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Journal of Chromatography B, 819 (2005) 315–322
JOURNAL OF CHROMATOGRAPHY B
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Development of simultaneous gas chromatography–mass spectrometric and liquid chromatography–electrospray ionization mass spectrometric determination method for the new designer drugs, *N*-benzylpiperazine (BZP), 1-(3-trifluoromethylphenyl)piperazine (TFMPP) and their main metabolites in urine

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Received 6 November 2004; accepted 24 February 2005
Available online 23 March 2005

Abstract

To prove the intake of recently controlled designer drugs, *N*-benzylpiperazine (BZP) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP), a simple, sensitive and reliable method which allows us to simultaneously detect BZP, TFMPP and their major metabolite in human urine has been established by coupling gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS). GC–MS accompanied by trifluoroacetyl (TFA) derivatization and LC–MS analyses were performed after the enzymatic hydrolysis and the solid phase extraction with OASIS HLB, and BZP, TFMPP and their major metabolites, 4′-hydroxy-BZP (*p*-OH-BZP), 3′-hydroxy-BZP (*m*-OH-BZP) and 4′-hydroxy-TFMPP (*p*-OH-TFMPP), have found to be satisfactorily separated on a semi-micro SCX column with acetonitrile–40 mM ammonium acetate buffer (pH 4) (75:25, v/v) as the eluent. The detection limits produced by GC–MS were estimated to be from 50 ng/ml to 1 μg/ml in the scan mode, and from 200 to 500 ng/ml in the selected ion monitoring (SIM) mode. Upon applying the LC–ESI-MS technique, the linear calibration curves were obtained by using the SIM mode for all analytes in the concentration range from 10 ng/ml to 10 μg/ml. The detection limits ranged from 5 to 40 ng/ml in the scan mode, and from 0.2 to 1 ng/ml in the SIM mode. These results indicate the high reliability and sensitivity of the present procedure, and this procedure will be applicable for proof of intake of BZP and TFMPP in forensic toxicology.
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Keywords: *N*-Benzylpiperazine; BZP; Hydroxy-*N*-benzylpiperazine; 1-(3-Trifluoromethylphenyl)piperazine; TFMPP; Hydroxy-1-(3-trifluoromethylphenyl) piperazine; GC–MS; LC–ESI-MS

1. Introduction

N-Benzylpiperazine (BZP) and 1-(3-trifluoromethylphenyl)piperazine (TFMPP), both of which have a piperazine ring moiety in their structures, are classified as a new class of designer drug piperazines, that also includes 1-(3-chlorophenyl)piperazine, 1-(4-methoxyphenyl) piperazine. The drugs have been sold in powder, liquid or tablet forms mainly on the Internet and in nightclubs.

BZP and TFMPP were first synthesized as a potential antiparasitic agent and chemical intermediate in pharmaceutical products, respectively. However, it has known that the drugs have a central serotoninomimetic action involving serotonin (5-HT) uptake inhibition and 5-HT$_1$ receptor agonistic effects [1–5]. Therefore, they have been widely used as pharmacological probe drugs for drug discrimination procedures in animals [6–8]. However, the safety for use of the drugs

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doi:10.1016/j.jchromb.2005.02.016

in humans has never been demonstrated and has no accepted medical use.

Recently, BZP and TFMPP have the reputation of producing amphetamine-like stimulant effects [9,10] and the psychoactive effects of methylenedioxymethamphetamine (MDMA) [11,12], respectively. In addition, their use was not controlled at that time. This caused the rapid spread of the abuse of these drugs as club-drugs or recreational drugs in the youth mainly in the United States in the late 1990s. In the last several years, such abuse has been even reported in Japan and Europe, and the fatal intoxication by BZP has also been reported [13–18]. These led to the temporary placement of BZP along with TFMPP into Schedule I of the Controlled Substance Act (CSA) in the United States in 2002 [19], followed by the final placement of BZP into Schedule I in 2004 [20]. Japan's authorities also controlled these substances under the Narcotics and Psychotropics Control Law in 2003, as the encountering of BZP and TFMPP was increasingly reported even in Japan. Thus, the establishment of a method to prove the intake of these drugs has been strongly required in forensic toxicology as well as in clinical chemistry.

For unequivocal proof of the intake of BZP and TFMPP, the identification of their principal metabolites in humans and the establishment of the determination procedures for their metabolites in biological specimens including urine and blood are indispensable. For the metabolism of BZP and TFMPP, Staack et al. reported on the basis of a GC–MS spectral interpretation that hydroxylation of the aromatic rings and degradation of the piperazine moiety were the principal metabolic pathways. However, they could not determine the exact position of the hydroxyl group on the principal hydroxylated metabolites [15,18].

More recently, the authors have achieved the synthesis of authentic standards of the hydroxylated metabolites of BZP, and isolation of the principal urinary metabolites in rats administered TFMPP. Based on these results, it has been demonstrated that the hydroxylated metabolites were the relevant target analytes to be detected to prove the intake of BZP and TFMPP, as well as the position of the hydroxy group on the main metabolites of both BZP and TFMPP which were at the have proved to be *para*-position (Fig. 1) [21,22].

Since BZP and TFMPP have often been encountered and taken in combination with each other, in the present study, we established a sensitive and reliable assay for the simultaneous determination of BZP, TFMPP and their main metabolites, 4′-hydroxy-BZP (*p*-OH-BZP) and 4′-hydroxy-TFMPP (*p*-OH-TFMPP) along with a minor BZP metabolite 3′-hydroxy-BZP (*m*-OH-BZP) in urine.

2. Experimental

2.1. Materials

N-Benzylpiperazine and 1-(3-trifluoromethylphenyl) piperazine were purchased from Aldrich (Milwaukee, WI, USA). *N*-(3-Hydroxybenzyl)piperazine and *N*-(4-hydroxybenzyl)piperazine were both chemically synthesized in the Forensic Science Laboratory of the Osaka Prefectural Police Headquarters as reported in our previous paper [21]. 1-(4-Hydroxy-3-trifluoromethylphenyl)piperazine was isolated and purified from rat urine in the our laboratory as described in our previous paper [22]. The standard stock solutions of BZP, *m*-OH-BZP, *p*-OH-BZP, TFMPP and *p*-OH-TFMPP were prepared in methanol (concentration of 1 mg/ml as their free bases), and adjusted to appropriate concentrations with distilled water or control human urine immediately prior to use. The internal standard (I.S.) 1-(4-chlorophenyl)piperazine (4CPP) was purchased from Aldrich (Tokyo, Japan), and the I.S. solution (concentration of 1 μg/ml) was prepared in the mobile phase for LC–MS.

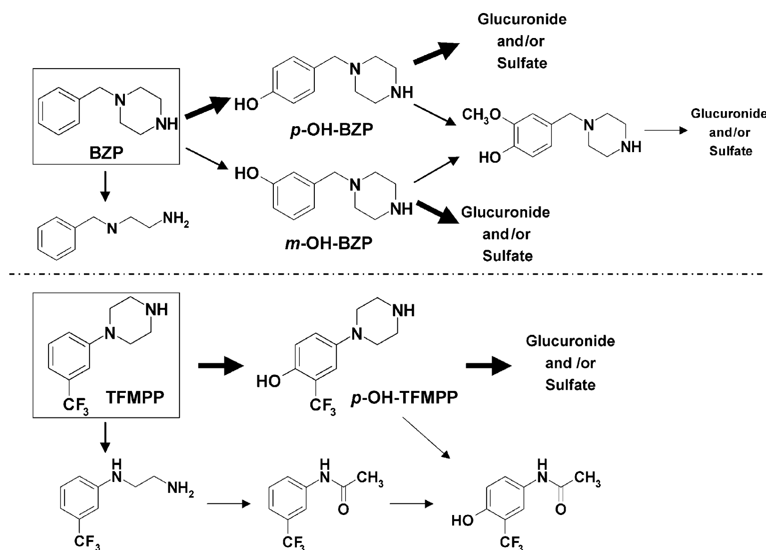

Fig. 1. Expected main metabolic pathways for BZP and TFMPP in humans.

Acetonitrile was of HPLC-grade, and all other chemicals used were of analytical grade.

Sulfatase/β-glucuronidase (*Helix pomatia*, Type H-1) was purchased from the Sigma (St. Louis, MO, USA). The OASIS HLB cartridges, hydrophilic polymer solid phase extraction cartridges, and OASIS MCX, mixed-mode cation-exchange reversed-phase extraction cartridges, were purchased from Waters Associates (Milford, MA, USA) and employed in the investigation of the solid phase extraction.

2.2. Enzymatic hydrolysis

To 1 ml of the rat urine sample was added 0.3 ml of 100 mM acetate buffer (pH 5.0), containing sulfatase/β-glucuronidase (100 and 2900 Fishman units/ml urine each). The mixture was then incubated for 12 h at 37 °C to hydrolyze the conjugates. After centrifugation, the supernatant solution was subjected to the solid phase extraction as described below.

2.3. Sample preparation

Extraction of the analytes was investigated using the following three procedures (procedures (a)–(c)). For the GC–MS analysis, the obtained extract residue was subjected to TFA derivatization as mentioned below and a 1 μl aliquot was injected into the GC–MS system. For the LC–MS analysis, the residue was reconstituted in 100 μl of the I.S. solution and filtered through a 0.22 μm membrane filter. A 5 μl aliquot was injected into the LC–MS system for quantitative analysis.

(a) Solid phase extraction with OASIS HLB.
 The OASIS HLB cartridge was successively prewashed with 1 ml of methanol and 1 ml of distilled water. The urine sample (1 ml) was loaded on the prewashed cartridge. Subsequently, the cartridge was rinsed with 0.5 ml of 5% methanol in water. The retained analytes were eluted with 1.5 ml of methanol. The eluate was then evaporated to dryness under a gentle stream of nitrogen.
(b) Solid phase extraction with OASIS MCX.
 The OASIS MCX cartridge was successively prewashed with 3 ml of methanol and 3 ml of distilled water. The urine sample (1 ml) was loaded on the prewashed cartridge. Subsequently, the cartridge was rinsed with 1 ml of 0.1% hydrochloric acid, 3 ml of distilled water and 3 ml of methanol. The retained analytes were eluted with 2 ml of 5% ammonia–methanol. The eluate was then evaporated to dryness under a gentle stream of nitrogen.
(c) Liquid–liquid extraction.
 A urine sample (1 ml) was adjusted to pH 9 with 28% aqueous ammonia and saturated with NaCl. The solution was extracted two times with 1 ml of a chloroform–2-propanol mixture (3:1, v/v), and the organic layer was separated after centrifugation. The extract was dried under a gentle stream of nitrogen.

2.4. Trifluoroacetyl (TFA) derivatization

TFA derivatization for GC–MS was performed by heating the dried extract residue with 200 μl of trifluoroacetyl anhydride and 200 μl of ethylacetate at 60 °C for 1 h. The reaction mixture was carefully evaporated to dryness under a gentle stream. The residue was reconstituted in 100 μl of ethylacetate, and a 1 μl aliquot was then injected into the GC–MS system.

2.5. Apparatus

2.5.1. GC–MS

GC–MS was carried out using a Shimadzu GCMS QP-2010. A fused-silica capillary column DB-5MS (column dimensions and film thickness: 30 m × 0.25 mm I.D. and 0.25 μm; J&W Scientific, Rancho Cordova, CA, USA) was employed for the separation. Injections were automatically done in the splitless mode at 270 °C. The column oven temperature was maintained at 80 °C for 1 min and then raised at 10 °C/min to 250 °C. The temperature of the transfer line was set at 250 °C, and high purity helium, at a flow rate of 1 ml/min, was used as the carrier gas. The EI operating parameters were as follows: source temperature, 200 °C; electron energy, 70 eV; ion multiplier gain, 1.2 kV. Data were collected from *m/z* 40 to 500 at a scan rate of 0.5 s/scan.

2.5.2. LC–MS

LC–MS was performed on a Shimadzu LCMS 2010A equipped with an ESI interface (Shimadzu, Kyoto, Japan). The probe voltage (interface voltage) was 4.5 kV and the curved desolvation line (CDL) voltage was set at 25 V. The Q-array voltage was kept at 15 V. The other ESI operating parameters were as follows: CDL temperature, 200 °C; nitrogen gas, 1.5 l/min; and multiplier gain, 1.7 V. Under these conditions, full scan spectra were acquired from *m/z* 80 to 300 in the positive mode for identification. The quantitative measurements were accomplished in the selected ion monitoring (SIM) mode by monitoring each protonated molecule (*m/z* 177 for BZP, *m/z* 193 for both *m*-OH-BZP and *p*-OH-BZP, *m/z* 231 for TFMPP, *m/z* 247 for *p*-OH-TFMPP, and *m/z* 197 for I.S.) using an internal standard method.

The chromatographic separation was carried out on a semi-micro SCX column (2.0 mm I.D. × 150 mm; Shiseido, Tokyo, Japan) with a mobile phase consisting of 40 mM ammonium acetate buffer (pH 4)–acetonitrile (25:75, v/v). The flow rate was set at 0.15 ml/min, and the entire effluent was introduced into the ESI source.

3. Results and discussion

3.1. Enzyme hydrolysis

The hydroxylated metabolites of BZP and TFMPP were expected to be extensively conjugated into their sulfates

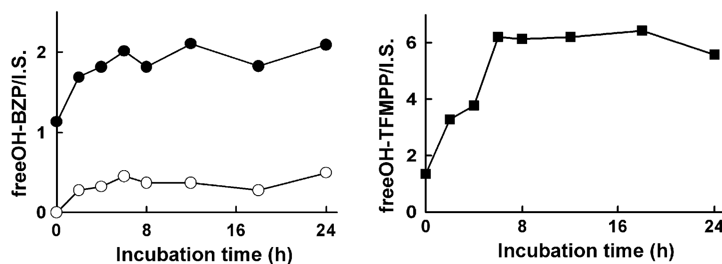

Fig. 2. Time-changes of the urinary levels of free *m*-OH-BZP, *p*-OH-BZP and *p*-OH-TFMPP during enzymatic hydrolysis at 37 °C and pH 5. Each value is calculated as the ratio of each peak area to that of I.S. Symbols: (○) *m*-OH-BZP; (●) *p*-OH-BZP; (■) *p*-OH-TFMPP.

and/or glucuronides in vivo and then excreted in the urine. In order to determine the total hydroxylated metabolites as free bases, hydrolysis of the conjugates was required before the extraction. For a rapid and complete hydrolysis, acid hydrolysis would often be efficient. However, it would also cause alteration or decomposition of some compounds during hydrolysis. Therefore, we chose the enzymatic hydrolysis and explored the optimization of the hydrolysis conditions for the conjugates of the hydroxylated metabolites in the present study.

As the hydrolysis enzyme, aryl sulfatase/β-glucuronidase from *H. pomatia* was selected, and the rat urine collected from rats administered a single intraperitoneal dose of 10 mg/kg each BZP or TFMPP was employed as the sample to be hydrolyzed. To the urine sample was added the enzyme (100 and 2900 Fishman units/ml urine each), the incubation time of the mixture was varied under the condition of 37 °C and pH 5 [23], and then the urinary levels of the free *p*-OH-BZP, *m*-OH-BZP and *p*-OH-TFMPP were measured.

As depicted in Fig. 2, the levels of the three metabolites were all increased with time up to approximately 6 h, and then showed no noticeable change. To confirm the complete hydrolysis of the conjugates, the glucuronides and sulfates of the three metabolites were monitored by measuring the ions corresponding to each conjugate (*m/z* 369 and 273 for glucuronides and sulfates of OH-BZPs, and *m/z* 423 and 327 for glucuronide and sulfate of *p*-OH-TFMPP, respectively) in the SIM mode [23–25]. Thus, we have found that neither glucuronide nor sulfate was detected in the urine samples hydrolyzed for 6 h. This suggests that the glucuronides and sulfates were completely hydrolyzed within 6 h under these conditions. Considering the urine sample containing higher levels of the conjugates, a 12 h incubation time was finally selected.

3.2. GC–MS following TFA derivatization

Derivatization is often a successful procedure for the sensitive GC–MS analysis of hydroxylated compounds. For derivatization of piperazine-derived drugs, heptafluorobutylation was successfully used by Peters et al. In our preliminary experiment, the TFA derivatization that allows easier sample preparation and is more popular for derivatization of both amino and hydroxyl groups in forensic and clinical toxicology was selected, and was applied to the hydroxylated metabolites of BZP and TFMPP. The TFA derivatization produced a significant improvement in the shapes of their peaks in the chromatogram accompanied by an increased sensitivity. Therefore, we further explored the optimization of the TFA derivatization of BZP, TFMPP and their three metabolites with trifluoroacetyl anhydride–ethylacetate (1:1, v/v) by varying reaction temperature and time. Based on the resultant derivatization efficiency, we finally selected the reaction temperature of 60 °C and reaction time of 1 h, where all the analytes were almost completely derivatized.

The extract obtained from the spiked urine sample after the SPE with HLB was subjected to GC–MS after TFA derivatization under the optimized condition. The extracted mass chromatograms and the mass spectra are depicted in Figs. 3 and 4, respectively. Under the present conditions, the TFA derivatives of the structural *m*-OH-BZP and *p*-OH-BZP isomers were detected as well-separated peaks, and easy distinction between the *m*-OH-BZP and *p*-OH-BZP was possible based on their retention times.

Each EI mass spectrum of the TFA derivatives of the five analytes was characterized by each molecular ion M^+ (*m/z* 272 for BZP, *m/z* 326 for TFMPP, *m/z* 384 for *m*-OH-BZP and *p*-OH-BZP, and *m/z* 438 for *p*-OH-TFMPP) and some distinctive fragment ions.

The mass spectra of the two isomers of OH-BZP were very similar, so the isomers were not distinguishable based only

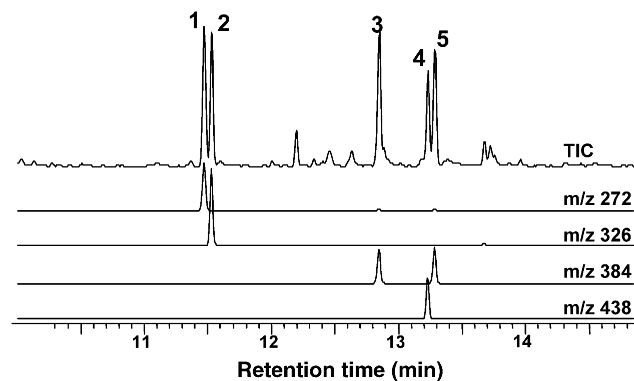

Fig. 3. Total ion chromatogram and extracted mass chromatograms by GC–MS obtained from a spiked urine sample. The concentrations of all the analytes added to the urine were 5 μg/ml. Peaks: (1) BZP; (2) TFMPP; (3) *p*-OH-BZP; (4) *p*-OH-TFMPP; (5) *m*-OH-BZP.

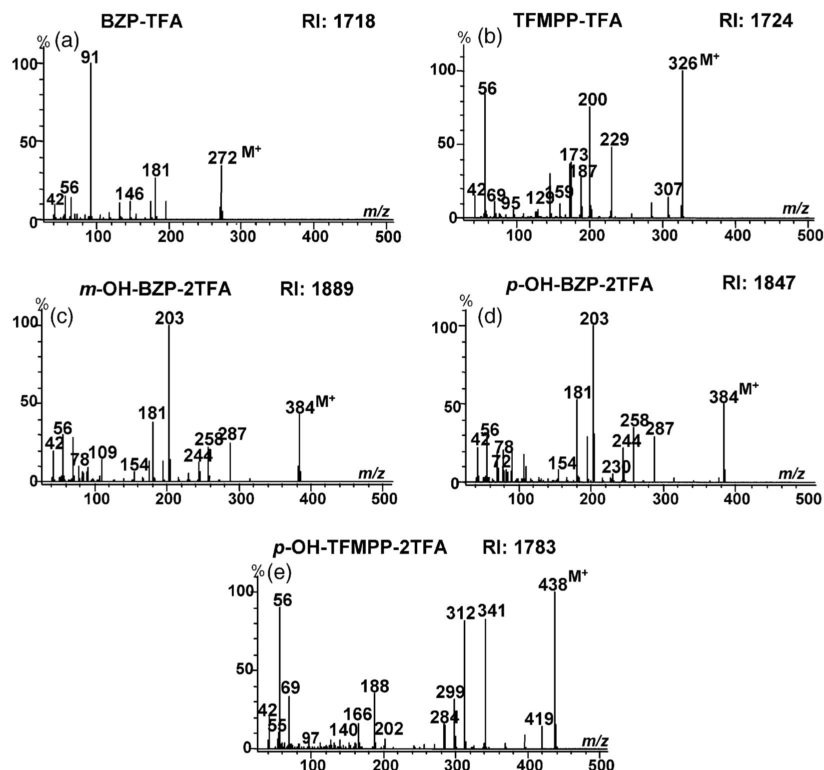

Fig. 4. EI mass spectra produced from peaks of TFA derivatives of BZP (a), TFMPP (b), *m*-OH-BZP (c), *p*-OH-BZP (d) and *p*-OH-TFMPP (e).

on their spectra. However, as described above, the retention times of the isomers will allow to readily discriminate *m*-OH and *p*-OH metabolites by GC–MS.

For the scan mode, the detection limits were estimated to be 50 ng/ml for BZP, 200 ng/ml for TFMPP, 750 ng/ml for *m*-OH-BZP and *p*-OH-BZP, and 1 μg/ml for *p*-OH-TFMPP, at which the three most intense ions in each mass spectrum could be observed and the intensity of noises from backgrounds were about the same as that of the least intense ion of the three target ions in each background-subtracted mass spectrum. When the selected ion monitoring technique, in which each molecular ion was selected, was applied, the limits were 20 ng/ml for BZP, 100 ng/ml for TFMPP, 500 ng/ml for *m*-OH-BZP and *p*-OH-BZP and *p*-OH-TFMPP based on a S/N ratio of ca. 3:1.

With further exploration, the GC–MS analysis after the TFA derivatization was found to show a relatively low reproducibility for the hydroxylated metabolites. This suggests that the GC–MS technique accompanied by TFA derivatization would not be preferable for the quantitative analysis of BZP, TFMPP and their metabolites.

3.3. Chromatographic condition for LC–MS

The mobile phase with a higher organic solvent concentration, which is also advantageous in achieving a higher sensitivity in ESI-MS, works more effectively on a SCX column than on an ODS column, especially when analyzing amines such as opiates [26] and amphetamines [27–29]. Therefore, an SCX-type column was employed in combination with the acetonitrile–ammonium acetate buffer (40 mM) mobile phase.

In order to simultaneously determine the five analytes, we explored the optimization of the eluent. The acetonitrile concentration was varied between 60 and 75% with the ammonium acetate buffer concentration in the fixed mobile phase, and their retention behavior was investigated. The five analytes eluted with a relatively good separation in the order of TFMPP, *p*-OH-TFMPP, BZP and OH-BZPs at all acetonitrile concentrations. At the acetonitrile concentration of 75%, all the analytes were eluted within 20 min. With this composition (40 mM ammonium acetate buffer–acetonitrile (25:75, v/v), referred to mobile phase B), the separation between TFMPP, *p*-OH-TFMPP and BZP was sufficient, but the two *m*-OH and *p*-OH-BZP isomers almost co-eluted.

In order to further improve the separation between *m*-OH-BZP and *p*-OH-BZP, the pH of ammonium acetate buffer was lowered by adding acetic acid to the mobile phase B. The satisfactory separations of all analytes and a relatively fast elution were achieved when the pH was approximately 4 (Fig. 5): the final composition was 40 mM ammonium acetate buffer (pH 4)–acetonitrile (25:75, v/v) (referred to as mobile phase A). When limited only to the analysis of TFMPP and *p*-OH-TFMPP, mobile phase B would be preferable for a better separation and a faster elution.

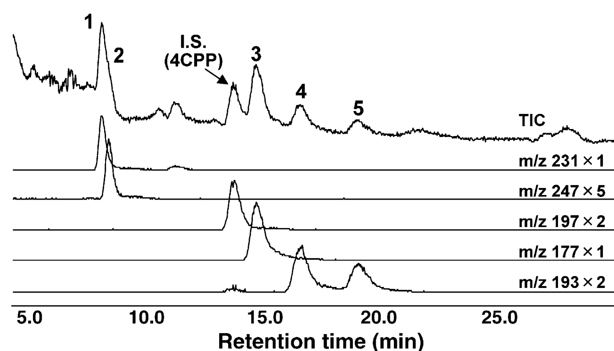

Fig. 5. Total ion chromatogram and extracted mass chromatograms by LC–ESI-MS obtained from a spiked urine sample. The concentrations of all analytes added to the urine were 1 μg/ml. Peaks: (1) TFMPP; (2) *p*-OH-TFMP; (3) BZP; (4) *p*-OH-BZP; (5) *m*-OH-BZP. The internal standard (I.S.) was 1-(4-chlorophenyl)-piperazine (4CPP).

3.4. Condition for ESI-MS

In order to optimize the operating parameters of ESI-MS for the five analytes, the probe, curved desolvation line (CDL) and Q-array voltages were varied, and the abundance of each protonated molecule $[M+H]^+$ was measured. At the probe, CDL and Q-array voltages of 4.5 kV, 25 V and 15 V, respectively, the highest abundance was achieved. Thus, we finally selected these parameters for the ESI-MS.

The mass spectra obtained under the optimized conditions are presented in Fig. 6. All of their mass spectra were characterized by each $[M+H]^+$ ion at *m/z* 177 for BZP, *m/z* 231 for TFMPP, *m/z* 193 for *m*-OH-BZP and *p*-OH-BZP, and *m/z* 247 for *p*-OH-TFMPP. Less intense ions corresponding to $[M+CH_3CN+H]^+$ (*m/z* 272 for TFMPP and *m/z* 288 for *p*-OH-TFMPP) also appeared in the mass spectra of TFMPP and *p*-OH-TFMPP. However, the mass spectra of *m*-OH-BZP and *p*-OH-BZP were very similar, and the retention characteristics will, therefore, be helpful in discrimination of the isomers.

3.5. Extraction recoveries

The simultaneous extraction of BZP, TFMPP and their hydroxylated metabolites, *m*-OH-BZP, *p*-OH-BZP and *p*-OH-TFMPP, was explored by solid phase extraction (SPE). In the present study, SPE with a hydrophilic polymer OASIS HLB cartridge [30,31] and a mixed-mode cation-exchange reversed-phase extraction OASIS MCX cartridge [32], and liquid–liquid extraction with chloroform–2-propanol mixture (3:1, v/v) were tested, and the resultant recoveries were compared. As urine samples, drug-free urine samples from a healthy volunteer in which the concentrations of the artificially added analytes were 1 and 10 μg/ml were employed. Extraction was conducted according to the method described in Section 2, and each extract was subjected to LC–MS in the SIM mode by monitoring each $[M+H]^+$ ion. Extraction recoveries were estimated by comparing the peak area ratios (analyte versus I.S.) from the spiked urine samples and standard aqueous solution for each analyte at each concentration.

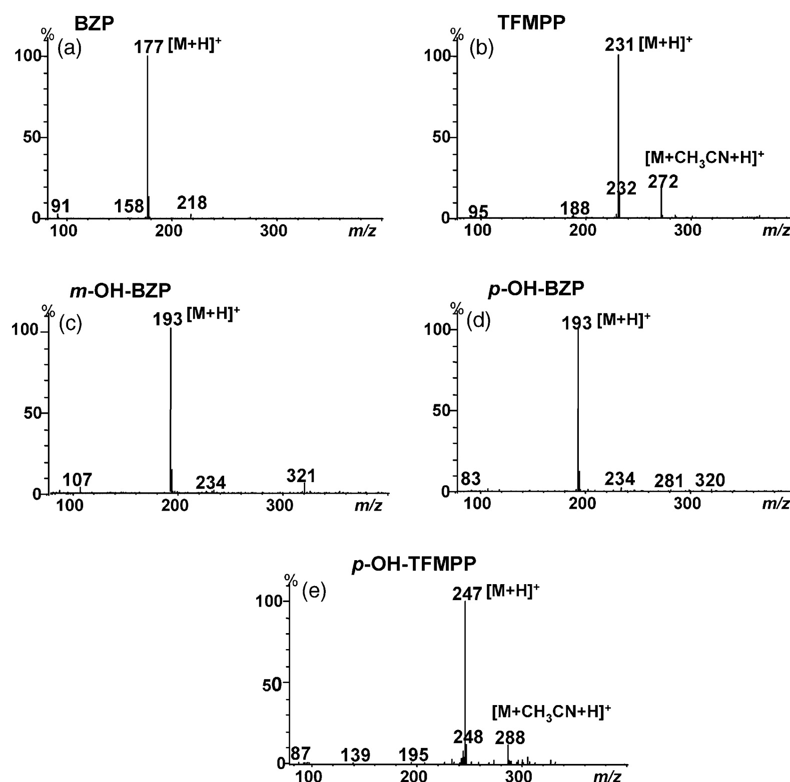

Fig. 6. ESI mass spectra produced from peaks of BZP (a), TFMPP (b), *m*-OH-BZP (c), *p*-OH-BZP (d) and *p*-OH-TFMPP (e).

Table 1
Extraction recoveries ($n = 5$)

Compound	Recoveries (%)					
	OASIS HLB		OASIS MCX		Liquid–liquid extraction	
	10 μg/ml	1 μg/ml	10 μg/ml	1 μg/ml	10 μg/ml	1 μg/ml
BZP	92	95	100	81	35	38
m-OH-BZP	83	87	102	85	6	6
p-OH-BZP	81	82	102	98	9	8
TFMPP	100	103	113	62	77	36
OH-TFMPP	109	83	103	20	20	7

As summarized in Table 1, the resultant recoveries by liquid–liquid extraction were considerably low for all the analytes. Especially, all three hydroxylated metabolites gave very low recoveries of less than 10%. This would be caused by the high hydrophilicity of the phenol moiety leading to their low distribution into the organic phase. Thus, the liquid–liquid extraction was ruled out.

On the other hand, the recoveries by SPE were relatively high: the recoveries for all the analytes using OASIS HLB and OASIS MCX cartridges ranged from 81 to 109% and from 20 to 113%, respectively. The recoveries of the hydroxylated metabolites at the concentration of 10 μg/ml, in particular, reached approximately 80% or more. The recoveries with MCX at the concentration of 1 μg/ml for TFMPP and *p*-OH-TFMPP were, however, significantly lower, though BZP and its hydroxylated metabolites had high recoveries at either concentration with MCX. The lower recoveries at the lower concentration for TFMPP and *p*-OH-TFMPP may be caused by adsorption on the sorbent. Based on these comparisons, the SPE procedure with OASIS HLB was finally chosen for the simultaneous extraction of BZP, TFMPP and their hydroxylated metabolites in the present study.

3.6. Quantitative analysis

Quantitative measurements were carried out in the presence of the internal standard, 4CPP using LC–MS in the SIM mode: The $[M + H]^+$ ions of each analyte (*m/z* 177 for BZP, *m/z* 231 for TFMPP, *m/z* 193 for *m*-OH-BZP and *p*-OH-BZP, *m/z* 247 for *p*-OH-TFMPP, and *m/z* 197 for 4CPP) were monitored, and the linearity and reproducibility were evaluated for drug-free urine samples spiked with known concentrations of analytes ($n = 5$).

The LC–ESI-MS showed a good linearity throughout the concentration range from 10 ng/ml to 10 μg/ml for all analytes (BZP: $y = 0.575x + 0.137$, $r^2 = 0.999$; TFMPP: $y = 0.293x + 0.00570$, $r^2 = 0.999$; *m*-OH-BZP: $y = 0.196x + 0.0840$, $r^2 = 0.998$; *p*-OH-BZP: $y = 0.120x + 0.0833$, $r^2 = 0.994$; *p*-OH-TFMPP: $y = 0.0591x + 0.0221$, $r^2 = 0.997$). As summarized in Table 2, the within-day relative standard deviations (RSDs) and the accuracy for all analytes ranged from 3.1 to 9.2% and from 89 to 108% at the concentration of 1 μg/ml, and from 3.2 to 8.0% and from 94 to 112% at 200 ng/ml, respectively. The between-day RSDs were calculated to be 1.2–11.1% at the concentration of 1 μg/ml.

Also, for LC–ESI-MS in the full scan mode, the detection limits were estimated to be 5 ng/ml for BZP, 10 ng/ml for TFMPP, *m*-OH-BZP and *p*-OH-BZP, and 40 ng/ml for *p*-OH-TFMPP, at which each $[M + H]^+$ ion could be observed and the intensity were about the same as those of noises from backgrounds in each background-subtracted mass spectrum. The SIM technique by monitoring each $[M + H]^+$ ion further reduced the detection limits to 0.2 ng/ml for BZP and TFMPP, 0.4 ng/ml for *m*-OH-BZP and *p*-OH-BZP, and 1 ng/ml for *p*-OH-TFMPP based on a S/N ratio of ca. 3:1. These results indicate that the present technique gave a satisfactorily high reliability and sensitivity.

Table 2
Precision and accuracy ($n = 5$)

Compound	Mean	SD	RSD (%)	Accuracy (%)
Within-day 1 μg/ml				
BZP	0.94	0.045	4.8	94
m-OH-BZP	0.89	0.028	3.1	89
p-OH-BZP	0.96	0.035	3.6	96
TFMPP	0.96	0.061	6.4	96
OH-TFMPP	1.08	0.099	9.2	108
Within-day 200 ng/ml				
BZP	211	6.9	3.2	106
m-OH-BZP	194	11.8	6.1	97
p-OH-BZP	224	17.9	8.0	112
TFMPP	216	14.9	6.9	108
OH-TFMPP	187	11.1	6.0	94

4. Conclusion

A sensitive and reliable analytical procedure for the simultaneous determination of BZP, TFMPP and their main metabolites *m*-OH-BZP, *p*-OH-BZP and *p*-OH-TFMPP, in urine was established by GC–MS and LC–ESI-MS analyses. A combination of the enzymatic hydrolysis, SPE with OASIS HLB, TFA derivatization for GC–MS, and LC separation on a semi-micro SCX column provided convenient pretreatment, good recoveries and high sensitivity.

GC–MS after TFA derivatization gave much more structural fragment ions that are favorable for the identification, but have found to show relatively low reproducibility. On the

other hand, LC–ESI-MS allowed accurate quantitation. In addition, identification of analyte for proof of the use of controlled drugs in forensic toxicology should be done by more than a technique, if possible. Thus, we strongly recommend the combination of GC–MS and LC–MS techniques for more reliable determination. The developed methods in the present study would serve as useful tools in drug enforcement that requires reliable proof of the intake of BZP and TFMP.

The abuse of new piperazine derivatives as recreational drugs will be encountered, and future studies on the development of analytical methods for these drugs and their metabolites will be required. However, the present procedure will be helpful for this requirement.

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