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Development and Validation of an HPLC Method for Determination of Purity of Sn-ADAM, a Novel Precursor of Serotonin Transporter SPECT Imaging Agent I-123-ADAM

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ABSTRACT

A reversed-phase high performance liquid chromatography (RP-HPLC) method for the purity assay of Sn-ADAM (2-((2-((Dimethylamino)methyl)phenyl)thio)-5-(tri-n-butyltin)-phenylamine) was developed and validated. Sn-ADAM is a precursor and free ligand of I-123-ADAM (I-123-2-([2-({dimethylamino}methyl)phenyl]thio)-5-iodophenylamine) that is a SPECT imaging agent for serotonin transporters (SERTs). The chromatographic separation for Sn-ADAM was achieved on a RP-18 column with an eluent consisting of methanol-acetonitrile-ammonium acetate (pH 7.0, 10 mM; 58.8 : 39.2 : 2, v/v/v). The absorbance at 220 nm against the concentration of Sn-ADAM was linear over the range of 9.9-317.8 ppm, with a correlation coefficient (r) of 0.99997. Method validation parameters, including specificity, linearity, precision, accuracy, LOD/LOQ, stability, robustness and suitability were evaluated, indicating the potential of this method in pharmaceutical quality control. Moreover, present results of precursor to product ion transitions of Sn-ADAM and I-127-ADAM by LC/ESI/MS/MS provided evidence for identification of fragmentation ions and proposition of the pathways of Sn-ADAM, I-127-ADAM and I-123-ADAM.

Key words: Sn-ADAM, RP-HPLC, LC/ESI/MS/MS, purity assay, method validation, fragmentation pathway

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) has been shown to be involved in a variety of physiological processes, including smooth muscle contraction, regulation of blood pressure and neurotransmission in both peripheral and central nervous systems (CNS)[(1)]. In mammals, serotonin is biosynthesized in a variety of organs such as the brain, spinal cord, gastrointestinal tract, bronchi, thyroid, pancreas and thymus, and is abundant in the gastrointestinal mucosa, serotonergic neurones, pineal gland and platelets[(1)]. In the CNS, serotonin is mainly found in the raphe nuclei which project into various regions throughout the brain, *i.e.* olfactory bulb, cerebral cortex, hippocampus and basal ganglia[(2-4)]. Serotonin transporters (SERTs) are macromolecular complexes located in the membrane of serotonergic neuron terminals which played a major role in the modulation of the serotonin content and serotonergic neuronal functions[(3-6)]. SERTs remove serotonin from the synaptic cleft and move it intact back into the neuronal cytoplasm, where it can be repackaged for reuse or metabolized. Most drugs and diseases induce compensatory changes in the transporter function before affecting the concentration of the postsynaptic serotonin receptors[(2,4,7)].

Serotonergic neuronal function acts as a neurotransmitter-neuromodulator in the CNS[(1-3)]. Abnormalities of processes involving serotonin in the CNS give rise to various pathological conditions, such as anorexia, anxiety, depression, schizophrenia, autism, obsessive-compulsive disorder, psychosis, eating disorders (bulimia nervosa), and substance abuse, whereas neurodegenerative disorders have been noted in Alzheimer's and Parkinson's diseases. Peripheral aberrations in processes involving serotonin have been implicated in drug-induced emesis, hypertension, migraine, genesis of cardiac

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arrhythmias, Raynaud's disease, fibrotic syndromes and symptoms of the carcinoid syndrome[1-4,8-10]. SERTs are also known to the molecular target of selective serotonin reuptake inhibitors (SSRIs) for psychiatric drugs (antidepressants) and anti-obesity drugs[2,3,6,11].

Studies of postmortem human brains showed a reduction in SERT binding in the subjects of suicides and depressant sufferers in comparison to healthy ones[2,10]. Noninvasive imaging of the SERTs by single photon emission computed tomography (SPECT) or positron emission tomography (PET) can provide information on the integrity of the serotonergic neurotransmission *in vivo*. These are valuable tools for early disease detection as well as for monitoring the progression of the serotonin pathogenesis of psychiatric and neurological disorders in living human brains[5,6,10]. Using SERTs imaging by both SPECT and PET can also have certain advantages, including the small amount of chemical tracer which needs to be injected (in a range of picogram to microgram) to achieve true tracer kinetics, giving a lower, even negligible, toxicity[11,12].

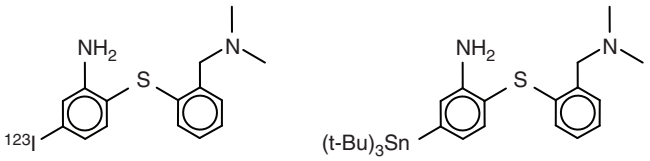
Many selective SPECT and PET imaging radiotracers for SERTs were heavily studied in the past, *i.e.* C-11-(+) McN5652, C-11-DASB, C-11-nor-β-CIT, C-11-MADAM, C-11-AFM, C-11-DAPA, F-18-ACF, F-18-AFM, I-123-5-iodo-6-nitroquipazine, I-123-IDAM, I-123-ODAM, I-123-β-CIT and I-123-nor-β-CIT[4,5,9,13-26].

Recently, a novel SPECT radio-ligand, I-123-ADAM (I-123-2-([2-({dimethylamino} methyl)phenyl] thio)-5-iodophenylamine) was developed by Oya *et al.*[5,8]. I-123-ADAM had been shown to be a highly promising tracer for SERTs, based on the studies of its partition coefficient, binding assays by LLC-PK1 cell membrane[5], biodistribution and microautoradiography of mice[3,27,28] and Sprague-Dawley (S. D.) rats[5,27], SPECT and radiation dosimetry of rabbits[11], baboon[5,8] and human[6,7,10,29-31]. Iodine-123 ($T_{1/2}$ = 13.27 hrs) is an ideal tracer with clear SPECT imaging[32]. Chemical structures of I-123-ADAM ($C_{15}H_{17}N_2SI$; MW = 380.28 for I-123 and 384.28 for I-127) and Sn-ADAM ($C_{27}H_{44}N_2SSn$; MW_{avg} = 547.43) are shown in Figure 1.

I-123-ADAM was synthesized by oxidative iododestannylation of tributyl tin precursor (Sn-ADAM, 2-((2-((Dimethylamino)methyl)phenyl)thio)-5-(tri-n-butyltin)-phenylamine) with iodine-123[3,10,11,27,28] as described below:

Sn-ADAM + I-123 + oxidizing agent + acid → I-123-ADAM

The purity of Sn-ADAM is crucial in synthesizing I-123-ADAM, since the presence of impurities in active pharmaceutical ingredients (APIs) can have significant effects on the quality, safety, stability and reaction yield of iododestannylation, especially in the case of a fast purification of a final product with a short half-life, removing reactants, by-products and decay products through solid phase extraction (SPE) [3,6,7,11,27-29,32]. To date, no method of purity assay of Sn-ADAM has been published. Therefore, our aim of this investigation was to develop and to validate a routine quality control HPLC method for the purity analysis of Sn-ADAM. In addition, the fragmentation pathways of the ($[M+H]^+$) ions generated from electrosprayed solutions of Sn-ADAM and I-127-ADAM were studied using an energy-variable collision activated dissociation (CAD) method to evaluate the integrity of Sn-ADAM as well as the feasible fragmentation of I-123-ADAM.



(a) (b)

Figure 1. Chemical structures of (a) I-123-ADAM and (b) Sn-ADAM.

MATERIALS AND METHODS

I. *Materials and Reagents*

Sn-ADAM, I-127-ADAM and I-123-ADAM were synthesized and prepared by the Isotope Application Division and Chemical Division of Institute of Nuclear Energy Research (INER), Taiwan. All chemicals and reagents were of analytical grade and used as received without further purification. Methanol (MeOH) and acetonitrile (ACN) (HPLC grade) were obtained from Merck (Darmstadt, Germany). Deionized water was purified using a Smart DQ3 reverse osmosis reagent water system (Millipore, MA, U.S.A.) with a 0.22-μm filter, TOC < 5 ppb, resistivity ≥ 18.2 MΩ-cm and endotoxin < 0.001 EU/mL. For safety, use of radioactive substances and liquid chromatography-tandem mass spectrometers (LC/MS/MS) were carried out in a radioactive operation area shielded by lead bricks. The operation license of the radioactive area, equipped with HEPA filter and operated under conditions of negative atmospheric pressure, was approved and supervised by the Atomic Energy Council (AEC), Taiwan.

II. *HPLC Instrumentation*

An Agilent 1100 series high performance liquid chromatography (HPLC) (Agilent, Palo Alto, CA, U.S.A.) was employed, consisting of an on-line degasser, binary pump, autosampler, thermostated column oven and photodiode-array detector (PDA). Data were acquired and processed with ChemStation (Agilent, Palo Alto, CA, U.S.A.). A C-18 reversed-phase column (Chromolith Performance RP-18e, 4.6 × 100 mm, Merck) was used for the separation of Sn-ADAM. An isocratic elution was achieved using

a mobile phase which consisted of methanol, acetonitrile and ammonium acetate (pH 7.0, 10 mM; 58.8 : 39.2 : 2, v/v/v). The flow-rate was 1.0 mL/min and the injection volume was 2 μL. The absorbance detection wavelength was 220 nm. For the separation of I-127-ADAM, a different C-18 column (Zorbox Eclipse XDB-C18, 4.6 × 50 mm, 1.8 μm, Agilent) was used. An isocratic elution was achieved using a mobile phase which consisted of methanol, acetonitrile and ammonium acetate (pH 7.0, 10 mM; 80 : 20 : 0.4, v/v/v). The flow-rate was 0.4 mL/min and the injection volume was 2 μL. The column temperature was set at 25°C in all experiments performed.

III. *LC/MS/MS Instrumentation*

Tandem MS analysis was carried out on a 4000 QTrap LC/MS/MS system with API Analyst software of version 1.4.2 (MDS Sciex, Ontario, Canada). Samples were introduced by a HPLC system (Agilent 1100 series HPLC system, Agilent, CA, U.S.A.) or a syringe pump at a flow rate of 10 μL/min (Harvard, Harvard Apparatus Inc., Holliston, MA, U.S.A.). Chromatography was performed as described in Section II on HPLC instrumentation. The samples were ionized by a turbo spray ion source (electrospray ionization) in the positive ion mode at 5000 V and 500°C. Mass spectra were obtained over the range of 50 or 100 to 1000 amu with unit resolution in Q1 and Q3. Other parameters are shown in Table 1. In all cases, nitrogen was used as the nebulization, curtain and collision gas.

Table 1. Tandem mass spectrometry working parameters for Sn-ADAM and I-127-ADAM analysis

Parameters	Sn-ADAM	I-127-ADAM
MRM pair (*m/z*)	549.7/123.0	385.0/212.5
Source Temperature (°C)	500	500
Scan Type	MRM	MRM
Polarity	Positive	Positive
Resolution (Q1&Q3)	Unit	Unit
Nebulizer Gas (NEB)	20	20
Curtain Gas (CUR)	10	10
IonSpray Voltage (IS, V)	5000	5000
Collision Gas (CAD)	Medium	Medium
Ion Energy 1 (IE1, V)	0.7	0.8
Ion Energy 3 (IE3, V)	-0.5	-0.5
Detector Parameters	Positive	Positive
Deflector (DF)	-	-
Channel Electron Multiplier (CEM, V)	2000	1950

IV. *Preparation of Standard Solutions and Quality Control Samples*

Stock solutions (1 mg/mL) were prepared by dissolving 10 mg of standards in 10 mL of methanol. Working solutions for HPLC and LC/MS/MS calibration curves were freshly prepared daily by diluting the stock solutions with methanol to obtain concentrations of 10 to 320 ppm and 1 to 256 ppb, respectively. Quality control (QC) samples at 15, 150 and 300 ppm were prepared in the same way as the working solutions.

V. *Purity*

The chromatographic purity (P, %) of Sn-ADAM was calculated as described below:

$$P\ (\%) = \frac{PA_{Sn\text{-}ADAM}}{PA_{total}} \times 100\% \qquad \text{eq. (1)}$$

where $PA_{Sn\text{-}ADAM}$ is the peak area of Sn-ADAM and PA_{total} is the total peak area of the chromatogram.

VI. *Resolution of Chromatogram*

The resolution (Rs) between two peaks was determined as follows:

$$Rs = \frac{t_{R2} - t_{R1}}{w_{half2} + w_{half1}} \times 1.175 \qquad \text{eq. (2)}$$

where t_{R2} and t_{R1} are the respective retention times and w_{half2} and w_{half1} are the respective peak widths at half height.

VII. *Method Validation*

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

(I) *Specificity (Selectivity)*

Forced degradation studies are used to facilitate the development of analytical methodology, to gain better understanding of active pharmaceutical ingredients (APIs) and the stability of drug products (DPs), and to provide information about degradation pathways and degradation products[32,33]. In this case, forced degradation studies were performed to evaluate the specificity (selectivity) of the purity assay method of Sn-ADAM. Samples of Sn-ADAM (0.5 mg) were dissolved in 0.5 mL of methanol and subjected to several stress conditions, *i.e.*, 0.25 mL of 1 M HCl, 0.25 mL of 1 M NaOH or 0.50 mL of 3% H_2O_2 for 30 min. Equivalent amounts of

Sn-ADAM (0.5 mg) were heated at 80°C in an oven over a period of 30 min. All forced degradation samples were neutralized using 1 M HCl or 1 M NaOH and diluted to 250 ppm with methanol before HPLC analysis.

(II) *Linearity*

The calibration curves of six concentrations (10 to 320 ppm) were obtained by plotting the respective peak areas against concentrations. The linearity was evaluated by the linear least square regression method with three determinations at each concentration.

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

The precision of the method was determined by performing three assays of the same batch of Sn-ADAM at six concentrations (10 to 320 ppm). 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 through the use of two different analysts.

(IV) *Accuracy (Recovery)*

The accuracy of the method was determined by the recovery test. Quality control (QC) samples of Sn-ADAM of low, medium and high concentration at 15, 150 and 300 ppm ($C_{nominal}$) were analyzed by the proposed method. Experimental values (C_{exp}) were obtained by interpolation to the linear least square regression equation of a fresh newly prepared calibration curve (10 to 320 ppm) and comparing with the theoretical values ($C_{nominal}$).

$$\text{Recovery (\%)} = \frac{C_{exp}}{C_{nominal}} \times 100\% \qquad \text{eq. (3)}$$

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

The LOD and LOQ of the method for impurities in Sn-ADAM were determined at signal to noise ratios of 3 and 10, respectively.

(VI) *Stability of Drug Solution*

The stability of the drug solution was examined using the QC samples (low, medium and high concentration at 15, 150 and 300 ppm) for bench-top stability study. The QC samples were kept in the autosampler at ambient temperature for HPLC analysis over three consecutive days. Experimental data were obtained by interpolation to the linear least square regression equation of a calibration curve (10 to 320 ppm) newly prepared each day. Retention time, recovery yield and purity of Sn-ADAM over three consecutive days were analyzed.

(VII) *Robustness*

The robustness of an analytical method is a basic measurement of its capacity to remain unaffected by small variations in method parameters. In this case, method robustness was evaluated through the effects of different columns (same type and manufacturer), column temperatures (± 5°C), pH values (± 0.5), components (± 5%) and flow rates of mobile phase (± 20%).

(VIII) *System Suitability*

The system suitability was assessed by six triplicate analyses of the drug in a concentration range of 10 - 320 ppm. The efficiency of the column was expressed in terms of the theoretical plates number (N), column capacity (k'), column selectivity (α), and tailing factor (t). The acceptance criterion for the percentage relative standard deviation (% R.S.D.) for the peak area and retention time of Sn-ADAM was ± 2%.

RESULTS AND DISCUSSION

I. *Method Development*

Instead of I-123-ADAM, Sn-ADAM and I-127-ADAM samples at concentrations of less than 100 ppm and 100 ppb were used to optimize conditions for HPLC and LC/ESI/MS/MS, respectively. The difference of retention time (t_R) of Sn-ADAM between HPLC (t_R = 4.40 min) and LC/ESI/MS/MS (t_R = 4.78 min) was due to the different flow rate and distance between the column and detectors.

Absorption spectra of Sn-ADAM and I-127-ADAM were recorded over the range of 200 to 300 nm by a post-column photodiode-array detector (PDA). A wavelength of 220 nm was found to be optimal for the detection and quantification of Sn-ADAM and I-127-ADAM in methanol-acetonitrile solution.

A Chromolith Performance RP-18e (4.6 × 100 mm, Merck) reversed-phase column was selected for the separation of Sn-ADAM based on the existence of the tributyltin functional group. The efficiency of the column was expressed in terms of the number of theoretical plates (N) and tailing factor (t).

The chromatographic separation of Sn-ADAM was achieved using a mobile phase which consisted of methanol, acetonitrile and ammonium acetate (pH 7.0, 10 mM; 58.8 : 39.2 : 2, v/v/v). The typical HPLC chromatogram of Sn-ADAM is shown in Figure 2. The peak at retention time (t_R) of 4.40 min was identified as a protonated Sn-ADAM ion ($[M+H]^+$) at *m/z* 549.7 by LC/ESI/MS. The peaks at t_R of 4.19 and 5.72 min were impurity A and

impurity B respectively. A protonated molecular ion with *m/z* 385.0 at t_R of 2.96 min was identified as I-127-ADAM.

For the determination of the major impurity based on the average percentage content, the percentage content of impurity A (t_R = 4.19 min) was 1.71%, whereas that for the total of other impurities was 1.52%. The calibration plot of peak area against concentration of Sn-ADAM showed a good linearity over the range of 9.9 to 317.8 ppm. A linear regression equation of y = 7.83x - 6.05 with a correlation coefficient of 0.99997 was obtained.

II. *Mass Spectrometric Analysis*

I-127-ADAM was used instead of I-123-ADAM during the investigation by ESI-MS/MS. The proposed mobile phases using methanol-acetonitrile-ammonium acetate (pH 7.0, 10 mM) were volatile and suitable for ESI-MS studies. Neither the component nor concentration ratio of mobile phases between HPLC and LC/MS/MS was changed.

Q1 full scans were achieved in a positive ion mode to optimize the electrospray ionization (ESI) conditions of Sn-ADAM and I-127-ADAM. The ESI spectra of Sn-ADAM and I-127-ADAM showed a protonated molecular ion ($[M+H]^+$) at *m/z* 549.7 and *m/z* 385.0 (Figure 3), respectively. The ten peaks that appeared in the protonated molecular ion ($[M+H]^+$) in the *m/z* range of 544 to 556 [see inset, Figure 3(a)] were due to the stable isotopes of Sn. The isotope pattern and isotope ratios were similar and coincident with the simulation result calculated by the software of API 'Isotopic Distribution Calculation' (Analyst, version 1.4.2, MDS Sciex, Ontario, Canada). The peak of the highest intensity was mainly contributed from the stable isotope Sn-120 with natural abundance of 32.4%.

Both product ion and precursor ion scans 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). The MS/MS parameters of precursor ions and product ions are summarized in Table 2. The product ion scanning spectra of $[M+H]^+$ showed fragment ions at *m/z* 291.1, 253.4, 234.9, 197.2, 179.5, 166.5, 135.0, 123.0, and 121.0 for Sn-ADAM and *m/z* 340.0, 212.5, 196.5, 184.5, 180.5, 165.6, and 152.5 for I-127-ADAM. The fragment ions of the highest and second highest intensities were found at *m/z* 549.7 to 123.0 and 549.7 to 234.9 for Sn-ADAM and at *m/z* 385.0 to 212.5 and 385.0 to 340.0 for I-127-ADAM.

The linearities of multiple reaction monitoring (MRM) transitions of Sn-ADAM and I-127-ADAM were studied. The linear least-square regression equations and correlation coefficients of MRM transitions summarized in Table 2 showed a good linearity over the calibration range. The correlation coefficients (r) were almost all above 0.995, indicating the stability of these fragmentations. Tandem mass spectrometry (MS/MS) experiments performed in a quadrupole linear ion trap (QTrap) were used to reveal the fragmentation behavior of Sn-ADAM and I-127-ADAM (Figure 4).

It was believed that there was no significant difference in the MS parameters between I-127-ADAM and I-123-ADAM. The fragmentation pathway of I-123-ADAM was similar to that of I-127-ADAM, except the drug stability of I-123-ADAM might be changed due to nuclide iodine-123 decaying into stable nuclide tellurium-123. We postulated that the product ions of I-123-ADAM are at *m/z* 336.0, 212.5, 196.5, 184.5, 180.5, 165.6, and 152.5. Based on the

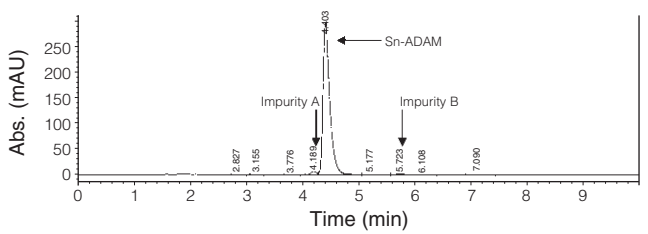


Figure 2. Typical HPLC chromatogram of Sn-ADAM and its major impurities.

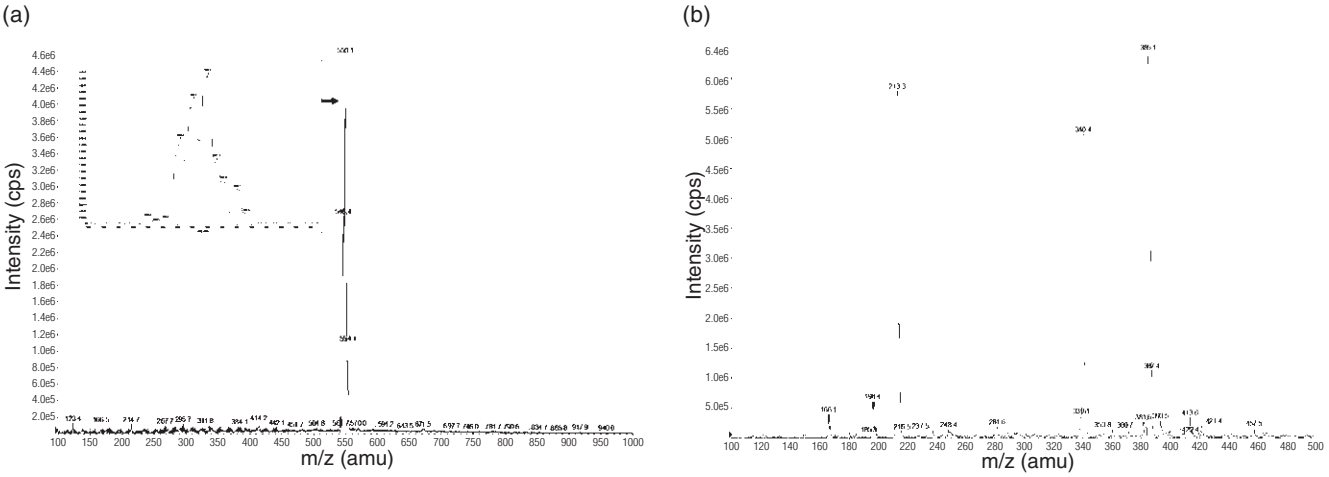


Figure 3. Typical ESI-MS/MS spectra of (a) Sn-ADAM and (b) I-127-ADAM.

Table 2. Optimized MS/MS parameters and linearity study of MRM transitions of Sn-ADAM and I-127-ADAM

Compound	t_R (min)	Precursor ion (*m/z*)	DP	EP	Product ion (*m/z*)	CE	CXP	L eq.	r	Range (ppb)
Sn-ADAM	4.78	549.7	105	10	291.1	28	18	$Y = 5.72 \times 10^1X + 2.67 \times 10^1$	0.9923	5-320
		549.7	105	10	234.9	31	14	$Y = 6.80 \times 10^2X - 6.32 \times 10^2$	0.9998	
		549.7	105	10	197.2	38	11	$Y = 1.69 \times 10^2X - 6.51 \times 10^2$	0.9992	
		549.7	105	10	179.5	41	9	$Y = 3.52 \times 10^2X - 1.09 \times 10^3$	0.9992	
		549.7	105	10	166.5	28	8	$Y = 2.42 \times 10^2X - 6.87 \times 10^2$	0.9992	
		549.7	105	10	123.0	77	21	$Y = 1.20 \times 10^3X - 1.76 \times 10^3$	0.9998	
		549.7	105	10	121.0	125	20	$Y = 3.74 \times 10^2X - 8.65 \times 10^2$	0.9991	
		178.9	170	10	123.0	16	6	$Y = 1.11 \times 10^3X + 1.13 \times 10^4$	0.9980	
		178.9	170	10	121.0	39	5	$Y = 2.03 \times 10^2X + 2.18 \times 10^3$	0.9973	
		177.1	171	8	121.0	16	6	$Y = 1.05 \times 10^3X + 8.17 \times 10^3$	0.9981	
		149.1	207	4	121.0	8	9	$Y = 1.13 \times 10^2X + 1.95 \times 10^3$	0.9965	
I-127-ADAM	2.96	385.0	71	13	340.0	23	9	$Y = 2.88 \times 10^4X + 3.47 \times 10^5$	0.9985	8.5-340
		385.0	71	13	212.5	32	12	$Y = 5.27 \times 10^5X + 9.55 \times 10^5$	0.9957	
		385.0	71	13	196.5	50	11	$Y = 2.73 \times 10^4X + 3.20 \times 10^5$	0.9982	
		385.0	71	13	184.5	108	8	$Y = 2.65 \times 10^3X + 2.05 \times 10^4$	0.9996	
		385.0	71	13	180.5	90	9	$Y = 6.95 \times 10^3X + 7.67 \times 10^4$	0.9984	
		385.0	71	13	165.6	26	8	$Y = 5.89 \times 10^3X + 6.84 \times 10^4$	0.9974	
		385.0	71	13	152.5	109	7	$Y = 1.44 \times 10^4X + 1.48 \times 10^5$	0.9991	

DP: Declustering potential; EP: Entrance potential; CE: Collision energy; CXP: Collision cell exit potential; L eq.: Linear regression equation; r: Correlation coefficient.

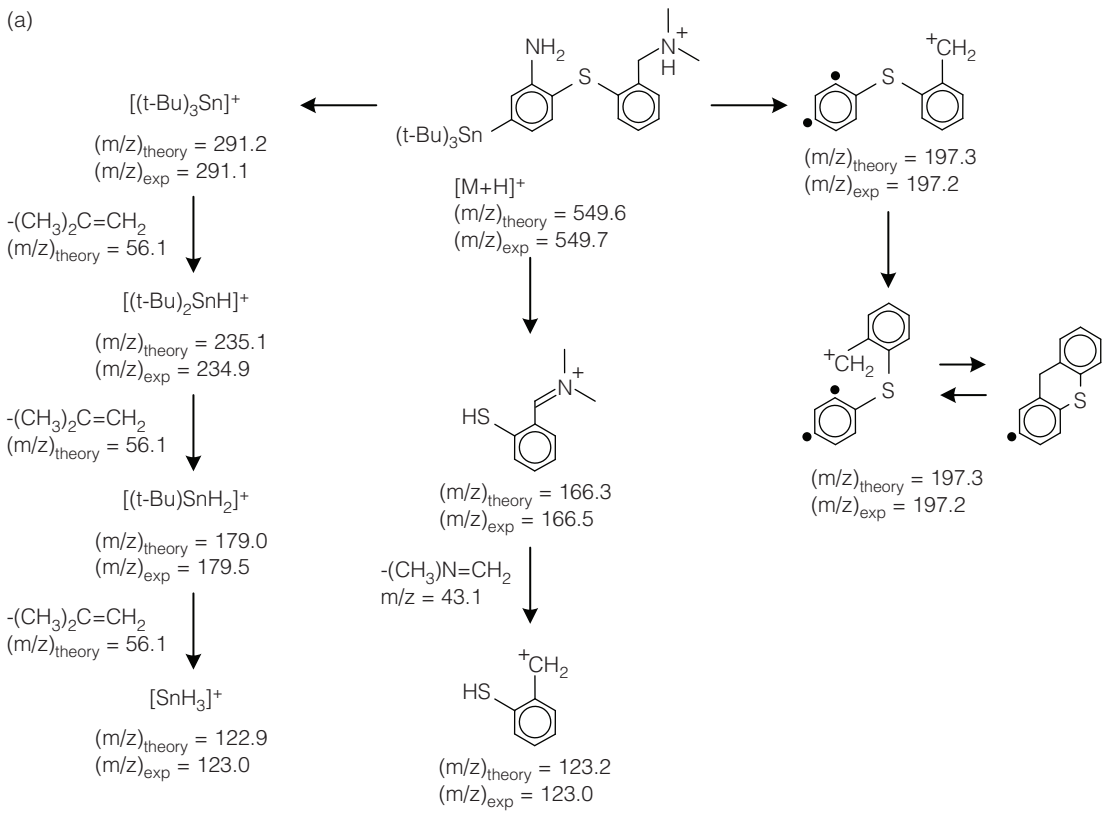


Figure 4. Proposed CAD fragmentation pathways of the protonated molecules of (a) Sn-ADAM at *m/z* 549.7 and (b) I-127-ADAM at *m/z* 385.0.

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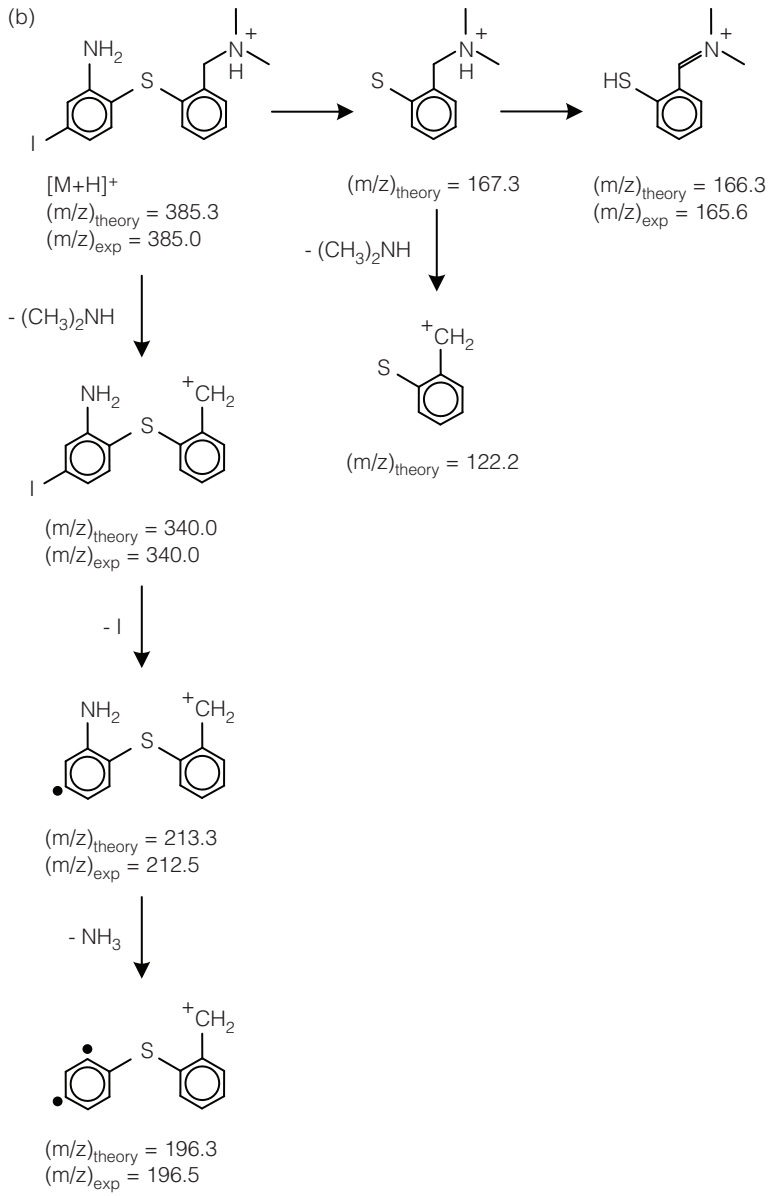

Figure 4. Proposed CAD fragmentation pathways of the protonated molecules of (a) Sn-ADAM at *m/z* 549.7 and (b) I-127-ADAM at *m/z* 385.0.

results, the fragmentation pathway, including the decay of I-123-ADAM was proposed (Figure 5).

III. *Validation of the Purity Assay Method*

(I) *Specificity (Selectivity)*

Significant degradations of 0.33 M HCl, 0.33 M NaOH and 1.5% hydrogen peroxide were noticed under stress conditions. Representative chromatograms from such studies are shown in Figure 6(a)-(c). Two major degradation products were noticed in the chromatograms at the retention time (t_R) of 1.55 min (FD1 and hydrogen peroxide) and 1.80 min (FD2). The resolutions between Sn-ADAM and its degradation peaks were greater than 17, indicating that the proposed method was sufficiently selective for its intended purpose. Figure 6(d) represents the chromatogram of a sample degraded at 80°C for 30 min and no significant degradation was found. However, the absolute peak area of the parent peak had declined by 5%.

(II) *Linearity*

Standard curves were constructed by plotting peak area against concentration of Sn-ADAM and were linear over the concentration range of 10 - 320 ppm. The linear least squares regression equation of the standard curve correlating the peak areas (PAs) to the drug concentration (X in ppm) in this range was Y = 7.83X - 6.05. The correlation coefficient (r) was 0.99997 (Table 3).

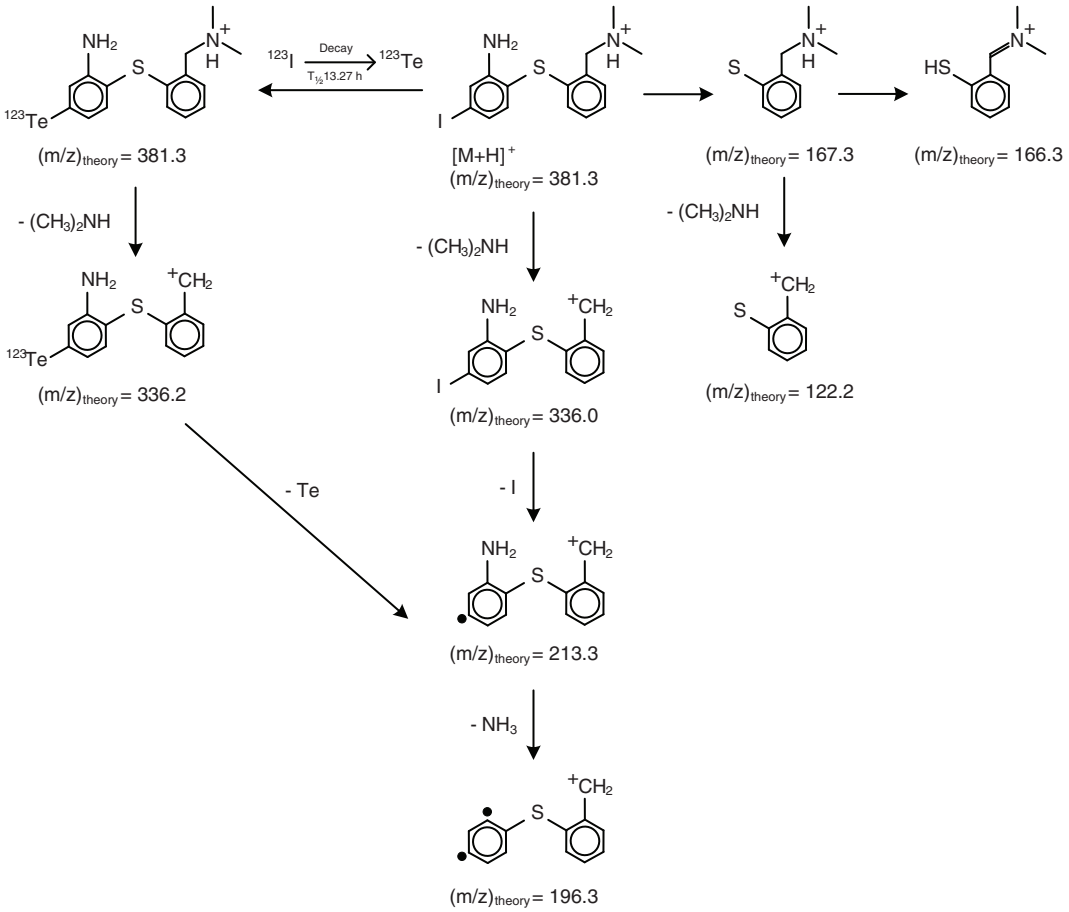

Figure 5. Proposed fragmentation pathway of I-123-ADAM.

Table 3. Intra-day precision of Sn-ADAM

Concentration of Sn-ADAM (ppm)	$t_{R,Sn\text{-}ADAM}$ (min)	$t_{R,A}$ (min)	Rs	P (%)
9.9†	4.44 ± 0.00 (0.10%)	4.19 ± 0.00 (0.10%)	1.25 ± 0.02 (1.71%)	-
19.9†	4.44 ± 0.00 (0.11%)	4.20 ± 0.01 (0.14%)	1.23 ± 0.00 (0.14%)	-
39.7†	4.44 ± 0.00 (0.11%)	4.20 ± 0.00 (0.09%)	1.21 ± 0.01 (0.88%)	-
79.4†	4.43 ± 0.01 (0.20%)	4.19 ± 0.01 (0.18%)	1.18 ± 0.00 (0.25%)	-
158.9†	4.42 ± 0.00 (0.09%)	4.19 ± 0.00 (0.09%)	1.14 ± 0.00 (0.27%)	-
317.8†	4.41 ± 0.00 (0.12%)	4.19 ± 0.00 (0.10%)	1.09 ± 0.00 (0.14%)	96.71 ± 0.05 (0.05%)
W_{half}‡	0.12 ± 0.00 (0.77%)	0.11 ± 0.00 (1.61%)	-	-
N‡	7085 ± 124 (1.75%)	8198 ± 261 (3.18%)	-	-
L eq.‡	Y = 7.83X - 6.05	Y = 0.14X - 0.15	-	-
r‡	0.99997	0.99999	-	-

$t_{R,Sn\text{-}ADAM}$: Retention time of Sn-ADAM; $t_{R,A}$: Retention time of impurity A; Rs: Resolution between Sn-ADAM and impurity A; P (%): Chromatographic purity; W_{half}: Peak width at half height; N: Number of theoretical plates; Linear range: 10 to 320 ppm.
† n = 3. ‡ n = 18.

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

The intra-day precision (repeatability), inter-day precision (reproducibility) and intermediate precision were demonstrated by analyzing Sn-ADAM at six concentrations (10 to 320 ppm). The results were summarized in Table 3. The numbers of theoretical plates of Sn-ADAM and impurity A were 7,085 and 8,198, respectively. Table 3 showed that there were no significant differences between the assays of either within-day or between-days, indicating the high precision of the proposed method.

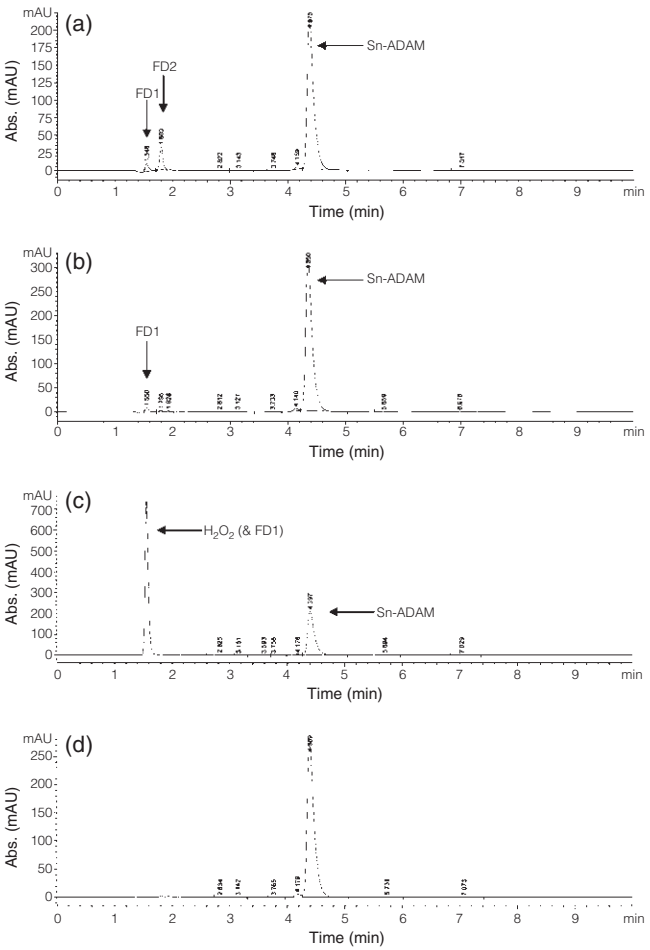

Figure 6. Forced degradation studies of 0.5 mg of Sn-ADAM with (a) 0.33 M HCl, (b) 0.33 M NaOH, (c) 1.5% hydrogen peroxide, and (d) 80°C. All forced degradation samples were neutralized and diluted to 250 ppm with methanol before HPLC analysis.

(IV) *Accuracy (Recovery)*

Recovery tests were achieved by comparing the concentration (C_{exp}) obtained from injection of QC samples to the nominal values ($C_{nominal}$). The intra-day and inter-day recoveries of Sn-ADAM at concentrations of 15, 150 and 300 ppm are summarized in Table 4. The recoveries were between 97 and 103%, indicating that there was sufficient accuracy in the proposed method. The R.S.D. for measurement of accuracy ranged from 0.15 to 0.99%.

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

The limits of detection (LOD, S/N = 3/1) and quantification (LOQ, S/N = 10/1) for the major impurity (impurity A, $t_{R,A}$ = 4.19 min, average abundance = 1.72%) in Sn-ADAM were found to be 0.12 ± 0.07 ppm (n = 3) and 0.41 ± 0.22 ppm (n = 3) respectively.

(VI) *Stability of Drug Solution*

The stability of Sn-ADAM solutions was examined by analyzing solutions over 3 days. The results of these studies are shown in Table 4, where the retention time of Sn-ADAM and the recovery and purity of QC samples were within the range of 97 - 103%. No significant degradation or reduction in the absolute peak area was observed within three days, indicating that Sn-ADAM standard solution would be stable for at least three days when kept on a bench top.

(VII) *Robustness*

The robustness of an analytical procedure is a measurement of its capacity to remain unaffected by small, but deliberate, variations in method parameters, and provides an indication of its reliability during normal

Table 4. Inter-day and intermediate precisions of Sn-ADAM

Parameters	t_R (min)‡	W_{half} (min)‡	N‡	L eq.‡	r‡	P (%)†
Analyst 1, Day 1	4.43 ± 0.01 (0.31%)	0.12 ± 0.00 (0.77%)	7085 ± 124 (1.75%)	Y = 7.83X - 6.05	0.99997	96.71 ± 0.05
Analyst 1, Day 2	4.44 ± 0.02 (0.34%)	0.12 ± 0.00 (0.58%)	7013 ± 100 (1.42%)	Y = 7.80X + 1.82	0.99997	96.94 ± 0.12
Analyst 2, Day 3	4.47 ± 0.03 (0.59%)	0.13 ± 0.00 (0.81%)	7019 ± 105 (1.50%)	Y = 8.14X - 19.50	0.99975	97.35 ± 0.04

$t_{R,Sn\text{-}ADAM}$: Retention time of Sn-ADAM; $t_{R,A}$: Retention time of impurity A; Rs: Resolution between Sn-ADAM and impurity A; P (%): Chromatographic purity; W_{half}: Peak width at half height; N: Number of theoretical plates; Linear range: 10 to 320 ppm.
† n = 3. ‡ n = 18.

Table 5. Intra-day accuracy, inter-day accuracy and stability studies in the analysis of Sn-ADAM

Day	Calibration range (ppm)†	L eq.†	r†	$C_{nominal}$ (ppm)‡	t_R (min)‡	Recovery (%)‡	P (%)‡
1	9.9-317.8	Y = 7.83X - 6.05	0.99997	15.3	4.42 ± 0.02 (0.37%)	97.18 ± 0.96 (0.99%)	96.77 ± 0.07 (0.07%)
				153		99.12 ± 0.14 (0.15%)	
				306		100.80 ± 0.38 (0.38%)	
2	10.1-324.2	Y = 7.80X + 1.82	0.99997	15.3	4.44 ± 0.02 (0.42%)	93.00 ± 0.77 (0.83%)	96.73 ± 0.06 (0.06%)
				153		100.28 ± 0.46 (0.46%)	
				306		102.36 ± 0.60 (0.59%)	
3	10.0-318.4	Y = 7.86X - 11.43	0.99961	15.3	4.45 ± 0.02 (0.55%)	101.96 ± 0.34 (0.33%)	96.70 ± 0.20 (0.20%)
				153		100.93 ± 0.32 (0.32%)	
				306		102.10 ± 0.33 (0.33%)	

† Data from freshly (daily) prepared calibration standards; ‡ Data from QC samples, n = 3.

usage[34]. In this case, robustness of the method was investigated by making small changes of column parameters, column temperature, mobile phase pH, composition and flow rate. The results of the robustness studies are shown in Table 5. All results were within acceptable range. No critical change in performance was found.

(VIII) *System Suitability*

The theoretical plates number (N), column capacity (k'), column selectivity (α), and tailing factor (t) were 7085 ± 124 (1.75%), 1.46 ± 0.01 (0.52%), 1.04 ± 0.01 (0.71%) and 1.56 ± 0.08 (5.30%), respectively. According to data from the robustness studies (Table 5), the repeatabilities (% R.S.D.) of retention time and peak area for triplicate analysis were within the acceptance criterion range (2%).

CONCLUSIONS

A new RP-HPLC method was developed and validated for the routine quality control analysis of the purity of Sn-ADAM. All validation studies met the predetermined acceptance criteria, indicating that the proposed method was adequate for the intended analysis. Moreover, the most sensitive precursor to product ion transitions of LC/ESI/MS/MS was found to be *m/z* 549.7 to 123.0 and 385.0 to 212.5 for Sn-ADAM and I-127-ADAM respectively. The present results provided a novel method for the identification of fragmentation ions and proposition of the pathways of Sn-ADAM and I-127-ADAM. Therefore, a feasible fragmentation pathway of I-123-ADAM as well as its multiple reaction monitoring (MRM) transitions were proposed.

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Table 6. Robustness study of Sn-ADAM

Parameters		t_R (min)	W_{half} (min)	N	P (%)	L eq.	R
Column†	#1	4.43 ± 0.01 (0.31%)	0.12 ± 0.00 (0.77%)	7085 ± 124 (1.75%)	96.71 ± 0.05	Y = 7.83X - 6.05	0.99997
	#2	5.11 ± 0.07 (1.33%)	0.15 ± 0.01 (4.47%)	6476 ± 712 (10.99%)	97.28 ± 0.02	Y = 7.95X + 2.15	0.99998
Temperature (°C)	25	4.43 ± 0.01 (0.31%)	0.12 ± 0.00 (0.77%)	7085 ± 124 (1.75%)	96.71 ± 0.05	Y = 7.83X - 6.05	0.99997
	30	4.24 ± 0.02 (0.42%)	0.12 ± 0.00 (0.68%)	6880 ± 118 (1.71%)	97.45 ± 0.01	Y = 8.16X - 18.53	0.99979
pH‡	6.5	4.40 ± 0.02 (0.34%)	0.12 ± 0.00 (0.78%)	7033 ± 134 (1.91%)	97.10 ± 0.11	Y = 7.95X + 2.01	0.99997
	7.0	4.43 ± 0.01 (0.31%)	0.12 ± 0.00 (0.77%)	7085 ± 124 (1.75%)	96.71 ± 0.05	Y = 7.83X - 6.05	0.99997
	7.5	4.40 ± 0.01 (0.29%)	0.12 ± 0.00 (0.64%)	7030 ± 111 (1.58%)	97.14 ± 0.10	Y = 7.90X + 2.25	0.99997
MeOH-ACN-NH_4Ac	63.7 : 34.3 : 2.0	4.55 ± 0.03 (0.73%)	0.13 ± 0.00 (0.95%)	6375 ± 81 (1.28%)	97.18 ± 0.18	Y = 7.90X - 4.47	0.99961
(pH 7.0†, 10 mM) (v/v/v)	58.8 : 39.2 : 2.0	4.43 ± 0.01 (0.31%)	0.12 ± 0.00 (0.77%)	7085 ± 124 (1.75%)	96.71 ± 0.05	Y = 7.83X - 6.05	0.99997
	53.9 : 44.1 : 2.0	4.83 ± 0.04 (0.85%)	0.14 ± 0.00 (0.58%)	6599 ± 88 (1.34%)	97.50 ± 0.06	Y = 7.95X - 6.04	0.99966
Flow rate (mL/min)	0.8	5.54 ± 0.02 (0.31%)	0.15 ± 0.00 (0.57%)	7458 ± 120 (1.61%)	97.18 ± 0.01	Y = 10.21X - 25.66	0.99978
	1.0	4.43 ± 0.01 (0.31%)	0.12 ± 0.00 (0.77%)	7085 ± 124 (1.75%)	96.71 ± 0.05	Y = 7.83X - 6.05	0.99997
	1.2	3.66 ± 0.01 (0.25%)	0.11 ± 0.00 (0.34%)	6515 ± 52 (0.79%)	97.49 ± 0.01	Y = 6.83X - 16.62	0.99979

† Column #1 and #2 refer to columns of same type, same manufacturer, but different batch.
‡ The pH value of the original aqueous component.

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