Journal of Food and Drug Analysis, Vol. 16, No. 5, 2008, Pages 28-38 藥物食品分析 第十六卷 第五期

Development and Validation of an HPLC Method for the Purity Assay of BZM, the Precursor of Striatal Dopaminergic D2/D3 Receptor SPECT Imaging Agent [^{123}I]IBZM (Iodobenzamide)

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(Received: October 23, 2007; Accepted: February 22, 2008)

ABSTRACT

We developed and validated a reverse-phase high performance liquid chromatography (RP-HPLC) method for the purity assay of BZM. BZM is a precursor and free ligand of [^{123}I]IBZM (iodobenzamide) that is a SPECT imaging agent for striatal dopaminergic D2/D3 receptor (D2R). The chromatographic separation was achieved on a Zorbax Eclipse XDB-C18 column with a gradient mobile phase consisting of 10 mM ammonium acetate buffer, pH 7.0 and acetonitrile at a flow rate of 0.5 mL/min. The absorbance at 254 nm against the concentration of BZM over the range 0.5-5.5 μg was linear, with a correlation coefficient above 0.9997. The limit of detection (LOD) and quantification (LOQ) of major impurity (impurity A, t_R = 3.85 min) in BZM were 0.028% and 0.094%, respectively. Method validation parameters, including specificity, precision, accuracy, linearity, LOD/LOQ, robustness and solution stability were evaluated, indicating the potential of this method in pharmaceutical quality control. Moreover, the most sensitive precursor to product ion transitions for the identification of BZM and 'cold' IBZM by LC-ESI-MS/MS were found to be m/z 279.0-112.0 and 405.0-112.0, respectively. The present results allow identification of fragmentation ions and proposition of the pathways of BZM and 'cold' IBZM, showing that the feasibility of this method for quantification of free ligand BZM in SPECT imaging agent [^{123}I]IBZM injection.

Key words: BZM, IBZM ([^{123}I]IBZM), RP-HPLC, LC-ESI-MS/MS, Purity assay, Method validation, MRM transition, Fragmentation pathway

INTRODUCTION

I-123-Iodobenzamide ([^{123}I]IBZM or (S)-2-hydroxy-3-[^{123}I]iodo-6-methoxy- *N*- [(1-ethyl-2-pyrrolidinyl)methyl]-benzamide, $C_{15}H_{21}N_2O_3I$, mw 400.24) (Figure 1), which has high uptake in the striatum and specific binding to the striatal dopaminergic D2/D3 receptor (D2R), is the first and still the most widely used radioligands for D2R[1]. A dose of 135~185 MBq, i.e. 15~21 pmole or 6.2~8.5 ng of [^{123}I]IBZM per patient is given by intravenous injection. The specific (striatum, caudate nucleus and putamen or basal ganglia) to nonspecific (cerebellum, frontal cortex or occipital cortex) equilibrium ratio or coefficient (V_3'') for quantification is calculated according to eq. (1)[2-15]:

$$V_3'' = \frac{A_{ROIs} - A_{ns}}{A_{ns}} = \frac{A_{ROIs}}{A_{ns}} - 1 \qquad \text{eq (1)}$$

where A_{ROIs} is the regions of interest (ROIs,) and A_{ns} is the nonspecific activity.

The clinical indication of [^{123}I]IBZM has been well developed for the studies of striatal dopaminergic disease or D2 receptor occupancy (D2 RO), including Huntington's disease[3], dopamine (DA) release following administration of amphetamine[4,16], obsessive- compulsive disorder (OCD)[5], cognitive performance[6] and fine motor activity[7] of schizophrenia, Cotard syndrome[8], prognosis in naive schizophrenic patients[9], distinction between nonidiopathic parkinsonism disorders (NIPS) and PD[10], as well as clinical efficacy of antipsychotic medica-

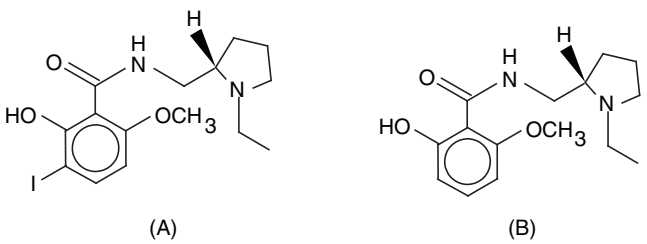

Figure 1. Chemical structures of (a) IBZM and its precursor (b) BZM.

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tions, i e., olanzapine, clozapine, haloperidol, sertindole, risperidone, quetiapine[11-14], extrapyramidal side effects (EPS)[2, 15] and acute psychosis exacerbation[2].

[^{123}I]IBZM was used as a single photon emission computed tomography (SPECT) imaging agent followed by radioiodination of BZM with Iodine-123 according to the eq. (2)[1, 17-22]. Iodine-123 is an ideal tracer because of its radioisotopic properties, i.e. the short half-life, clear SPECT images, ease of handling, shielding and dispose[17-18]. Synthesis of [^{123}I]IBZM was developed firstly by Kung *et al*.[17-20] and later by Zea-Ponce *et al*.[21-22] as well as by Baldwin *et al*.[1]. BZM ((S)-(-)-2-Hydroxy -6-methoxy-N-[(1-ethyl-2-pyrrolidinyl) methyl]benzamide, $C_{15}H_{22}N_2O_3$, mw 278.35) is the free ligand and precursor of [^{123}I]IBZM (Figure 1).

$$\text{BZM} + \text{I-123} + \text{oxidizing agent} \rightarrow [^{123}\text{I}]\text{IBZM} \qquad \text{eq (2)}$$

Purity of BZM is essential since the presence of impurities in active pharmaceutical ingredients (API) can have a significant effect on their quality, safety and stability of radioiodination yield, especially in the case of purification of final product from reactants and by-products by solid phase extraction (SPE)[23]. Till date, neither method or method validation for the purity assay of BZM before it is labeled with I-123, nor quantification of free BZM in [^{123}I]IBZM injection has been published. This study was aimed to develop and to validate a HPLC method for the purity assay of BZM for the purpose of pharmaceutical CMC quality control. Moreover, using the precursor to product ion transitions for the identification of BZM and IBZM, LC-ESI-MS/MS was developed to evaluate the feasibility for the quantification of free BZM in [^{123}I]IBZM injection.

EXPERIMENTAL

I. *Materials and Reagents*

BZM, 'cold' IBZM and [^{123}I]IBZM were synthesized and prepared by Radiation Application Technology Center, Institute of Nuclear Energy Research (INER, Taiwan). All chemicals and reagents were of analytical grade and used as received without further purification. Dimethyl sulfoxide (DMSO for spectroscopy), methanol and acetonitrile (HPLC grade) were obtained from Merck (Darmstadt, Germany). Deionized water was purified using a Smart DQ3 reverse osmosis reagent water system (Millipore, MA, USA) with 0.22 μm filter, TOC < 5 ppb, resistivity ≥ 18.2 MΩ-cm and endotoxin < 0.001 EU/mL. For safety, radioactive substances and LC-MS/MS were carried out in a radioactive area and shielding by lead bricks. The operation license of radioactive area, where is equipped with HEPA filter and is operated under negative atmospheric pressure condition, is approved and regulated by Atomic Energy Council (AEC), Taiwan.

II. *HPLC Instrumentation*

An Agilent 1100 series HPLC (Agilent, Palo Alto, CA, USA), consisted of on-line degasser, binary pump, auto-sampler, thermostated column oven and photodiode-array detector (PDA) was employed. Data were acquired and processed with ChemStation (Agilent, Palo Alto, CA, USA). A C-18 reversed phase column (Zorbax Eclipse XDB-C18, 4.6 × 50 mm, 1.8 μm, Agilent) was used for the separation. The column temperature was held at 25°C. A step gradient elution was achieved using the mobile phase consisting of eluent A (10 mM ammonium acetate buffer, pH 7.0) and eluent B (acetonitrile). The gradient was 10%-95% B in 0.5 min and kept at 95% B for 5.5 min, and back to 10% B in 4 min. The flow-rate was 0.5 mL/min. The absorbance detection wavelength was 254 nm.

III. *HPLC-MS/MS Instrumentation*

Tandem MS analysis was carried on a 4000 QTrap LC-MS/MS System and API Analyst software of version 1.4.1 (MDS Sciex, Ontario, Canada). Sample was introduced by HPLC system (Agilent 1100 series HPLC system, Agilent, CA, USA) or a syringe pump at a flow rate of 10 μL/min (Harvard, Harvard Apparatus Inc., MA, USA). Chromatography was performed as described in Section of HPLC instrumentation. The samples were ionized by a turbo spray ion source (electrospray ionization) in the positive ion mode at 5500 V and 500°C. Mass spectra were obtained over the range from 50 or 100 amu to 1000 amu with unit resolution in Q1 and Q3. Other parameters were shown in Table 1. Nitrogen was used in all cases, i.e. for the nebulization, curtain and collision.

IV. *Preparation of the Sample Solutions*

All sample solutions for HPLC experiments were prepared by dissolving BZM and IBZM in DMSO with final concentration of 500 ppm. For LC-MS/MS experiments, the BZM and IBZM stock solutions were diluted with acetonitrile to a final concentration of 100 ppb. Mixtures of BZM and IBZM were further diluted with acetonitrile.

V. *Purity*

The purity (P, %) of BZM was calculated as below:

$$\text{P (\%)} = \frac{\text{P A}_{\text{BZM}}}{\text{P A}_{\text{total}}} \times 100\% \qquad \text{eq (3)}$$

Where PA_{BZM} is the peak area of BZM and PA_{total} is the total peak area of chromatogram.

VI. *Resolution of Chromatogram*

Resolution (Rs) between two peaks was determined as below:

Table 1. Main working parameters of tandem mass spectrometry

Parameters	MS2 scan
Source Temperature	0
Scan Type:	Product Ion (MS2)
Polarity	Positive
Resolution (Q1&Q3)	Unit
Nebulizer Gas (NEB)	11
Curtain Gas (CUR)	10
IonSpray Voltage (IS)	5500 V
Collision Gas (CAD)	Medium
Ion Energy 1 (IE1)	0.7 V
Ion Energy 3 (IE3)	-0.3 V
Detector Parameters	Positive
Deflector (DF)	-100
Channel Electron Multiplier (CEM)	2200 V

$$Rs = \frac{t_{R2} - t_{R1}}{w_{half2} + w_{half1}} \times 1.175 \qquad \text{eq (4)}$$

Where t_{R2} and t_{R1} are the respective retention time, w_{half2} and w_{half1} are the respective width of the peaks at half height.

VII. *Method Validation*

The method was validated according to the International Conference on Harmonization (ICH) guidelines for the validation of analytical method, including specificity, linearity, accuracy, precision, LOD/LOQ, robustness and solution stability.

(I) *Specificity (Selectivity)*

Forced degradation studies are used to facilitate the development of analytical methodology, to gain a better understanding of active pharmaceutical ingredient (API) and drug product (DP) stability, and to provide information about degradation pathways and degradation products[26]. Forced degradation studies were also performed to evaluate the specificity (selectivity) of purity assay method of BZM. Solid state samples or solutions of BZM were subjected to several stress conditions, i.e., acid, base, heat, UV light and oxidation for different periods of time. Samples were neutralized and diluted to 500 ppm of BZM concentration before HPLC analysis.

(II) *Linearity*

The calibration curves of sample injection amount were plotted against the peak areas. The linearity was evaluated by the linear least square regression method with five or three determinations at each injection.

(III) *Precision (Repeatability, Reproducibility and Intermediate)*

The precision of the method was determined by performing five assays of the same batch of BZM at four injection amounts (0.54-5.4 μg). The intra-day precision (repeatability) and inter-day precision (reproducibility) were evaluated by one analyst within one day and two different days, respectively. The intermediate precision was achieved by using different columns (same type and different batch) and different analysts.

(IV) *Accuracy (Recovery)*

The accuracy was determined by the recovery test. The samples were spiked with two known amounts of BZM (A_{spiked}) and analyzed (A_{exp}) by the proposed method.

$$\text{Recovery (\%)} = \frac{A_{exp}}{A_{spiked}} \times 100\% \qquad \text{eq (5)}$$

(V) *Limit of Detection (LOD) and Limit of Quantification (LOQ)*

LOD and LOQ of HPLC of BZM and Impurities were determined at signal to noise ratios of 3 and 10, respectively.

(VI) *Robustness*

Method robustness was evaluated with effects of different column temperatures, pH values and flow rates of mobile phase.

(VII) *Stability of Drug Solution*

The stability of the drug solution was determined on the basis of the retention time of BZM, resolution between BZM and impurity A, purity of BZM, slope and correlation coefficient of the calibration curves. The drug solution was kept at room temperature (around 25cC) and the calibration curves over the injection range (0.53 - 3.71 μg) for three consecutive days were obtained.

RESULTS AND DISCUSSION

I. *Method Development*

The BZM and 'cold' IBZM, instead of [^{123}I]IBZM samples at the concentrations of 500 ppm and 100 ppb were used to optimize conditions for HPLC and LC-ESI-MS/MS, respectively. No significant difference was found in the chromatographic retention time and MS

Journal of Food and Drug Analysis, Vol. 16, No. 5, 2008

parameters between [^{127}I]IBZM and [^{123}I]IBZM.

Absorption spectra of BZM and IBZM were recorded over the wavelength range of 200-300 nm. It was found that their absorbance near 210 nm is interfered by DMSO. A wavelength of 254 nm was found optimal for the detection and quantification of the purity assay of BZM in DMSO solution.

The Zorbax Eclipse XDB-C18 (4.6 × 50 mm, 1.8 μm) column was selected for the separation of BZM, IBZM, impurities and forced degradation compounds. The efficiency of the column was demonstrated by number of theoretical plates (N) and tailing factor.

The chromatographic separations of BZM and 'cold' IBZM were monitored at 254 nm using mobile phases consisting of various mixtures of ammonium acetate, methanol and acetonitrile. Although the resolution between BZM and impurity A (the nearest peak) was best at pH 6.50, pH 7.00 was more suitable to achieve the separation of forced degradation compounds. Finally, a mobile phase consisting of eluent A (10 mM ammonium acetate, pH 7.0) and eluent B (acetonitrile) with gradient of 10% - 95% B in 0.5 min and kept at 95% B for 5.5 min, then back to 10% B in 4 min.

The typical HPLC chromatogram of BZM was shown in Figure 2. The peaks at retention time (t_R) of 1.24 min, 3.10 min, 3.85 min and 4.38 min were for solvent, impurity B, impurity A and impurity C, respectively. The peak at 3.45 min was identified as protonated BZM ion at m/z 279.4 by LC-ESI-MS. The protonated molecular ion ([M+H]$^+$) with m/z 405.2 at the retention time of 4.06 min was identified for 'cold' IBZM (spectrum not shown).

As to the average content percentage of major impurity, impurity A (t_R = 3.85 min) was 1.30%, whereas those for impurity B (t_R = 3.10 min) and impurity C (t_R = 4.38 min) were less than 0.01% - 0.02%. The calibration plot of peak area against BZM amount showed good linearity over the injection range of 0.54 - 5.40 μg. A linear regression equation of y = 3919x -11 with a correlation coefficient of 1.0000 was achieved.

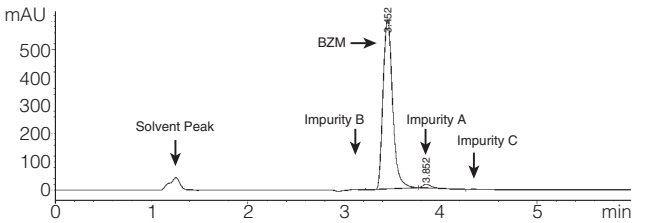

Figure 2. Typical HPLC chromatogram of BZM and its major impurities.

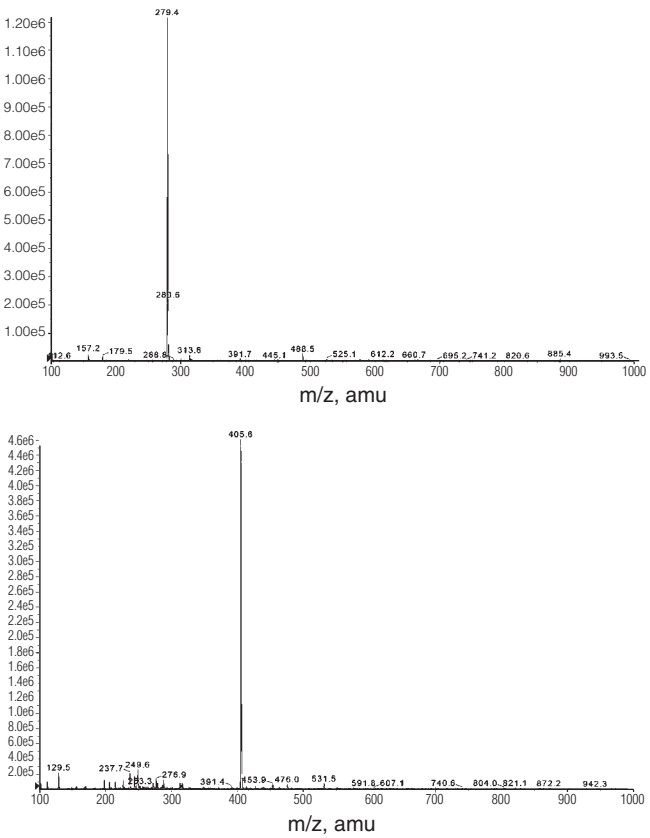

Figure 3. ESI spectra of (a) BZM and (b) 'cold' IBZM.

II. *Mass Spectrometric Analysis*

'Cold' IBZM ([^{127}I]IBZM) was used instead of [^{123}I]IBZM for the MS/MS method development. The proposed mobile phase consisting of 10 mM ammonium acetate and acetonitrile was volatile and suitable for ESI-MS study. Neither component or concentration ratio of mobile phase was changed.

In order to optimize the ESI conditions of BZM and 'cold' IBZM, Q1 full scans were achieved in a positive ion mode. The ESI spectra of BZM and 'cold' IBZM (Figure 3) showed a protonated molecular ion, [M+H]$^+$ at m/z 279.4 and m/z 405.2, respectively. Significant impurity was not found from the Q1 scan of BZM and 'cold' IBZM.

Product ion scan and precursor ion scan were then carried out at different collision activated dissociation (CAD) conditions to optimize the declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) (Table 2). The product ion scanning spectra of [M+H]$^+$ showed fragment ions at m/z 151.1, 136.1, 129.2, 112.2, 108.1 for BZM and at m/z 276.0, 262.0, 234.0, 150.1, 129.2, 112.2, 107.1 for 'cold' IBZM (Figure 4). The fragment ions of the highest and second high intensity were found at m/z 112.2 and 129.2 for both compounds.

Linearity of multiple reaction monitoring (MRM) transitions were studied from m/z 279.0 to 151.0, 136.0, 129.0, 112.0 and 108.0 for BZM, and from m/z 405.0 to 276.0, 262.0, 234.0, 150.0, 129.0, 112.0, 107.0 for 'cold' IBZM, respectively. The linear least square regression equations and correlation coefficients of MRM transitions of BZM and 'cold' IBZM (Table 3) showed good

Table 2. Retention times and optimized mass spectrometry parameters for the determination of BZM and 'cold' IBZM

Compound	t_R (min)	Precursor ion (m/z)	DP	EP	Product ion (m/z)	CE	CXP
BZM	3.38	279.0	65	10	151.0	40	11
					136.0	61	12
					130.0	29	12
					129.0	28	12
					112.0	36	19
					108.0	76	17
					121.0	56	21
					105.0	63	12
					261.0	26	17
'cold' IBZM	4.06	405.0	97	8	276.0	46	17
					262.0	62	14
					234.0	80	10
					150.0	67	6
					129.0	34	10
					122.0	69	6
					112.0	46	9
					107.0	90	18

t_R: retention time; DP: declustering potential ; EP: entrance potential; CE: collision energy; CXP: collision cell exit potential

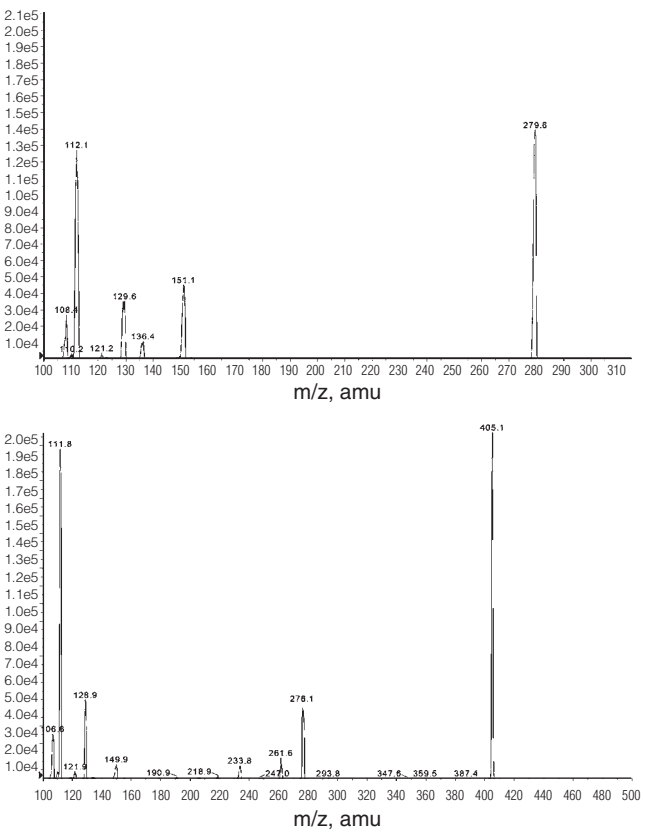

Figure 4. MS/MS spectra of (a) BZM ion at m/z 279.0 and (b) 'cold' IBZM ion at m/z 405.0.

linearity over the calibration range. The correlation coefficients were all above 0.9970. The similarity of fragmentation ions confirmed thereby that similar structures of BZM and 'cold' IBZM. Again, the most sensitive mass transition was from m/z 279.0 to 112.0 for BZM and from m/z 405.0 to 112.0 for cold IBZM, respectively.

The MS/MS fragmentation ions and pathway of BZM and 'cold' IBZM were proposed as shown in Figure 5. We believed that there is no significant difference in the MS parameters between [^{127}I]IBZM and [^{123}I]IBZM. Although drug stability of [^{123}I]IBZM might change due to the decay reaction of iodine-123 into tellurium-123, the fragmentation pathway of [^{123}I]IBZM is similar to that of 'cold' IBZM. We postulated that the product ions of [^{123}I]IBZM are at m/z 272.0, 258.0, 230.0, 150.1, 129.2, 112.2 and 107.1. The MRM transition of m/z 279.0 to 112.0 for BZM and that of m/z 401.0 to 112.0 for [^{123}I]IBZM indicated the feasibility for the simultaneous quantification of free ligand BZM and [^{123}I]IBZM in SPECT imaging agent [^{123}I]IBZM injection.

III. *Purity Assay Method Validation*

(I) *Specificity (Selectivity)*

Results in the stress study were summarized in Table 4. No significant degradation was observed under stress conditions of 1M HCl, 1M NaOH, 80°C (in an oven) and

Table 3. Linearity studies of MRM transitions of BZM and 'cold' IBZM

Compound	MRM transition	Linear regression eq.	r
BZM	279.0/151.0	$Y = 8.33x10^4X + 3.53\ x10^4$	0.9987
	279.0/136.0	$Y = 1.78x10^4X + 4.10\ x10^3$	0.9971
	279.0/129.0	$Y = 1.29x10^5X + 8.01\ x10^4$	0.9973
	279.0/112.0	$Y = 1.96x10^5X + 1.02\ x10^5$	0.9973
	279.0/108.0	$Y = 2.54x10^4X + 1.50\ x10^4$	0.9974
'cold' IBZM	405.0/276.0	$Y = 6.81x10^5X + 2.11\ x10^5$	0.9974
	405.0/262.0	$Y = 1.59x10^5X + 9.09\ x10^3$	0.9980
	405.0/234.0	$Y = 1.14x10^5X + 1.10\ x10^4$	0.9985
	405.0/150.0	$Y = 1.23x10^5X + 5.55\ x10^3$	0.9985
	405.0/129.0	$Y = 1.47x10^6X + 3.26\ x10^5$	0.9990
	405.0/112.0	$Y = 2.34x10^6X + 1.01\ x10^6$	0.9974
	405.0/107.0	$Y = 2.93x10^5X + 7.43\ x10^4$	0.9976

linear range: 0.135 - 1.350 ng (n = 3) for BZM and 0.126 - 1.260 ng (n = 3) for 'cold' IBZM; r: correlation coefficient

Table 4. Forced degradation study results of BZM

Stress tests	Weight or concentration of BZM	Stress conditions	Neutralization or dissolution before analysis by HPLC	Duration time (hrs)	Purity of BZM (%)[d]
Acid	1.0 mg/0.4 mL DMSO	0.4 mL HCl, RT	NaOH	4	98.25
	0.6 mg/0.4 mL DMSO	0.4 mL HCl, RT	NaOH	24	98.65
	0.6 mg (solid state)	0.4 mL HCl, RT	NaOH and DMSO	24	98.55
Base	1.0 mg/0.4 mL DMSO	0.4 mL NaOH, RT	HCl	4	98.56
	0.6 mg/0.4 mL DMSO	0.4 mL NaOH, RT	HCl	24	98.62
	0.6 mg (solid state)	0.4 mL NaOH, RT	HCl and DMSO	24	98.08
Heat	1.0 mg (solid state)	50°C	DMSO	4	98.62
	0.6 mg (solid state)	50°C	DMSO	24	98.42
Acid & Thermal	0.6 mg (solid state)	0.4 mL HCl, 80°C	NaOH and DMSO	4	98.72
	0.6 mg (solid state)	0.4 mL HCl, 80°C	NaOH and DMSO	24	98.10
Base & Thermal	0.6 mg (solid state)	0.4 mL NaOH, 80°C	HCl and DMSO	4	98.36
	0.6 mg (solid state)	0.4 mL NaOH, 80°C	HCl and DMSO	24	98.86
Acid & UV	0.6 mg (solid state)	0.4 mL HCl, UV	NaOH and DMSO	4	84.59
	0.6 mg (solid state)	0.4 mL HCl, UV	NaOH and DMSO	72	97.98
Base & UV	0.6 mg (solid state)	0.4 mL NaOH, UV	HCl and DMSO	4	95.53
	0.6 mg (solid state)	0.4 mL NaOH, UV	HCl and DMSO	72	98.18
Oxidation	0.6 mg (solid state)	0.4 mL 3% H_2O_2	DMSO	4	80.72
	0.6 mg (solid state)	0.4 mL 3% H_2O_2	Diluted with 3% H_2O_2	24	44.10
	0.6 mg (solid state)	0.4 mL 30% H_2O_2	DMSO	4	91.16
	0.6 mg (solid state)	0.4 mL 30% H_2O_2	Diluted with 30% H_2O_2	24	70.48
	0.6 mg (solid state)	1.2 mL 3% H_2O_2		4	85.82
	0.6 mg (solid state)	1.2 mL 3% H_2O_2		24	50.16
	0.6 mg (solid state)	1.2 mL 30% H_2O_2		4	96.75
	0.6 mg (solid state)	1.2 mL 30% H_2O_2		24	90.61
	0.6 mg/0.4 mL DMSO	0.8 mL 3% H_2O_2		4	74.99
	0.6 mg/0.4 mL DMSO	0.8 mL 3% H_2O_2		24	32.96
	0.6 mg/0.4 mL DMSO	0.8 mL 3% H_2O_2		72	9.57
	0.6 mg/0.4 mL DMSO	0.8 mL 30% H_2O_2		4	71.66
	0.6 mg/0.4 mL DMSO	0.8 mL 30% H_2O_2		24	61.14
	0.6 mg/0.4 mL DMSO	0.8 mL 30% H_2O_2		72	60.77

[HCl] = 1.0M; [NaOH] = 1.0M; RT：room temperature

UV light over 4 - 72 hrs. However, 3% hydrogen peroxide decomposed the BZM most effectively. For example, when 0.63 mg of solid BZM sample was dissolved in 1.2 mL 3% hydrogen peroxide, 50% degradation of BZM was observed at room temperature in a period of 24 hrs (Figure 6). The HPLC resolution between BZM and its main forced degradation compound (t_R = 3.02 min, the nearest peak) was 1.88, indicating that none of the degradation products interfered with the quantification of BZM.

(II) *Linearity*

The calibration curve for BZM purity assay showed good linearity over the injection range of 0.54 - 5.4 μg.

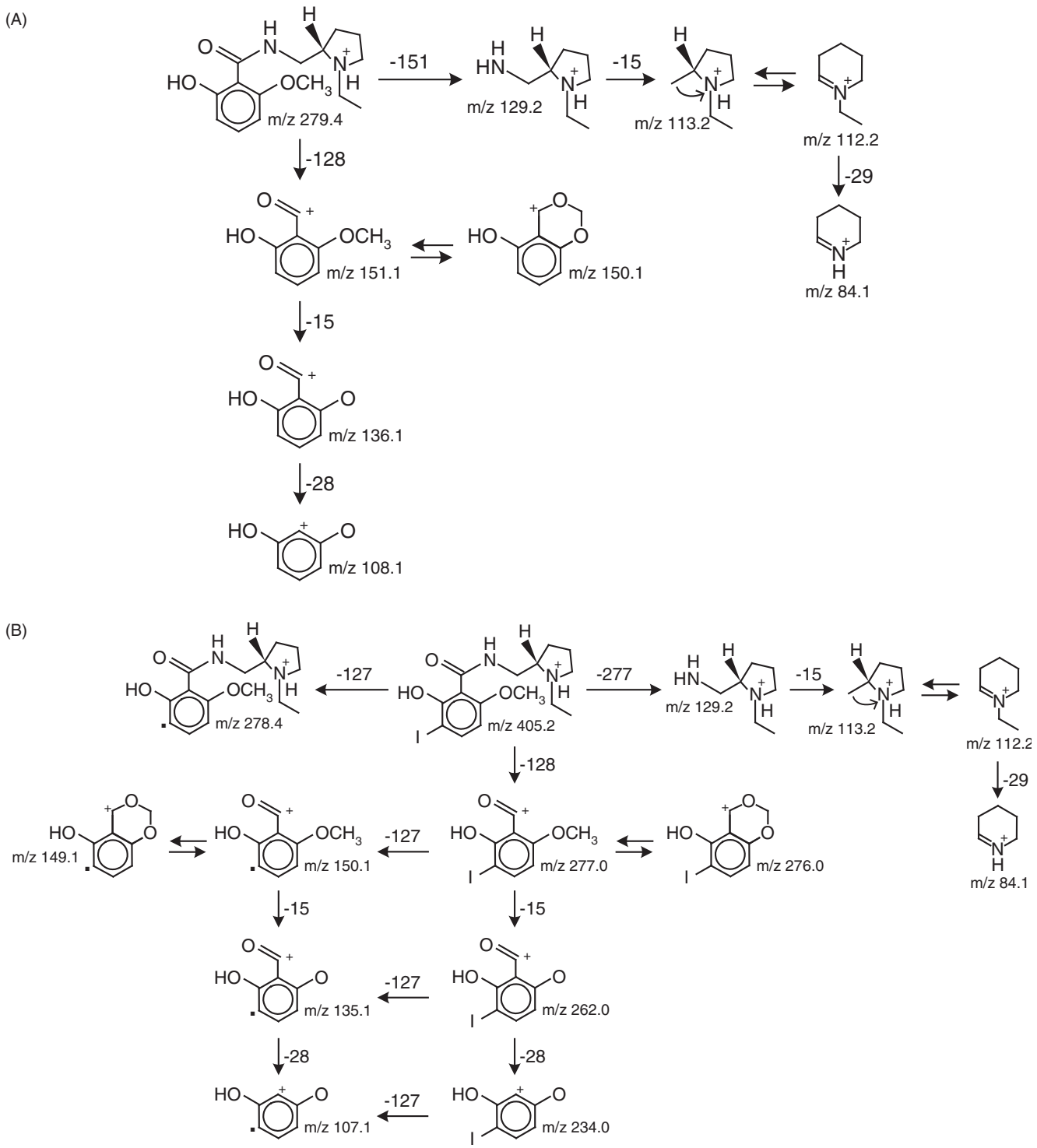

Figure 5. Proposed fragmentation pathways of (a) BZM and (b) 'cold' IBZM.

The linear least square regression equation was Y = 3919X - 11, with a correlation coefficient of 1.0000 (Table 5).

(III) *Precision (Repeatability, Reproducibility and Intermediate)*

The intra-day precision values of retention time of BZM, impurity A (t_R - 3.85 min) and purity were 0.28%, 0.23% and 0.40%, respectively (Table 5). The resolution between BZM and impurity A was 2.13 ± 0.61 (RSD = 28.78%, n = 20). The numbers of theoretical plates of BZM and impurity A were 4,100 and 21,000, respectively.

The inter-day precision of retention time of BZM, resolution between BZM and impurity A, purity of BZM and slope of the calibration curve were 0.34%, 3.93%, 0.88% and 3.12%, respectively (Table 6). The correlation coefficients were 1.0000.

The intermediate precision which was determined by retention time of BZM, resolution between BZM and impurity A, purity of BZM and slope of the calibration curve were 0.58%, 2.90%, 0.49% and 10.44% for different columns and 0.34%, 10.81%, 0.73% and 2.90% for different analysts, respectively. The correlation coefficients of BZM were above 0.9990.

(IV) *Accuracy (Recovery)*

Two known amounts, i.e. 3 μL and 7 μL of BZM (400 ppm) in DMSO (blank solution) were analyzed by the proposed method. The peak areas of spiked samples were interpolated by the calibration curve linear regression equation. The accuracy results were summarized in Table 7. The recoveries were between 95-105%, indicating that the developed method was sufficiently accurate.

(V) *Limit of Detection (LOD) and Limit of Quantification (LOQ)*

It was found that the major impurity was impurity A (t_R = 3.85 min) with average abundance of 1.30%, whereas impurity B (t_R = 3.10 min) and impurity C (t_R = 4.38

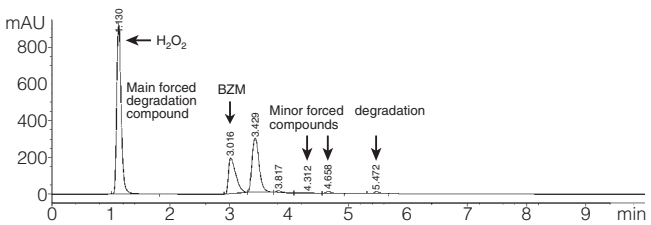

Figure 6. Forced degradation study with 3% hydrogen peroxide. 0.63 mg of BZM was dissolved in 1.2 mL of 3% hydrogen peroxide for 24 hrs at room temperature (25°C) and analyzed by HPLC.

Table 5. Intra-day precision of BZM

Injection weight (μg)	$t_{R, BZM}$ (min)[c]	$t_{R, impurity\ A}$ (min)[c]	P (%)
0.54	3.44 ± 0.00 (RSD = 0.03%)	3.86 ± 0.00 (RSD = 0.03%)	98.54 ± 0.006 (RSD = 0.01%)
1.08	3.45 ± 0.00 (RSD = 0.04%)	3.85 ± 0.00 (RSD = 0.03%)	98.39 ± 0.004 (RSD = 0.00%)
2.70	3.46 ± 0.00 (RSD = 0.05%)	3.84 ± 0.00 (RSD = 0.05%)	98.46 ± 0.007 (RSD = 0.01%)
5.40	3.46 ± 0.00 (RSD = 0.05%)	3.84 ± 0.00 (RSD = 0.04%)	99.35 ± 0.006 (RSD = 0.01%)
Average[e]	3.45 ± 0.01 (RSD = 0.28%)	3.85 ± 0.01 (RSD = 0.23%)	98.69 ± 0.400 (RSD = 0.40%)

[BZM] = 540 ppm; linear range: 0.54 - 5.40 μg (n = 5); t_R: retention time (n = 5); P (%): purity of BZM (n = 5); Average n = 20

Table 6. Inter-day precision and intermediate precision of BZM

Parameters		$t_{R, BZM}$ (min)	R	P (%)	L eq	r
Day	#1	3.45 ± 0.01 (0.28%)	2.13	98.69 ± 0.40 (0.40%)	Y = 3919X - 11	1.0000
	#2	3.46 ± 0.01 (0.20%)	2.02	98.36 ± 0.77 (0.78%)	Y = 4096X + 72	1.0000
Column	#1	3.45 ± 0.01 (0.28%)	2.13	98.69 ± 0.40 (0.40%)	Y = 3919X - 11	1.0000
	#2	3.45 ± 0.02 (0.51%)	2.22	99.80 ± 0.28 (0.28%)	Y = 3380X + 368	0.9994
Analyst	#1	3.45 ± 0.01 (0.28%)	2.13	98.69 ± 0.40 (0.40%)	Y = 3919X - 11	1.0000
	#2	3.45 ± 0.01 (0.20%)	1.83	98.74 ± 0.60 (0.61%)	Y = 4083X + 108	1.0000

[BZM] = 540 ppm; linear range: 0.54 - 5.40 μg (n = 5); $t_{R, BZM}$: retention time of BZM (n = 20); R: resolution between BZM and impurity A (n = 20); P (%): purity of BZM (n = 20); Leq: linear regression equation; r: correlation coefficient

min) were less than 0.01% - 0.02%. The limit of detection (LOD) and quantification (LOQ) of BZM were estimated to be 0.183 ± 0.021 ng (RSD = 11.23%, n = 4) and 0.610 ± 0.068 ng (RSD = 11.23%, n = 4), respectively. The LOD and LOQ of major impurity (impurity A) in BZM were 0.028 ± 0.003% (RSD = 11.14%, n = 4) and 0.094 ± 0.010% (RSD = 11.14%, n = 4), respectively, which were sufficient to meet the requirement of threshold of 0.1% impurity[24].

(VI) *Robustness*

Results in the robustness study were presented in Table 8. The purity measurements after deliberate alterations of column temperature, flow rate and buffer pH remained unaffected.

Table 7. Accuracy study of the determination of BZM

W_{spiked} (μg)	A_{spiked} (ppm)	A_{exp} (ppm)	Recovery (%)
1.23	400	408.41	102.10 (n=3)
2.85	400	407.70	101.92 (n=3)

W_{spiked}: Known amount of BZM were spiked into DMSO; A_{spiked}: Concentration of BZM spiked solution; A_{exp}: Measured concentration of BZM;

$$\text{Recovery (\%)} = \frac{A_{exp}}{A_{spiked}} \times 100\%$$

(VII) *Stability of Drug Solution*

The three days' percentage relative standard deviation of the retention time of BZM, resolution between BZM and impurity A, purity and slope of the calibration curves were 0.34%, 2.89%, 0.20% and 1.92%, respectively (Table 9). The correlation coefficients were all above 0.9990. No significant degradation was observed within three days, indicating that BZM standard solution (in DMSO) is stable for at least three days at room temperature.

CONCLUSIONS

A reverse-phase HPLC method for the BZM purity assay was developed. The method validation parameters were evaluated for the specificity, precision, accuracy, linearity, LOD/LOQ, robustness and solution stability. The proposed method is proved practical for pharmaceutical quality control. The most sensitive precursor to product ion transitions of LC-ESI-MS/MS was found to be m/z 279.0-112.0 for BZM and 405.0-112.0 for 'cold' IBZM, respectively. The present results allow identification of fragmentation ions and proposition of the pathways of BZM and 'cold' IBZM, showing the feasibility of this method for quantification of free ligand BZM in SPECT imaging agent [^{123}I]IBZM injection. To our best

Table 8. Robustness study results of the HPLC method developed

Parameters	Modification	$t_{R, BZM}$ (min)	R	P (%)	L eq	r
Column	25°C	3.45 ± 0.01 (0.28%)	2.13	98.69 ± 0.40	Y = 3919X - 11	1.0000
temperature	30°C	3.52 ± 0.01 (0.37%)	2.26	97.01 ± 0.84	Y = 3928X - 176	1.0000
Flow rate	0.4	4.28 ± 0.00 (0.12%)	2.38	97.38 ± 0.77	Y = 4890X - 216	1.0000
(mL/min)	0.5	3.45 ± 0.01 (0.28%)	2.13	98.69 ± 0.40	Y = 3919X - 11	1.0000
	0.6	2.96 ± 0.00 (0.07%)	2.16	97.11 ± 1.06	Y = 3255X - 208	1.0000
Buffer pH	6.50	3.38 ± 0.01 (0.18%)	5.83	97.38 ± 0.82	Y = 3614X - 4	1.0000
	7.00	3.45 ± 0.01 (0.28%)	2.13	98.69 ± 0.40	Y = 3919X - 11	1.0000
	7.50	3.54 ± 0.01 (0.23%)	1.71	99.10 ± 0.68	Y = 4089X + 216	0.9996

Linear range: 0.54 - 5.40 μg (n=5); $t_{R, BZM}$: retention time of BZM (n=20); R: resolution between BZM and impurity A (n = 20); P (%): purity of BZM (n = 20); Leq: linear regression equation; r: correlation coefficient

Table 9. Stability study of BZM solution

Day	$t_{R, BZM}$ (min)[b]	R[c]	P (%)[d]	L eq[e]	r[f]	n
1	3.45 ± 0.01 (0.22%)	1.72	99.06 ± 0.006 (0.60%)	Y = 3682X + 56	1.0000	12
2	3.47 ± 0.00 (0.05%)	1.79	98.76 ± 0.006 (0.63%)	Y = 3560X + 280	0.9997	20
3	3.47 ± 0.00 (0.09%)	1.82	98.69 ± 0.006 (0.63%)	Y = 3565X + 251	0.9997	20

[BZM] = 530 ppm; linear range: 0.53 - 3.71 μg; $t_{R, BZM}$: retention time of BZM; R: resolution between BZM and impurity A; P(%): purity of BZM; Leq: linear regression equation; r: correlation coefficient

knowledge, these are the first MRM data that allow identification and quantification of free BZM and [^{123}I]IBZM in [^{123}I]IBZM injection.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Kun-Chuan Chang, Department of Neurosurgery of Cathay General Hospital (Taiwan), who provided precious medical opinion on this study.

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