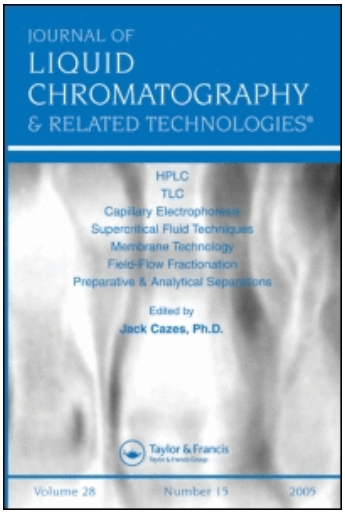

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Identification of Drugs by Ultra Fast Liquid Chromatography/Electrospray Ionization-Quadrupole Ion Trap/Time-of-Flight Mass Spectrometry

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Abstract: Hybrid quadrupole ion trap/time-of-flight mass spectrometry (QIT/TOFMS) was applied for detection of ultra fast liquid chromatography (UFLC). The system performance for fast qualitative analysis was evaluated using test drugs. The test drugs were separated on a C_{18} column (30 mm × 2.0 mm I.D., particle size: 2.2 μm) with fast linear gradient elution using 0.1% formic acid and acetonitrile as mobile phase. The flow rate was set at 0.5 mL/min and the analysis cycle time was 4.5 min. Relative standard deviations (RSDs) of retention time and peak area for each drug (200 pg each) were 0.2% or better, lower than 3%, respectively. Mass accuracy of each compound was found to be 3.2 ppm or better. Rapid positive to negative polarity switching mode was demonstrated, showing good mass accuracy below 5 ppm. Moreover, an MS^3 measurement was carried out and the formulae of compounds were confirmed using formula prediction software.

Keywords: Drug, Liquid chromatography, Time-of-flight mass spectrometry, Mass accuracy, Tandem mass spectrometry, Formula prediction

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INTRODUCTION

Recently, in liquid chromatography, a lot of effort is being put into high throughput analysis. Among many approaches for faster analysis, one of the most important requirements is column design. Although sub-2 μm particles show successful performance,[1] conventional LC systems cannot be used because of high backpressure, up to 100 MPa. Specially designed LC systems are adopted for such an analysis method. If the backpressure is limited under 30 MPa, which is acceptable for conventional LC systems, with minimal replacement of some parts such as tubings or valves for auto-samplers, the best performance is obtained using 2.0–2.5 μm particles.[2] If the system is operated with the optimum linear velocity (υ_{opt}) at the minimum HETP (height equivalent to a theoretical plate) for each particle size (e.g., 29.0 cm/min for 2.0 μm particle, 19.4 cm/min for 3.0 μm particle, 11.6 cm/min for 5 μm particle) obtained from van Deemter curves, the maximum column length (L_{max}) is limited, resulting in limited theoretical plate numbers. The effect of this limitation is larger, especially for sub-2 μm particles. Resolution (Rs) of two peaks is expressed as the following equation:

$$\begin{aligned} Rs &= 1/4 \cdot \{(\alpha - 1)/\alpha\} \cdot N^{1/2} \cdot \{k/(k+1)\} \\ &= 1/4 \cdot \{(\alpha - 1)/\alpha\} \cdot N^{1/2} \cdot (1 - t_0/t_R) \end{aligned} \quad (1)$$

(α: separation factor, N: column plate number, k: retention factor, t_0: column dead time, t_R: retention time).

As α is constant in this case, t_R is effective for better Rs value. To reach $Rs = 2.0$, which means complete separation of peaks, longer analysis time (t_R) is required under limited N conditions for sub-2 μm particles. Equation (1) is as follows because $t_0 = L_{max}/\upsilon_{opt}$.:

$$Rs = 1/4 \cdot \{(\alpha - 1)/\alpha\} \cdot N_{max}^{1/2} \cdot \{1 - L_{max}/(\upsilon_{opt} \cdot t_R)\}$$

Figure 1 shows an example of the relationship between particle size and time needed for the separation of two compounds. Longer column length can be a choice with 2.0–2.5 μm particles because of lower backpressure. The performance and the risk of high backpressure are balanced with the 2.2 μm particles. Numerous resources of conventional LC systems are applied for faster analysis with minimal modification and analysis time reduced to 10–20% compared with that using 5 μm particles. Thus the UFLC system has been developed for higher throughput keeping compatibility with conventional LC instruments. From a point of view of detection, target compounds are eluted in narrower peak band widths with a higher flow rate.

The choice of detection method is also an important issue for higher throughput analysis. MS is the most powerful detection method for drug analysis by LC and has contributed both to fast quantitative analysis[3,4] and qualitative analysis.[5] Throughput of analysis is accelerated by MS with its high specificity.

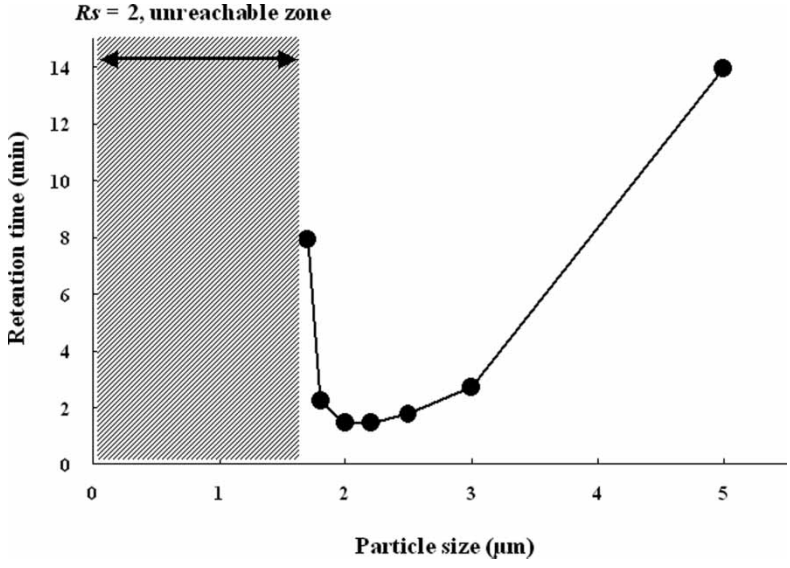

Figure 1. An example of the relationship between particle size and separation time to obtain $Rs = 2$ under 30 MPa backpressure.

The first hybrid QIT/TOF instrument with ESI interface was introduced and resolution and sensitivity were enhanced by Lubman et al.[6] The role of the QIT was to store and integrate the ion signal, and to cool ions. MS^n function was not performed with the system. On the other hand, the matrix assisted laser desorption/ionization (MALDI)-QIT/TOFMS instrument, which is capable of MS^n analysis was developed by Tanaka et al.[7] Structural analyses of peptide mixtures,[8] oligosaccharides,[9] polyethers,[10] and glycosphingolipids[11] in MS^n mode were presented, revealing multiple stage mass spectrometry using MALDI-QIT/TOFMS instruments as a useful method. With high mass accuracy, high sensitivity, and MS^n function, technology for the combination of QIT and TOF was successfully introduced to LC/ESI-MS.[12,13] LC/ESI-QIT/TOFMS was applied for identification of drug candidates,[14] modified peptides,[15] and natural products.[16] Recent research proves the complementary use of a quadrupole-TOFMS (Q-TOFMS) system and a ion trap MS system are very effective for structural elucidation of drugs.[17–21] In these studies, advantages of mass accuracy of Q-TOFMS and MS^n capability of ion trap MS were fully demonstrated. Samples were introduced to each MS instrument separately by the infusion technique. QIT/TOFMS has the potential to support MS^n analysis with high mass accuracy in a single chromatographic run.

The present work describes the application and evaluation of the QIT/TOFMS system as a UFLC detector, especially for qualitative drug analysis. The structures of prepared test drugs are shown in Figure 2. Accurate mass measurements of these drugs were carried out under several analytical

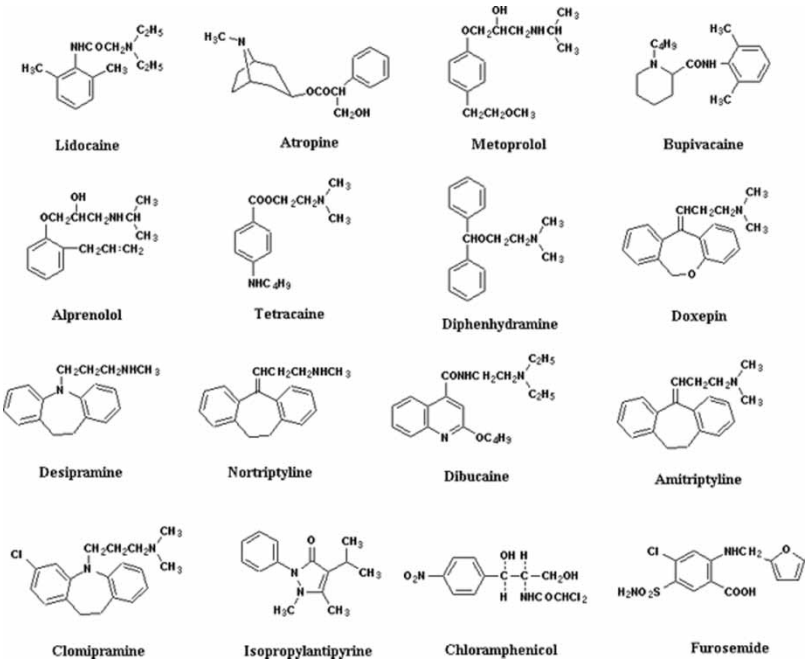

Figure 2. Structures of the test compounds.

conditions. Moreover, stability of mass accuracy was determined because of its importance for a mass spectrometer.

EXPERIMENTAL

Materials

Lidocaine, atropine, metoprolol, bupivacaine, alprenolol, tetracaine, diphenhydramine, doxepin, desipramine, nortriptyline, dibucaine, amitriptyline, clomipramine, isopropylantipyrine, chloramphenicol, furosemide, HPLC grade formic acid, LCMS grade acetonitrile (ACN), and LCMS grade methanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Water for sample dilution and mobile phase was prepared using a Millipore (Tokyo, Japan) Milli-Q Gradient system.

Sample Preparation

Standard stock solutions (1000 μg/mL) of drugs were prepared in water (atropine, diphenhydramine, doxepin, desipramine, nortriptyline,

amitriptyline, and clomipramine) or methanol (lidocaine, metoprolol, bupivacaine, alprenolol, tetracaine, dibucaine, isopropylantipyrine, chloramphenicol, and furosemide). Analytical standards were prepared by mixing stock solutions and diluting appropriately with water. Concentrations of drugs were 100 ng/mL for MS or MS/MS analysis, or 1000 ng/mL for MS^3 analysis. A 1 ng/mL mixture was also prepared.

Chromatographic Conditions

The liquid chromatograph was a Shimadzu (Kyoto, Japan) Prominence UFLC system with a CBM-20A communications bus module, an SIL-20ACHT autosampler, two LC-20AD pumps, a DGU-$20A_3$ vacuum degasser, and a CTO-20AC column oven. A Shim-pack XR-ODS (30 mm × 2.0 mm, 2.2 μm, Shimadzu) analytical column was kept at 40°C. Mobile phase A was 0.1% formic acid, and mobile phase B was ACN. The following gradient condition was used: 5% ACN (0 min)–70% ACN (2.5 min)–5% ACN (2.51–4.5 min). The flow rate of the mobile phase was set at 0.5 mL/min. The injection volume was 2, or 5 μL.

Mass Spectrometry

Analyses were performed using a Shimadzu LCMS-IT-TOF hybrid quadrupole ion trap/time-of-flight mass spectrometer equipped with an ESI interface in positive ion mode or in positive negative switching mode. Tuning of the QIT/TOF instrument was carried out with sodium trifluoroacetate as an external standard.[22] The ESI probe voltage was set at +4.5 kV for positive ion detection, or −4.0 kV for negative ion detection. The curved desolvation line temperature and the block heater temperature were both set at 300°C. A nitrogen generator (model 20E; System Instruments, Tokyo, Japan) supplied nitrogen (purity: >99%) for the ESI probe (flow rate: 1.5 L/min) and the drying gas (pressure: 0.15 MPa). Argon (>99.99%, Koike Sanso Kogyo (Tokyo, Japan)) was used for the collision gas. Pressure of the argon was set at 0.02 Pa. The flight tube temperature was kept at 40°C. Analytical conditions for MS were as follows: mass range, m/z 150–500; ion accumulation time, 30 msec; loop time, 100 msec. MS/MS and MS^3 measurements were carried out using an automatic MS^n function. Precursor ions were automatically selected according to intensity. MS/MS conditions were as follows: mass range for parent ion, m/z 150–500; ion accumulation time, 30 msec; precursor isolation time, 20 msec; mass range for product ion, m/z 50–350; ion accumulation time for product ion, 30 msec; total loop time, 400 msec. MS^3 conditions (MS/MS → MS3) were as follows: precursor isolation time, 20 msec; mass range for product ion, m/z 50–350; ion accumulation time, 30 msec, total loop time, 600 msec. Conditions for

positive negative detection were as follows: mass range for positive ion mode, m/z 150–500; ion accumulation time for positive ion mode, 30 msec; mass range for negative ion mode, m/z 150–500; ion accumulation time for negative ion mode, 30 msec; total loop time, 400 msec. Data processing

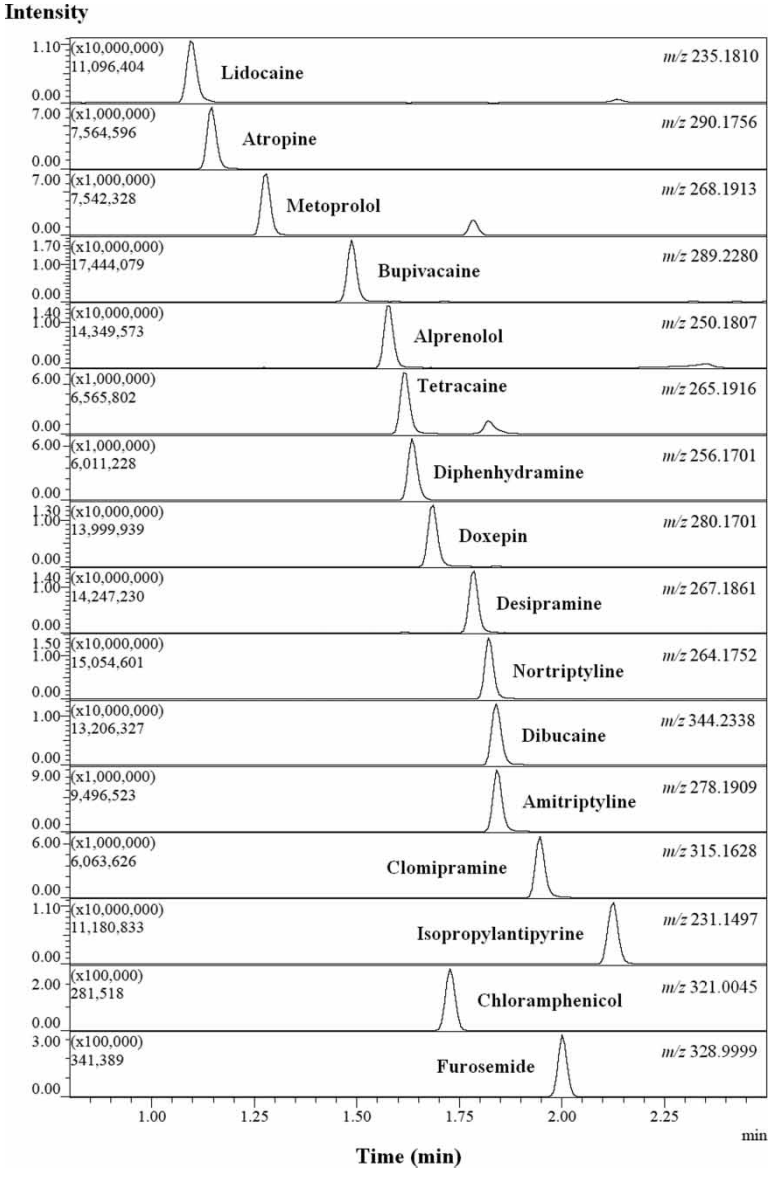

Figure 3. Representative mass chromatograms of test drugs using the positive negative polarity-switching mode (200 pg each).

was performed using the LCMS Solution version 3.40 software with the Formula Predictor software.[23,24]

RESULTS AND DISCUSSION

Accurate Mass Measurement

The optimum flow rate of mobile phase is 0.4–0.5 mL/min for the 2.2 μm particles in a 2.0 mm I.D. column. The flow rate was set at 0.5 mL/min and all compounds were eluted within 2.5 min (Figure 3). As shown in Table 1, repeatability of retention time and peak area for each drug was determined by 6 consecutive injections. The RSDs for retention time (t_R) were 0.20% or better and those for peak area did not exceed 3%. In positive mode (200 pg each), protonated molecules were detected for all compounds, except for chloramphenicol and furosemide. As peak band width at baseline varied from 3 to 6 sec, more than 30 spectra were obtained for each drug. Mass accuracies were summarized in Table 2, showing good performance with <4 ppm error. The standard mixture at a low concentration (1 ng/mL each) was also injected (5 μL). Deterioration of mass accuracies was minimized with <5 ppm error. Simultaneous positive negative detection was also demonstrated. Even though the instrument was operated in rapid polarity switching mode, mass accuracies were sufficient. Chloramphenicol (t_R: 1.727 min), furosemide (t_R: 2.000 min) gave negative ions at m/z 321.0039

Table 1. Repeatability of retention time and peak area (n = 6, 200 pg each)

	Retention time		Peak area	
Compound	Average (min)	RSD (%)	Average	RSD (%)
Lidocaine	1.102	0.20	18310219.7	2.12
Atropine	1.151	0.11	10130893.3	1.62
Metoprolol	1.284	0.09	10827167.8	1.28
Bupivacaine	1.493	0.08	24925174.8	2.43
Alprenolol	1.583	0.08	22358365.2	1.00
Tetracaine	1.623	0.13	10027581.8	1.47
Diphenhydramine	1.641	0.09	9131774.5	2.51
Doxepin	1.690	0.08	21733707.8	1.87
Desipramine	1.789	0.10	21384620.5	1.12
Nortriptyline	1.827	0.09	20661572.5	1.54
Dibucaine	1.844	0.06	20843671.8	1.62
Amitriptyline	1.847	0.11	13194265.3	1.98
Clomipramine	1.951	0.07	9225889.3	1.96
Isopropylantipyrine	2.128	0.09	21278278.8	1.06

Table 2. Mass accuracy of drugs

Compound	Theoretical m/z	Positive mode (200 pg each) Measured m/z	Positive mode (200 pg each) Error (ppm)[a]	Positive mode (5 pg each) Measured m/z	Positive mode (5 pg each) Error (ppm)[a]	Positive-negative mode (200 pg each) Measured m/z	Positive-negative mode (200 pg each) Error (ppm)[a]
Lidocaine	235.1810	235.1811	−0.26	235.1802	3.57	235.1819	−3.66
Atropine	290.1756	290.1752	1.45	290.1746	3.52	290.1757	−0.28
Metoprolol	268.1913	268.1912	0.26	268.1907	2.13	268.1914	−0.48
Bupivacaine	289.2280	289.2276	1.35	289.2269	3.77	289.2279	0.31
Alprenolol	250.1807	250.1810	−1.16	250.1801	2.44	250.1804	1.24
Tetracaine	265.1916	265.1914	0.79	265.1913	1.17	265.1918	−0.72
Diphenhydramine	256.1701	256.1700	0.55	256.1704	−1.01	256.1707	−2.19
Doxepin	280.1701	280.1699	0.86	280.1693	3.00	280.1690	4.07
Desipramine	267.1861	267.1858	1.24	267.1850	4.23	267.1862	−0.26
Nortriptyline	264.1752	264.1749	1.25	264.1742	3.90	264.1749	1.25
Dibucaine	344.2338	344.2333	1.48	344.2349	−3.17	344.2341	−0.84
Amitriptyline	278.1909	278.1905	1.37	278.1902	2.44	278.1910	−0.43
Clomipramine	315.1628	315.1625	0.95	315.1621	2.22	315.1621	2.22
Isopropylantipyrine	231.1497	231.1490	3.20	231.1486	4.93	231.1492	2.34
Chloramphenicol	321.0045	—	—	—	—	321.0039	1.87
Furosemide	328.9999	—	—	—	—	329.0004	−1.55

[a]Error (ppm) = {(theoretical m/z) − (measured m/z)}/(measured m/z) × 10^6.

Table 3. MS/MS, MS^3 results (base peak)

	MS/MS			MS^3		
Compound	Measured m/z	Error (ppm)[a]	Proposed ion	Measured m/z	Error (ppm)[a]	Proposed ion
Lidocaine	86.0977	−8.13	$C_5H_{12}N^+$	ND	NA	NA
Atropine	124.1127	−0.81	$C_8H_{14}N^+$	93.0708	−4.30	$C_7H_9^+$
Metoprolol	116.1076	−0.86	$C_6H_{14}NO^+$	ND	NA	NA
Bupivacaine	140.1445	−4.28	$C_9H_{18}N^+$	98.0964	6.12	$C_6H_{12}N^+$
Alprenolol	116.1081	−5.17	$C_6H_{14}NO^+$	ND	NA	NA
Tetracaine	176.1083	−4.54	$C_{11}H_{14}NO^+$	120.0447	1.67	$C_7H_6NO^+$
Diphenhydramine	167.0848	7.78	$C_{13}H_{11}^+$	ND	NA	NA
Doxepin	235.1111	5.10	$C_{17}H_{15}O^+$	ND	NA	NA
Desipramine	236.1423	6.78	$C_{17}H_{18}N^+$	208.1113	6.25	$C_{15}H_{14}N^+$
Nortriptyline	233.1333	−1.29	$C_{18}H_{17}^+$	218.1077	8.71	$C_{17}H_{14}^+$
Dibucaine	271.1453	−2.21	$C_{16}H_{19}N_2O_2^+$	215.0806	6.97	$C_{12}H_{11}N_2O_2^+$
Amitriptyline	233.1329	0.43	$C_{18}H_{17}^+$	218.1085	5.04	$C_{17}H_{14}^+$
Clomipramine	270.1059	−3.33	$C_{17}H_{17}NCl^+$	ND	NA	NA
Isopropylantipyrine	189.1018	5.29	$C_{11}H_{13}N_2O^+$	131.0733	1.53	$C_9H_9N^+$

[a]Error (ppm) = {(theoretical m/z) − (measured m/z)}/(measured m/z) × 10^6; ND: not detected; NA: not available.

(error: 1.87 ppm), or m/z 329.0004 (error: −1.55 ppm), respectively. As priority was taken for minimization of peak tailing of basic drugs, formic acid was chosen for the mobile phase in the present study. The sensitivity of the compounds that gave negative ions decreased instead of leading to better retention onto the ODS surface at low pH, but the amount of 200 pg was enough to detect and demonstrate accurate mass measurement of these two drugs. Error (ppm) for each compound was comparable to that of the positive only mode. Detection with the polarity switching mode in an analysis run is effective for screening of various drugs.[25] In that case, higher pH ($4 < \text{pH} < 7$) of mobile phase would be more appropriate. The rapid polarity switching mode has the potential to enhance or to double the throughput of UFLC. This mode is more efficient at the analytical method development stage.

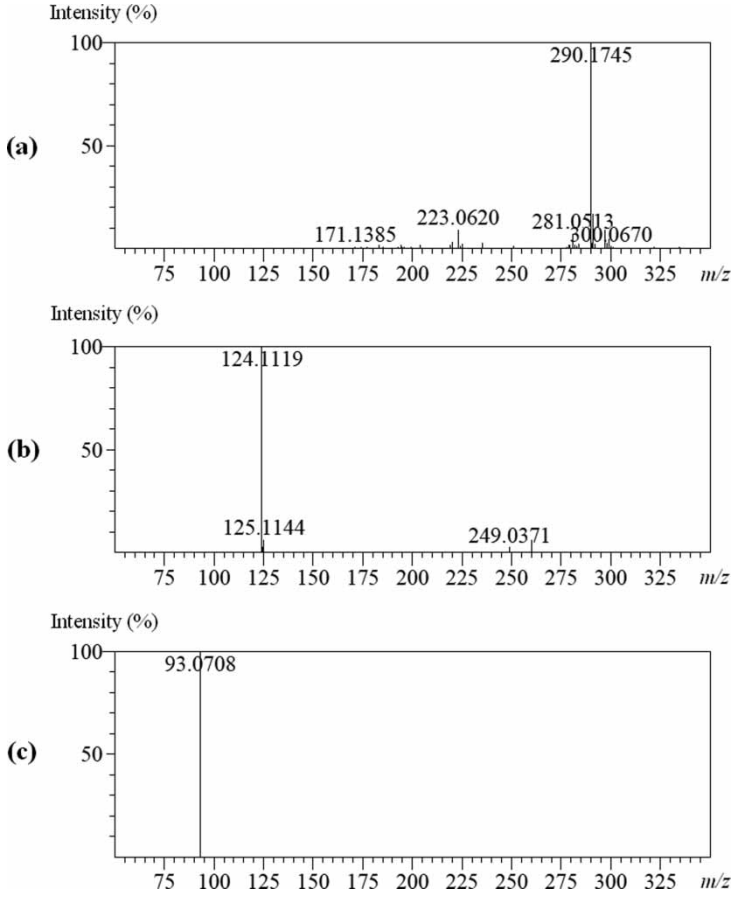

Figure 4. Mass spectra of atropine: (a) MS, (b) MS/MS, (c) MS^3.

MS/MS, MS^n Capability

MS/MS or MS^3 capability was determined. The base peak m/z of the MS/MS or MS^3 spectrum and the corresponding proposed ion for each drug is shown in Table 3. The precursor ion for MS/MS was the protonated molecule of each

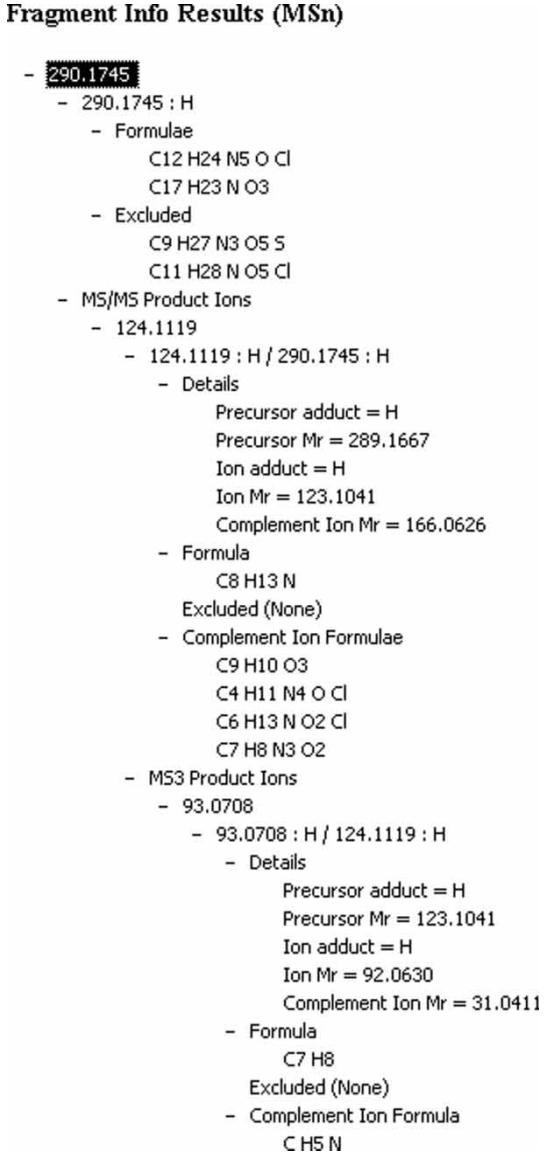

Figure 5. MS^n results of atropine by using the Formula Predictor software.

Table 4. Reproducibility of retention time during 400-injections and mass accuracy after 34 hours

	Retention time		Mass accuracy	
Compound	Average (min)	RSD (%)[a]	Measured m/z	Error (ppm)[b]
Lidocaine	1.093	0.25	235.1812	−0.68
Atropine	1.144	0.19	290.1752	1.45
Metoprolol	1.276	0.16	268.1904	3.24
Bupivacaine	1.481	0.11	289.2272	2.73
Alprenolol	1.573	0.10	250.1806	0.44
Tetracaine	1.612	0.14	265.1908	3.05
Diphenhydramine	1.630	0.13	256.1699	0.94
Doxepin	1.679	0.12	280.1692	3.36
Desipramine	1.778	0.10	267.1850	4.23
Nortriptyline	1.798	0.08	264.1741	4.28
Dibucaine	1.833	0.08	344.2326	3.52
Amitriptyline	1.836	0.12	278.1900	3.16
Clomipramine	1.940	0.10	315.1614	4.44
Isopropylantipyrine	2.127	0.07	231.1487	4.50

[a]RSD (%): data from 5th, 100th, 200th, 300th, and 400th injection (n = 5).
[b]Error (ppm) = {(theoretical m/z) − (measured m/z)}/(measured m/z) × 10^6.

drug. Mass accuracies (error: ppm) were better than 10 ppm. All ion species were proposed using the Formula Predictor software. Scores and formula candidates were calculated with accurate mass, isotopic patterns, and other parameters, e.g., elements, adduct ions, double bond equivalents, hydrogen to carbon ratio, nitrogen rule, and fragment information of MS^n. In MS^3 measurement, atropine, bupivacaine, tetracaine, desipramine, nortriptyline, dibucaine, amitriptyline, and isopropylantipyrine gave fragment ions, which originated from base peak ions of MS/MS. Error (ppm) for each compound was better than 10 ppm. Figure 4 shows representative mass spectra of atropine. The fragment information result by the Formula Predictor is shown in Figure 5. Information on the parent ion, the MS/MS product ion, and the MS^3 product ion are summarized. Predicted formulae are also given.

Stability

Stability of mass accuracy is critical for a mass spectrometer, especially in accurate mass measurement. Higher throughput LC is preferred from the view of productivity and also variation of mass accuracy. A Thirty-four hour analysis was carried out to evaluate stability of mass accuracy. After more

than 400 consecutive injections, a mixture (200 pg each) was injected and accuracies for drugs were calculated (Table 4). Good mass accuracies were maintained within 5 ppm using external calibration. As the entire flight tube is thermostatically controlled within $\pm 0.3^{\circ}$C, deviation of mass accuracy is minimized. Representative accuracies (error: ppm) of diphenhydramine at several runs during the 34 hour analysis were as follows: 5th run, 0.94; 100th run, 1.33; 200th run, −3.75; 300th run, 1.33; 400th run, 0.94. Those of amitriptyline were as follows: 5th run, −2.59; 100th run, 1.37; 200th run, 4.24; 300th run, 3.16; 400th run, 3.16. Overall accuracies of the drugs were comparable to those of sodium trifluoroacetate clusters (error: <3 ppm, 24 hours, m/z 928).[13] Robustness of the chromatographic performance is also shown in Table 4. Reproducibility of retention time for each compound was also maintained, giving RSDs better than 0.3%.

CONCLUSION

QIT/TOFMS was applied for detection of UFLC and showed a successful performance on accurate mass measurement of the test drugs. UFLC enhances its performance in conjunction with QIT/TOFMS and the formula prediction software. UFLC/ESI-QIT/TOFMS accelerates not only speed of the analysis run, but also the entire process of qualitative analysis. Accuracy of each drug was maintained in MS, MS/MS analysis. Deterioration on accuracies of test compounds was minimized in the rapid polarity switching mode and analysis at a low concentration, giving results of $<5\%$ error. Even though the instrument is tuned by external calibration, stability of mass accuracy was proven with minimal deviation. UFLC/ESI-QIT/TOFMS has the potential for extensive applications, such as structural elucidation of drug candidates, impurity analysis, drug screening, and metabolite analysis.

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