequential 0.5-cm segments. As shown in Fig. 6, segmentation revealed higher concentration of clonazepam at 0.5–1 cm followed by 1–1.5 cm while for 7-aminoclonazepam, higher concentrations were observed at the segment lengths corresponding to 0–0.5 and 0.5–1 cm.

4. Discussion

The LC–MS/MS method developed for simultaneous determination of 18 benzodiazepines in hair samples is able to extend the screening range in DFC [18,24]. With its high sensitivity, it is a useful testing method for confirming the

Please cite this article in press as: P. Xiang, et al., Segmental hair analysis using liquid chromatography–tandem mass spectrometry after a single dose of benzodiazepines, Forensic Sci. Int. (2010), doi:10.1016/j.forsciint.2010.04.046

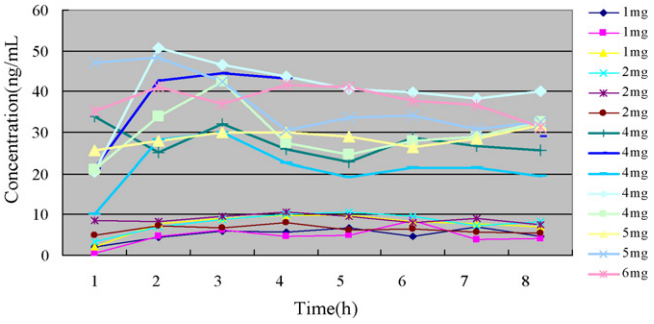

Fig. 2. The concentration–time curve in blood samples of volunteers who took single dose of estazolam.

presence of trace amounts of benzodiazepines in hair after a single dose.

Benzodiazepines are the most frequently used drugs in DFC. Triazolam was the most popular one in China several years ago, which led to a severe social problem. So, in 2005, triazolam was listed by the State Food and Drug Administration (SFDA) as a Class A mental-illness drug, with rigid controls on production, sale and consumption. While cases involving triazolam were decreasing greatly, other benzodiazepines were being abused instead. Estazolam is a benzodiazepine-type drug that is used mainly in China in treating insomnia. It possesses anxiolytic, anticonvulsant, sedative and skeletal muscle relaxant properties. It has been shown in some cases to be more potent than diazepam or nitrazepam [25]. Most of studies on estazolam were from Asian countries [26–28], with few reports from Western countries [29].

There were 14 volunteers involved in the present estazolam experiments. To our knowledge, this study is the first to investigate the distribution of estazolam in human hair after a single dose. Previous studies to document the windows of detection of zolpidem [9], zopiclone [20], bromazepam [13], clonazepam [13] and lorazepam [30] in hair after a single dose based on 2 or 3 volunteers. Negrusz et al. [31] determined flunitrazepam and its major metabolite 7-aminoflunitrazepam in hair collected from ten healthy volunteers after they received a single 2 mg dose of Rohypnol®.

The data about threshold dosage which is related to the sensitivity of method can be useful in the interpretation of results of hair analysis. With a LOD of 0.2 pg/mg and a LOQ of 0.5 pg/mg, our method is demonstrated to be able to detect a single 1 mg estazolam administration after a month.

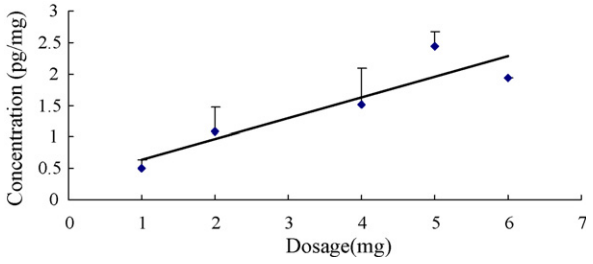

Fig. 3. Estazolam concentrations in hair at different doses.

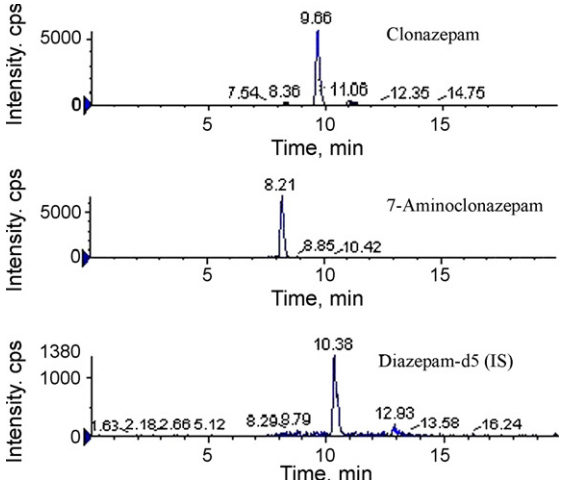

Fig. 4. Chromatogram of the proximal segment of V#1.

The mechanisms of drug incorporation into hair have not been established [32]. Three models for incorporation have been proposed [33]. Drugs can enter the hair through: (1) active or passive diffusion from the bloodstream feeding the dermal papilla; (2) diffusion from sweat and other secretions bathing the growing or mature hair fiber, or (3) external drug from vapors or powders diffusing into the mature hair fiber. Indeed, a combination of these routes is probably the most realistic model. No estazolam was detected in the last dichloromethane wash. With oral administration and the washing procedure used, the external contamination factor can be excluded in our study.

Except at low dosage, the 2–4 cm segment hair tested positive in both control experiments and DFSA cases. With increasing

Table 5
Concentrations of estazolam in hair 1 month after a single dose.

No.	Age	Sex	Weight (kg)	Hair length (cm)	Dosage (mg)	Concentration (pg/mg)			
						0–2 cm	2–4 cm	4–6 cm	Distal 2 cm
1	26	Female	50	20 cm	1	0.56	+[a]	–[b]	–
2	26	Female	44	12 cm	1	0.61	–	–	–
3	24	Male	58	4 cm	1	+	–	/[c]	
4	27	Male	57.5	2 cm	2	0.67	/	/	
5	24	Female	80	20 cm	2	1.45	–	–	–
6	27	Male	60	4 cm	2	1.11	–	/	
7	27	Male	57.5	4 cm	4	1.52	0.77	/	
8	26	Female	44	12 cm	4	1.12	0.83	+	–
9	24	Male	58	4 cm	4	2.45	0.71	/	
10	26	Female	47	15 cm	4	0.94	+	+	–
11	23	Female	42	22 cm	4	1.49	+	–	–
12	25	Male	59	4 cm	5	2.60	+	/	
13	26	Male	60	4 cm	5	2.28	+	/	
14	27	Male	59	4 cm	6	1.94	+	/	

[a] +: detected, but below LOQ.
[b] –: not detected.
[c] /: sample is absent.

Please cite this article in press as: P. Xiang, et al., Segmental hair analysis using liquid chromatography–tandem mass spectrometry after a single dose of benzodiazepines, Forensic Sci. Int. (2010), doi:10.1016/j.forsciint.2010.04.046

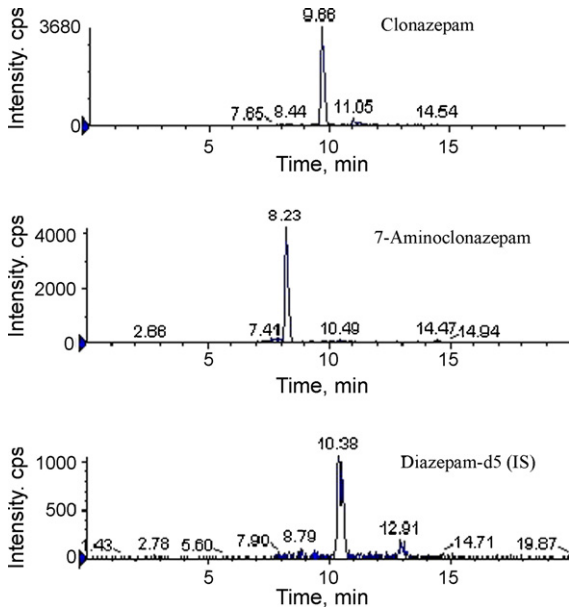

Fig. 5. Chromatogram of the proximal segment of V#2.

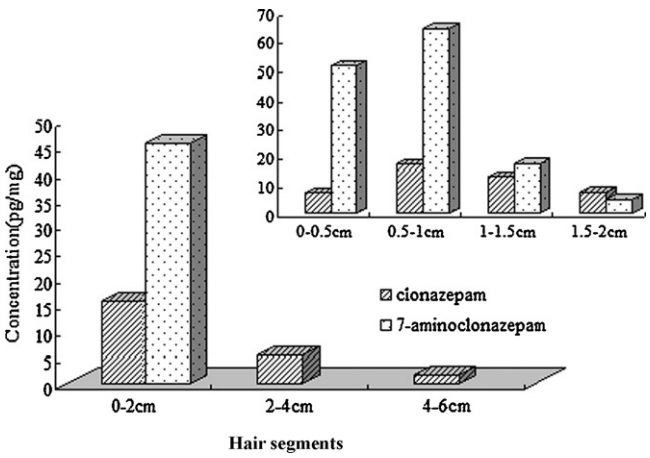

Fig. 6. Hair Segment Analysis of V#1.

dosage, estazolam can be detected in some of 4–6 cm segments. There was a significant difference between Kintz et al.'s findings [15,17–20] and our results. Although experimental error such as collecting and sectioning the hair samples might have been a factor, the results of successive 0.5 cm segments was consistent with that of 2 cm segments. In addition, there was an increase of clonazepam and 7-aminoclonazepam concentrations at the corresponding time according to normal hair growth rate. Therefore, we believe that the segments data presented above are creditable on the whole.

In our DFSA cases, the blood samples revealed low concentrations of clonazepam, i.e. 1.05 and 0.22 ng/ml, the concentrations of 7-aminoclonazepam were 20.60 and 15.34 ng/ml, respectively because there was 18 h delay and individual difference in metabolism. Although the administration dosage was not known, there were significant poisoning symptoms for the two victims. They were involved in deep coma after drinking. For instance, when V#1 was taken to the living place by suspects, one of her legs was burnt badly by the outlet pipe of motorcycle without known. The 0–2 cm segment of V#1 hair sample was positive at levels of 15.47 pg/mg for clonazepam and 45.30 pg/mg for 7-aminoclonazepam. The same segment of V#2 was at levels of 11.93 pg/mg for clonazepam and 33.47 pg/mg for 7-aminoclonazepam. Both clonazepam and its metabolite were detected in our research. Chèze et al. [13] reported a sensitive method using LC–MS/MS with a LOD of 0.5 pg/mg for clonazepam and of 1 pg/mg for 7-aminoclonazepam. After single exposure of 2 mg clonazepam, 7-aminoclonazepam was present in powdered hair at 22 pg/mg in the first 1-cm segment, while no clonazepam was detectable. This method was applied in one forensic case which showed only the presence of 7-aminoclonazepam at about 3.2 pg/mg in axillary hair 4 months later. The difference results may be based on inter-individual differences in dosage, blood concentrations, drug incorporation rates, hair pigmentation, physical state of the hair, age, gender, body weight, etc [32–37].

Another explanation for broadening the band of positive hair from a single dose is that drugs and metabolites are incorporated into hair during formation of the hair shaft via diffusion from sweat or other secretions [33,38–40]. Henderson et al. [39] reported that deuterated cocaine was found in multiple segments after a single dose, supporting sweat or other secretions as a route for drug deposition in hair. Raul et al. [40] suggested that cortisol and cortisone incorporated into hair not through the bloodstream, but mainly through diffusion from sweat. Stout and Ruth [41] evaluated the incorporation of cocaine, flunitrazepam, and nicotine and demonstrated insignificant deposition of the drugs into the hair from sebum. Negrusz et al. [31] have shown that 7-aminoflunitrazepam was detected 24 h after flunitrazepam administration and remained in hair throughout the entire 28-day study period (0.6–8.0 pg/mg) in five of ten healthy volunteers, although the study used NCI-MS. Actually, Kintz and coworkers have encountered such circumstances. In one DFSA case, they detected bromazepam, the target analyte, 'in the range 2–7 pg/mg in the four other consecutive segments', which made "a single exposure statement difficult". They considered it probably resulted from diffusion [19].

Our finding that the concentration of 7-aminoclonazepam was significant higher than that of clonazepam in hair after a single

Table 6
Concentrations of clonazepam and 7-aminoclonazepam in two victims' hair.

Hair segments from proximal	V#1		V#2	
	Clonazepam (pg/mg)	7-Aminoclonazepam (pg/mg)	Clonazepam (pg/mg)	7-Aminoclonazepam (pg/mg)
0–2 cm	15.47	45.30	11.93	33.47
2–4 cm	5.31	–[a]	1.31	–
4–6 cm	1.63	–	–	–
6–8 cm	+[b]	–	–	–
8–10 cm	–	–	–	–
10–12 cm	–	–	–	–
12–14 cm	–	–	–	–
14–16 cm	–	–	–	–

[a] –: not detected.
[b] +: detected, but below LOQ.

Please cite this article in press as: P. Xiang, et al., Segmental hair analysis using liquid chromatography–tandem mass spectrometry after a single dose of benzodiazepines, Forensic Sci. Int. (2010), doi:10.1016/j.forsciint.2010.04.046

dose was consistent with that reported by Chèze et al. [13]. Nakahara et al. [34] demonstrated that the different concentrations of the drugs in hair depend on their physicochemical properties and on the functional groups [42]. Amino substitution on the benzene ring raised the drug incorporation into hair and hydroxy substitution showed a negative effect.

In summary, our research subjects are all Chinese with black hair. A single 1 mg dose of estazolam can be detectable in the proximal segments (0–2 cm). There was a good correlation between the dosage and 0–2 cm segment concentration with narrow variations for subjects at the same dosage. Until these mechanisms of drug incorporation into hair are better understood and the reasons for the intersubject variability clarified, it needs more substantial guidelines to use segmental hair analysis in drug-facilitated crime.

Acknowledgments

The authors would like to express their gratitude to National Natural Science Foundation and National Institute Scientific Program for financial support (No. 20975070 and No. GY0903).

References

[1] Z.M. Guo, Review: drugs facilitated in robbery cases, Chin. Forensic Sci. Technol. 2 (2003) 44–45.
[2] Z.L. Jiang, J.Y. Tan, L.J. Yao, Screening analysis of benzodiazepine medicines and selected metabolites in plasma and urine using gas chromatography with nitrogen phosphorus detection, J. Anal. Sci. 21 (2005) 639–642.
[3] Y. Zhu, J.Y. Tan, Detection of benzodiazepines and the metabolites in urine using GC–MS, Guangdong Police Technol. 70 (2003) 21–25.
[4] J.H. Xiong, Determination of triazolam in human urine by GC-uECD, Chin. J. Forensic Med. 21 (2006) 101–102.
[5] M. LeBeau, W. Andollo, W.L. Hearn, et al., Recommendations for toxicological investigations of drug-facilitated sexual assaults, J. Forensic Sci. 44 (1999) 227–230.
[6] B. Marc, Current clinical aspects of drug-facilitated sexual assaults in sexually abused victims examined in a forensic emergency unit, Ther. Drug Monit. 30 (2008) 218–224.
[7] H. Schloegl, S. Dresen, K. Spaczynski, M. Stoertzel, F.M. Wurst, W. Weinmann, Stability of ethyl glucuronide in urine, post-mortem tissue and blood samples, Int. J. Legal Med. 120 (2006) 83–88.
[8] M. Pavlic, K. Libiseller, P. Grubwieser, H. Schubert, W. Rabl, Medicolegal aspects of tetrazepam metabolism, Int. J. Legal Med. 121 (2007) 169–174.
[9] M. Villain, M. Chèze, A. Tracqui, B. Ludes, P. Kintz, Windows of detection of zolpidem in urine and hair: application to two drug facilitated sexual assaults, Forensic Sci. Int. 143 (2004) 157–161.
[10] J.P. Goullé, M. Chèze, G. Pépin, Determination of endogenous levels of GHB in human hair. Are there possibilities for the identification of GHB administration through hair analysis in cases of drug-facilitated sexual assault? J. Anal. Toxicol. 27 (2003) 574–580.
[11] G. Frison, D. Favretto, L. Tedeschi, S.D. Ferrara, Detection of thiopental and pentobarbital in head and pubic hair in a case of drug-facilitated sexual assault, Forensic Sci. Int. 133 (2003) 171–174.
[12] G. Pepin, M. Chèze, G. Duffort, F. Vayssette, Interest of hair and tandem mass spectrometry for chemical submission: about nine cases, Toxicol. Anal. 14 (2002) 395–406.
[13] M. Chèze, M. Villain, G. Pépin, Determination of bromazepam, clonazepam and metabolites after a single intake in urine and hair by LC–MS/MS, application to forensic cases of drug facilitated crimes, Forensic Sci. Int. 145 (2004) 123–130.
[14] P. Kintz, V. Cirimele, C. Jamey, B. Ludes, Testing for GHB in hair by GC/MS/MS after a single exposure: application to document sexual assault, J. Forensic Sci. 48 (2003) 195–200.
[15] P. Kintz, M. Villain, M. Chèze, G. Pépin, Identification of alprazolam in hair in two cases of drug-facilitated incidents, Forensic Sci. Int. 153 (2005) 222–226.
[16] M.P. Juhascik, A. Negrusz, D. Faugno, et al., An estimate of the proportion of drug-facilitation of sexual assault in four U.S. localities, J. Forensic Sci. 52 (2007) 1396–1400.
[17] P. Kintz, Bioanalytical procedures for detection of chemical agents in hair in the case of drug-facilitated crimes, Anal. Bioanal. Chem. 388 (2007) 1467–1474.
[18] M. Villain, M. Concheiro, V. Cirimele, P. Kintz, Screening method for benzodiazepines and hypnotics in hair at pg/mg level by liquid chromatography–mass spectrometry/mass spectrometry, J. Chromatogr B: Analyt Technol. Biomed. Life Sci. 825 (2005) 72–78.
[19] M. Villain, M. Chèze, V. Dumestre, P. Kintz, Hair to document drug-facilitated crimes: four cases involving bromazepam, J. Anal. Toxicol. 28 (2004) 516–519.
[20] M. Villain, M. Chèze, B. Ludes, P. Kintz, Testing for zopiclone in hair application to drug-facilitated crimes, Forensic Sci. Int. 145 (2004) 117–121.
[21] M. Shen, P. Xiang, B.H. Shen, D. Ma, Screening for 132 drugs in blood by LC–MS/MS with multiple-reaction monitoring, Chin. J. Forensic Sci. 1 (2006) 14–20.
[22] F. Peters, O. Drummer, F. Musshoff, Validation of new methods, Forensic Sci. Int. 165 (2007) 216–224.
[23] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[24] R.C. Irving, S.J. Dickson, The detection of sedatives in hair and nail samples using tandem LC–MS–MS, Forensic Sci. Int. 166 (2007) 58–67.
[25] http://pubchem.ncbi.nlm.nih.gov/summary/summary.cgi?sid=7847377 (accessed 29 Sep 2009).
[26] B.H. Shen, M. Shen, X.Y. Zhuo, Screening of benzodizepines and their metabolites in urine, Fa Yi Xue Za Zhi 18 (2002) 22–25.
[27] Y. Harahap, L. Sasongko, B. Prasaja, et al., Comparative bioavailability of two estazolam tablet formulations in Indonesian healthy volunteers, Arzneimittelforschung 58 (2008) 501–504.
[28] M. Nakamura, T. Ohmori, Y. Itoh, M. Terashita, K. Hirano, Simultaneous determination of benzodiazepines and their metabolites in human serum by liquid chromatography–tandem mass spectrometry using a high-resolution octadecyl silica column compatible with aqueous compounds, Biomed. Chromatogr. 23 (2009) 357–364.
[29] P. Adamowicz, M. Kała, Date-rape drugs scene in Poland, Przegl Lek 62 (2005) 572–575.
[30] P. Kintz, M. Villain, V. Cirimele, G. Pépin, B. Ludes, Windows of detection of lorazepam in urine, oral fluid and hair, with a special focus on drug-facilitated crimes, Forensic Sci. Int. 145 (2004) 131–135.
[31] A. Negrusz, C.M. Moore, K.B. Hinkel, et al., Deposition of 7-aminoflunitrazepam and flunitrazepam in hair after a single dose of Rohypnol, J. Forensic Sci. 46 (2001) 1143–1151.
[32] F.B. Musshoff, B. Madea, New trends in hair analysis and scientific demands on validation and technical notes, Forensic Sci. Int. 165 (2007) 204–215.
[33] P. Kintz, Analytical and practical aspects of drug testing in hair Taylor & Francis Group, LLC, 2007.
[34] Y. Nakahara, K. Takahashi, R. Kikura, Hair analysis for drugs of abuse. X. Effect of physicochemical properties of drugs on the incorporation rates into hair, Biol. Pharm. Bull. 18 (1995) 1223–1227.
[35] G.L. Henderson, M.R. Harkey, C. Zhou, R.T. Jones, P. Jacob, Incorporation of isotopically labeled cocaine into human hair: race as a factor, J. Anal. Toxicol. 22 (1998) 156–165.
[36] P. Xiang, M. Shen, X.Y. Zhuo, Hair analysis for ketamine and its metabolites, Forensic Sci. Int. 162 (2006) 131–134.
[37] R. Wennig, Potential problems with the interpretation of hair analysis results, Forensic Sci. Int. 107 (2000) 5–12.
[38] E.J. Cone, Mechanisms of drug incorporation into hair, Ther. Drug Monit. 18 (1996) 438–443.
[39] G.L. Henderson, M.R. Harkey, C. Zhou, R.T. Jones, P. Jacob, Incorporation of isotopically labeled cocaine and metabolites into human hair. 1. Dose–response relationships, J. Anal. Toxicol. 20 (1996) 1–12.
[40] J.S. Raul, V. Cirimele, B. Ludes, P. Kintz, Detection of physiological concentrations of cortisol and cortisone in human hair, Clin. Biochem. 37 (2004) 1105–1111.
[41] P.R. Stout, J.A. Ruth, Deposition of [3H] cocaine, [3H] nicotine, and [3H] flunitrazepam in mouse hair melanosomes after systemic administration, Drug Metab. Dispos. 27 (1999) 731–735.
[42] Y. Nakahara, R. Hanajiri, Hair analysis for drugs of abuse XXI. Effect of para-substituents on benzene ring of methamphetamine on drug incorporation into rat hair, Life Sci. 66 (2000) 563–574.

Please cite this article in press as: P. Xiang, et al., Segmental hair analysis using liquid chromatography–tandem mass spectrometry after a single dose of benzodiazepines, Forensic Sci. Int. (2010), doi:10.1016/j.forsciint.2010.04.046