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High-throughput liquid chromatography for drug analysis in biological fluids: investigation of extraction column life

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Abstract

A novel method was developed and assessed to extend the lifetime of extraction columns of high-throughput liquid chromatography (HTLC) for bioanalysis of human plasma samples. In this method, a 15% acetic acid solution and 90% THF were respectively used as mobile phases to clean up the proteins in human plasma samples and residual lipids from the extraction and analytical columns. The 15% acetic acid solution weakens the interactions between proteins and the stationary phase of the extraction column and increases the protein solubility in the mobile phase. The 90% THF mobile phase prevents the accumulation of lipids and thus reduces the potential damage on the columns. Using this novel method, the extraction column lifetime has been extended to about 2000 direct plasma injections, and this is the first time that high concentration acetic acid and THF are used in HTLC for on-line cleanup and extraction column lifetime extension.
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Keywords: Drug analysis; Column lifetime

1. Introduction

Determination of pharmaceutical compounds and their metabolites in biological fluids is a very important aspect of drug discovery and development in the pharmaceutical industry. High-performance liquid chromatography with tandem mass spectrometry (LC–MS/MS) has been widely utilized for the bioanalysis due to its selectivity and excellent sensitivity. An accurate and reliable LC–MS/MS assay, however, demands an effective sample cleanup procedure, such as solid-phase extraction (SPE) [1], liquid–liquid extraction (LLE) [2] and solid–liquid extraction (using diatomaceous earth) [3]. These optimized cleanup methods efficiently remove the potentially interfering components, e.g. proteins, lipids, salts, etc., and provide a relatively clean sample for LC–MS/MS analysis; but they require multiple operation steps and long sample preparation time. Automated parallel off-line methods, such as SPE and LLE in a 96-well format, have been successfully used to reduce the sample preparation time. However, the time-consuming evaporation and reconstitution steps are often not eliminated. Therefore, a rapid, accurate and precise bioanalytical method is needed to keep pace with accelerated drug development process.

High-throughput liquid chromatography (HTLC), introduced by Cohesive Technologies (Franklin, MA, USA) in the late 1990s [4], makes it possible to directly inject biological fluids onto a narrow bore large particle size extraction column (50 mm × 1.0 mm, 60 μm) using a high flow rate (4–8 ml/min) aqueous mobile phase. Under these conditions, proteins and salts are flushed away while the small molecules are retained on the hydrophobic surface inside the porous particles [5–12]. The retained analytes are subsequently eluted from the extraction column using an organic mobile phase onto an analytical column for the chromatographic separation. Thus, HTLC has the potential to eliminate off-line sample cleanup procedures for clinical sample analysis; and our group has successfully employed HTLC/LC–MS/MS to determine a new Merck compound in human urine and dialysate [13] without the off-line sample cleanup.

For human plasma samples, it was difficult to develop a robust and cost effective method using HTLC due to the short lifetime of the extraction column (the number of injections undertaken for each HTLC extraction column). In some publications, the extraction column was reported to last approximately 300–600 injections for animal plasma samples

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[5–7]. In other published research papers, the lifetime of the extraction column was not mentioned at all [8,9]. Based on our experience, the extraction column did not perform well after as few as 160 human plasma sample injections (5–10 μl direct plasma injection) when the traditional mobile phase (less than 1% of the acetic acid or formic acid in the water) was used. The accelerated drug development required that 200–400 or even more human plasma samples be analyzed in a daily run; and with such short extraction column lifetime, HTLC did not seem to be suitable as a high throughput method for human plasma sample analysis. Furthermore, the extraction column lifetime is not only related to the cost, but also to the robustness of a bioanalytical method. In this paper, we report a novel method that significantly extends the HTLC extraction column lifetime. And we believe that this new method will make HTLC a feasible and important technology for the determination of pharmaceutical compounds in human plasma samples.

2. Experimental

2.1. Materials

Compounds I and II were obtained from Merck Research Labs. (West Point, PA, USA). Compound I is a primary amine. The pK_a and the aqueous solubility of compound I are 9.4 and 5.9 mg/ml, respectively. Compound II is a chemical analogue of the compound I and was used as the internal standard for this assay. The purity of compounds I and II were 99.8 and 99.2%, respectively. Fig. 1 shows the partial structure of compounds I and II. HPLC grade acetonitrile (ACN), HPLC grade tetrahydrofuran (THF) and HPLC grade glacial acetic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (98–100%) and ethylamine (70%) were purchased from Sigma (St. Louis, MO, USA). Control human plasma was purchased from Biological Specialty Corp. (Colmar, PA, USA).

2.2. Instrumentation

A Cohesive 2300 HTLC turboflow system (Cohesive Technologies Inc., Franklin, MA, USA) was used for on-line extraction which included a quaternary pump, a binary pump and a valve module. A LEAP HTS PAL autosampler (CTC Analytics, Zingen, Switzerland) was used to inject the sample. A Sciex API 4000 mass spectrometer (Toronto, Canada) with a Sciex TurboionSpray Interface was used as the detector. A Packard MultiPROBE II HT EX robotic liquid handling system (Meriden, CT, USA) was used in all pipetting steps for the sample preparation. The data were collected and processed using Analyst software v. 1.1 (Sciex, Toronto, Canada).

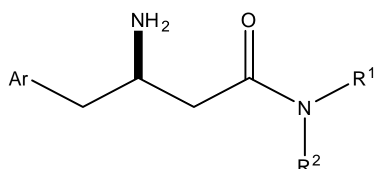

Fig. 1. Partial structure of compounds I and II.

2.3. Preparation of calibration standard and quality control (QC) samples

A stock solution of compound I was prepared at 100 μg/ml in 50% ACN (ACN:water, 50:50, v/v). Working standard solutions from 2.5 to 5000 ng/ml (2.5, 5, 25, 100, 500, 2500, 4000 and 5000 ng/ml) in 10% ACN (ACN:water, 10:90, v/v) were prepared by serial dilution from the stock solution (100 μg/ml). Plasma standards were prepared by mixing 50 μl of each working standard with 250 μl of control human plasma, 50 μl of working IS solution and 50 μl of 20% acetic acid in a 96-well plate.

A stock solution of compound II which served as the internal standard (IS) was prepared at 100 μg/ml in 50% ACN. The working IS solution was prepared at 500 ng/ml in 10% ACN.

A quality control (QC) stock solution of compound I, prepared from a separate weighing, was prepared at 100 μg/ml in 50% ACN. A secondary QC stock solution was prepared at 5 μg/ml in 10% ACN by dilution of the stock solution (100 μg/ml). A tertiary QC stock solution was prepared at 100 ng/ml in 10% ACN by dilution from the secondary QC stock solution (5 μg/ml). QC samples were prepared by adding appropriate volumes of QC stock solutions into volumetric flask and diluting to the mark with control human plasma to achieve the desired concentrations of 1.5, 100 and 800 ng/ml for low, medium and high QCs, respectively. Four hundred micoliters of each QC sample was transferred into conical polypropylene tubes which were capped and stored at −20 °C.

A number of QC samples were processed along with the unknown clinical samples during each analytical run. The number of QC samples in an analytical run depended on the total number of samples analyzed in the run. The minimum number of QC samples should be at least 5% of the number of unknown samples analyzed in a given run or six total QCs, whichever is greater [14].

2.4. Sample preparation

A volume of 250 μl of QC or human clinical sample was pipetted directly into a 96-well plate followed by 50 μl of working IS solution and 50 μl of 20% acetic acid. Then, 50 μl of 10% ACN was added to make up the volume.

2.5. Chromatographic conditions

A Cyclone HTLC column (50 mm × 1.0 mm, 60 μm particle size) from Cohesive Technologies Inc. (Franklin, MA, USA) and a BDS Hypersil C18 column (30 mm × 2.1 mm,

3 μm particle size) from ThermoHypersil-Keystone (Bellefonte, PA, USA) were used as extraction and analytical columns, respectively. Four solutions, A: 2.5 mM ethylamine, 0.1% formic acid (FA) aqueous solution; B: 0.1% FA acetonitrile (ACN) solution; C: 15% acetic acid aqueous solution; D: 90% tetrahydrofuran solution (THF:ACN, 90:10, v/v), were used as mobile phases. The retention times for I and II were about 2.80 and 2.83 min (peak width at half height of compound I for 1000 ng/ml sample is about 0.06 min), respectively, and the total run time was 5 min. Fig. 2 shows the chromatograms of analyte (compound I) and internal standard (compound II).

2.6. Mass spectrometry detection conditions

Precursor ions for the analyte (compound I) and internal standard (compound II) were determined from Q1 spectra obtained during infusion of neat solution of each compound, via the turbo ion spray (TIS) source into the mass spectrometer operated in positive ionization mode with the collision gas off. Under these conditions, the analyte and internal standard yielded predominantly protonated molecules at *m/z* 408 and *m/z* 422. Each of the precursor ions was subjected to collision induced dissociation (CID) in order to generate product ions. The product ion of compound I at *m/z* 235 and compound II at *m/z* 249 were chosen for the selected reaction monitoring (SRM). Experiment parameters were optimized during the infusion of compound I through the TIS interface. The ionspray voltage was 3000 V and the TIS interface temperature was maintained at 700 °C. Nitrogen was used as nebulizer, curtain and collision gas. The declustering potential was 52 V, entrance potential was 8 V, collision energy was 26 V and collision cell exit potential was 12 V. Instrument settings were adjusted to maximize the response for the compound I precursor/product ion transitions of *m/z* 408 → 235.

3. Results and discussions

3.1. On-line extraction procedure and human urine sample analysis

Table 1 shows the on-line extraction methods A, B and C which were developed for the determination of compound I in human urine and plasma samples. From Table 1, the on-line extraction method A can be described as four general steps: (1) the loading step (cleaning step), where the biological fluid is directly injected from the autosampler onto a narrow bore large particle size extraction column (50 mm × 1.0 mm, 60 μm), the matrix components are rapidly washed away and analytes are retained using an aqueous mobile phase at a high flow rate (5 ml/min); (2) the transfer step, where analytes are eluted from the extraction column and transferred onto the reversed-phase analytical column using a high organic mobile phase; an aqueous mobile phase is teed into the high organic mobile phase just before the analytical column allowing the analyte to focus on the head of

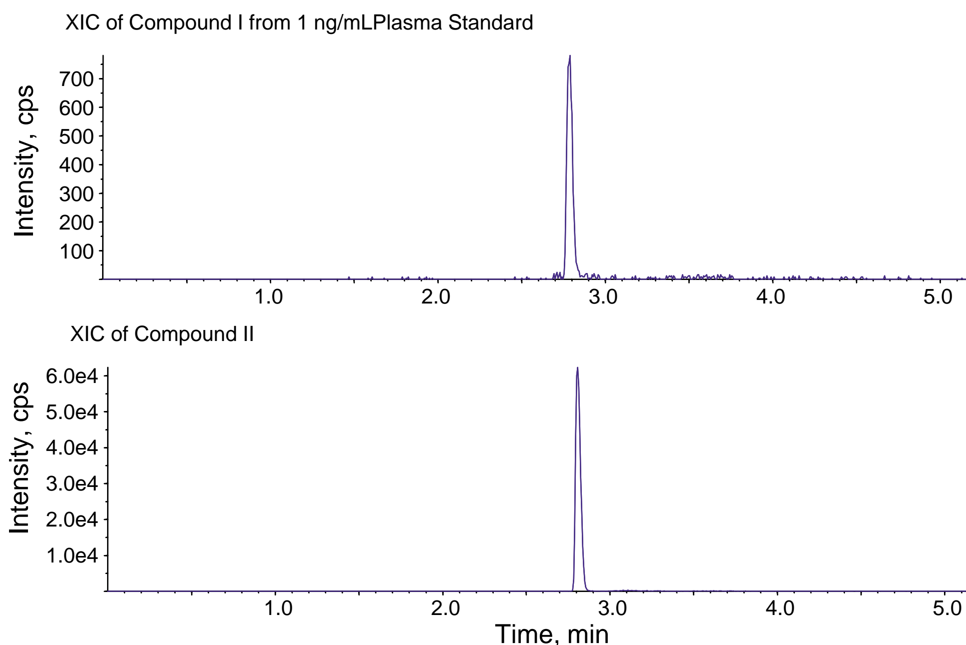

Fig. 2. Chromatograms obtained after the injection of 10 μl of a plasma standard sample include 1 ng/ml of analyte and 100 ng/ml of internal standard using method C. Experimental conditions: cohesive 2300 HTLC turboflow system; extraction column, Cyclone HTLC column (50 mm × 1.0 mm, 60 μm), analytical column, BDS Hypersil C18 column (30 mm × 2.1 mm, 3 μm). Four solutions, A: 2.5 mM ethylamine, 0.1% formic acid (FA) aqueous solution; B: 0.1% FA acetonitrile (ACN) solution; C: 15% acetic acid aqueous solution; D: 90% tetrahydrofuran (THF) solution (THF:ACN, 90:10, v/v), were used as mobile phases.

Table 1
On-line HTLC method A, B and C

	Loading pump								Eluting pump			Method
	Time (s)	Flow (ml/min)	Grad	%B[a]	%C[b]	%D[c]	Tee	Loop	Flow (ml/min)	Grad	%B	
Loading	15	5.0	Step	0	100	0		Out	0.4	Step	0	B,C
Loading	30	5.0	Step	0	0	0		Out	0.4	Step	0	A, B, C
Transfer	60	0.2	Step	0	0	0	T	In	0.25	Step	0	A, B, C
Eluting	12	4.0	Step	0	0	0		In	0.4	Step	40	A
Eluting	12	4.0	Step	0	100	0		In	0.4	Step	40	B, C
Eluting	30	4.0	Step	100	0	0		In	0.4	Ramp	70	A, B
Eluting	36	4.0	Ramp	70	0	0		In	0.4	Step	70	A, B
Eluting	33	4.0	Step	80	0	0		In	0.4	Ramp	70	C
Eluting	30	4.0	Step	0	0	100		In	0.4	Step	70	C
Eluting	24	0.8	Step	0	0	100	T	In	0.0	Step	100	C
Eluting	18	4.0	Step	70	0	0		In	0.4	Ramp	100	A, B
Eluting	24	5.0	Step	40	0	0		In	0.4	Ramp	50	A, B
Equilibrate	12	2.0	Step	0	0	0		Out	0.4	Ramp	10	A, B
Eluting	21	5.0	Step	90	0	0		In	0.5	Ramp	80	C
Eluting	24	5.0	Step	40	0	0		In	0.8	Ramp	50	C
Equilibrate	10	2.0	Step	0	0	0		Out	0.6	Ramp	10	C

[a] Mobile phase A: 2.5 mM ethylamine, 0.1% formic acid (FA) aqueous solution; mobile phase B: 0.1% FA acetonitrile (ACN) solution.
[b] Mobile phase C: 15% acetic acid aqueous solution.
[c] Mobile phase D: 90% tetrahydrofuran (THF) solution (THF:ACN, 90:10, v/v).

the analytical column; (3) the elution step, where the analytes are separated on the analytical column and eluted to the mass spectrometer for determination; and (4) the equilibration step, where the HPLC system is equilibrated for the next injection.

As mentioned previously, one of the advantages of the HTLC technique is the high washing efficiency. For example, the dead volume of the extraction column is about 20 μl; when 2.5 ml of aqueous mobile phase passes over the extraction column in the first 30 s (5 ml/min flow rate), 125× the column dead volume of the mobile phase passes over the column during the initial loading step. If the matrix is highly soluble in the aqueous mobile phase, it will be washed away with the aqueous mobile phase in the loading step.

Based on method A, satisfactory results were obtained for human urine sample assay. The accuracy of the intraday validation ranged from 96.9 to 105.5% of nominal value and precision (R.S.D.%, $n = 5$) ranged from 2.3 to 6.5% which met requirements of the FDA's guidance [14]. The accuracy of the QC samples and standard curve samples also met requirements of the FDA's guidance in the routine clinical human urine sample analysis. The lifetime of the extraction column was more than 2000 injections. These results demonstrate that this is a very simple, fast and rugged method for "direct inject" human urine sample analysis.

3.2. Human plasma sample analysis

When human plasma samples were analyzed using method A, the peak areas of the analyte and internal standard rapidly declined. The peak areas of the analyte and the internal standard at 104th injection were about one-half of the initial peak areas and were approximately one third of the initial peak areas at 163th injections when five sets of plasma standard samples were injected multiple times. These results suggested that some matrix components such as proteins were not washed away with a 0.1% FA aqueous mobile phase, and accumulated on the extraction column affecting the column performance. Because the volume of the extraction column (50 mm × 1.0 mm) is very small, the amount of the stationary phase in the column is limited and therefore if even a trace amount of the matrix is not washed away, it could dramatically affect column performance. It was proposed that any method which would more effectively wash away matrix from the extraction column should extend the lifetime of the column.

3.3. Effect of loading times at the loading step using an aqueous mobile phase containing 0.1% FA for human plasma samples

In an attempt to lengthen extraction column lifetime for plasma samples, the loading step using 0.1% FA aqueous mobile phase was extended from 30 to 60 s (5 ml/min flow rate). However, even a loading step of 250× the column dead volume of the mobile phase did not extend the extraction column lifetime. This is probably due to the fact that the volume of the mobile phase passing through the extraction column in 30 s is already high enough compared to the dead volume of the extraction column. Therefore, simply increasing the washing volume at the loading step is not an effective way to extend the lifetime of the extraction column.

3.4. Effect of mobile phase during the loading step for human plasma samples

Since the single 0.1% FA aqueous mobile phase was not sufficient to adequately clean the column for plasma sam-

ples, it was proposed that to find a mobile phase suitable to increase solubility of human matrix components would be a better approach to solve this problem. The major difference between human plasma and urine samples is the presence of proteins in the former, but not the latter [15]. The short lifetime of the extraction column for human plasma samples is, therefore, likely caused by proteins; and increasing protein solubility in the mobile phase will help extend the extraction column lifetime.

Several strategies can be used to increase the solubility of proteins, such as 10% sodium dodecyl sulfate (SDS) aqueous solution, 6 M guanidine buffered at pH 6–8, 6 M urea/5% acetic acid, 5–80% acetic acid and 0.1–0.5 M perchloric acid [16]. However, most solutions are not suitable for on-line extraction because they are difficult to be removed from the HPLC system in a suitable time and affect the quantitation of the analyte by the mass spectrometer. After careful investigation of the steps following the loading step, it was determined that 15% acetic acid would be a good choice as a mobile phase to remove proteins from the extraction column. The 15% acetic acid solution could be easily eluted from the extraction column during the second loading step by 0.1% FA aqueous mobile phase, and the residual acetic acid does not affect the retention of the analyte on the analytical column and the quantitation of the analyte by the mass spectrometer.

Table 1 provides a protocol for on-line extraction and chromatographic steps for method B with an additional loading step using the 15% acetic acid mobile phase to solubilize proteins in the plasma so that these proteins could be efficiently eluted from the extraction column. The intraday validation, followed by clinical sample analyses, for human plasma samples was performed based on this method, and its accuracy and precision as well as the accuracy of QC and standard samples in the routine human plasma clinical sample analysis, met the FDA's guidance [14] requirements. The peak areas of the analyte (100 ng/ml) and internal standard are 2.28×10^5 and 1.41×10^5 at 8th injection and 2.06×10^5 and 1.33×10^5 at 938th injection, respectively; and their reduction is insignificant after nearly 1000 injections. Compared with traditional mobile phase (less than 1% of the formic acid or acetic acid aqueous mobile phase), this 15% acetic acid mobile phase has significantly extended the extraction column lifetime, probably by weakening the interactions between proteins and the stationary phase.

Further injection was not performed on the extraction column after nearly 1000 injections because the peak started to broaden. This suggested that other components in the plasma still could not be cleaned by aqueous mobile phase as well as 100% acetonitrile mobile phase; and further investigation suggested that these components are lipids (triglycerides, phospholipids, and cholesterol) [15,17]. A thin layer of lipids on the surface of some human plasma clinical samples were frequently observed after centrifuging. Removing the lipids from individual samples could potentially solve this problem, but this could be too labor intensive and time consuming to be performed routinely.

If no additional sample pretreatment, other than the centrifugation, is used to sediment particulates and fibrinogen in the plasma prior to using robotic liquid handling system to pipette samples into the 96-well plate, it is possible that lipids in the plasma samples can be transferred into the 96-well plate. Due to their hydrophobicity, lipids are harmful to reversed-phase HPLC columns. Once lipids are introduced into the HPLC system with a highly aqueous mobile phase, they can potentially modify the functionality of the stationary phase, adversely affecting column performance and causing retention times to vary. It is also very difficult to wash lipids away from the reversed-phase HPLC column by using even 100% acetonitrile mobile phases in the suitable time. Therefore, to protect the columns and to guarantee good reproducibility and ruggedness of the assay, finding a very strong mobile phase that can easily wash away lipids from the extraction column and analytical column is very important when using HTLC methods for human plasma clinical sample analysis.

Tetrahydrofuran is a commonly used HPLC mobile phase with high eluting power for hydrophobic compounds (e.g. lipids). Therefore, using 90% THF as the mobile phase to clean the extraction and analytical column could greatly reduces the potential and cumulative effect of the lipids on the column.

Table 1 details method C which is based on method B. In method C, two more steps were added to wash the extraction and analytical column using 90% THF. Because the analytes had been eluted from the analytical column into the mass spectrometer before 90% THF was used, 90% THF did not affect the quantitation of the analytes and could be applied to any compound analysis.

The intraday validation and clinical sample analysis for human plasma samples were performed based on method C, and its accuracy and precision, as well as the accuracy of the QC and standard samples in the routine human plasma clinical sample analysis, met FDA's guidance [14] requirements. The peak areas of the analyte and internal standard are 2.58×10^5 and 1.62×10^5 at 8th injection and 3.27×10^5 and 1.89×10^5 at 1842th injection, respectively; and they did not change significantly after nearly 2000 injections. In addition, the peak shapes as well as the retention times of the analyte and internal standard were consistent from initial injection to about the 2000th injection. This indicates that the 90% THF mobile phase is able to effectively wash away the lipids from the extraction and analytical columns, reduce the potential effect of lipid accumulation on the columns and the 15% acetic acid mobile phase could increase the solubility of proteins and effectively wash away proteins from the extraction column. Overall, the column lifetime was increased 12-fold using method C as compared to method A.

Table 2
Calculated concentrations of the compound I plasma standard samples of six independent analytical runs on column 1 (S/N: 125505, Lot#: 27084) and column 2 (S/N: 228909, Lot#: 37107) using method C

	Calculated concentration (ng/ml)								Slope	Intercept	Number of injection[b]
	0.5[a]	1	5	20	100	500	800	1000			
Column 1											
Run 1	0.503	1.21	4.80	19.1	103	506	843	990	0.0196	0.00133	351–620
Run 2	0.512	0.965	4.75	18.8	107	511	829	996	0.0175	0.00349	621–850
Run 3	0.482	1.08	4.82	18.9	104	501	815	987	0.0154	0.00426	851–1170
Run 4	0.518	0.934	4.90	18.9	105	505	850	998	0.0177	0.00284	1171–1500
Run 5	0.497	1.02	4.95	19.1	103	492	836	983	0.0155	0.00176	1501–1830
Run 6	0.492	1.04	4.86	18.4	103	512	835	990	0.0151	0.00187	1831–2030
Mean	0.501	1.04	4.85	18.9	104	505	835	991	0.0168	0.00259	
S.D.	0.013	0.098	0.072	0.258	1.60	7.34	12.0	5.57	0.00177	0.00114	
R.S.D. (%)	2.64	9.38	1.49	1.37	1.54	1.46	1.44	0.563			
Accuracy (%)	100.1	104.2	96.9	94.3	104.2	100.9	104.3	99.1			
Column 2											
Run 1	0.483	1.08	4.86	19.0	102	507	822	967	0.0134	0.00160	371 –720
Run 2	0.500	1.21	5.03	19.4	103	496	816	984	0.0155	0.00206	721–980
Run 3	0.483	1.09	4.60	18.8	105	504	839	972	0.0152	0.00337	981–1240
Run 4	0.485	1.07	4.80	19.0	103	500	836	982	0.0160	0.00211	1241–1570
Run 5	0.486	1.06	4.91	19.0	104	501	835	940	0.0165	0.00201	1571–1720
Run 6	0.511	0.96	5.01	18.2	101	508	858	1010	0.0143	0.00161	1721–2070
Mean	0.491	1.08	4.87	18.90	103	503	834	976	0.0152	0.00213	
S.D.	0.012	0.080	0.158	0.395	1.41	4.55	14.7	23.0	0.00114	0.000649	
R.S.D. (%)	2.35	7.41	3.24	2.09	1.37	0.90	1.76	2.36			
Accuracy (%)	98.3	107.8	97.4	94.5	103.0	100.5	104.3	97.6			

[a] Nominal concentration.
[b] Injection 1–350 were used for the method development for column 1 and injection 1–370 were used for other compound assay for column 2.

3.5. Evaluation of the performance of the extraction column

To evaluate the performance of the extraction column using method C for the determination of compound I in human plasma clinical samples, the results of standard and QC samples in six different analytical runs on the same extraction column and those on different extraction columns (different lots) were compared. Two Cyclone HTLC extraction columns with different lot numbers (column 1: Lot#: 27084, S/N#: 125505; column 2: Lot#: 37107, S/N#: 228909) were used for these evaluations.

Table 3
Calculated concentrations of the compound I quality control (QC) samples of six independent analytical runs on column 1 (S/N: 125505, Lot#: 27084) and column 2 (S/N: 228909, Lot#: 37107) using method C

	Number of QC	Low QC				Medium QC				High QC				Number of injection[a]
		Mean (ng/ml)	S.D. (ng/ml)	R.S.D. (%)	Accuracy (%)	Mean (ng/ml)	S.D. (ng/ml)	R.S.D. (%)	Accuracy (%)	Mean (ng/ml)	S.D. (ng/ml)	R.S.D. (%)	Accuracy (%)	
Column 1														
Run 1	5	1.524	0.155	10.2	101.6	105.6	1.82	1.72	105.6	828.8	15.9	1.92	103.6	351–620
Run 2	4	1.580	0.093	5.87	105.3	96.6	4.27	4.42	96.6	797.5	46.3	5.80	99.7	621–850
Run 3	3	1.510	0.044	2.89	100.7	105.0	3.00	2.86	105.0	837.3	18.3	2.19	104.7	851–1170
Run 4	5	1.622	0.143	8.79	108.1	109.6	4.04	3.68	109.6	924.8	64.2	6.95	115.6	1171–1500
Run 5	5	1.356	0.045	3.32	90.4	101.4	4.06	4.00	101.4	792.4	16.7	2.11	99.1	1501–1830
Run 6	5	1.482	0.090	6.08	98.8	102.1	5.14	5.03	102.1	799.2	34.8	4.36	99.9	1831–2030
Column 2														
Run 1	4	1.515	0.059	3.91	101.0	104.0	2.45	2.36	104.0	803.8	69.3	2.40	100.5	371–720
Run 2	4	1.475	0.126	8.55	98.3	110.8	4.57	4.13	110.8	897.8	16.5	1.83	112.2	721–980
Run 3	4	1.535	0.077	5.03	102.3	110.0	6.06	5.50	110.0	907.0	5.94	0.66	113.4	981–1240
Run 4	5	1.512	0.086	5.68	100.8	108.4	2.30	2.12	108.4	848.0	12.5	1.47	106.0	1241–1570
Run 5	2	1.515	0.134	8.87	101.0	100.0	7.07	7.07	100.0	763.0	35.4	4.63	95.4	1571–1720
Run 6	5	1.436	0.114	7.95	95.7	99.8	3.77	3.78	99.8	791.0	41.5	5.25	98.9	1721–2070

[a] Injection 1–350 were used for the method development for column 1 and injection 1–370 were used for another assay for column 2.

Table 2 shows the results of the standard samples of six independent analytical runs (from 351th to 2030th injection for column 1 and from 371th to 2070th injection for column (2) on extraction columns 1 and 2, respectively. There was no significant difference in the calculated concentrations from six independent analytical runs. This means that the performance of the extraction column did not change significantly from analytical runs 1 to 6 and there was not any significant difference between the performance of columns 1 and 2. Thus, the performance of the extraction column was consistent from the first to the last injection.

To further evaluate significance, a *t*-test (two-tail test) was employed. The mean of the slopes and intercepts of six independent analytical runs for columns 1 and 2 were compared. The experimental t values for the slope and intercept are 1.86 and 0.868, respectively, smaller than the critical *t*-value of 2.23 ($P = 0.05$). This means that the slopes and intercepts of six independent analytical runs for columns 1 and 2 are not significantly different.

Table 3 shows the results of the QC samples of six independent analytical runs (from 351th to 2030th injection for column 1 and from 371th to 2070th injection for column 2) on extraction columns 1 and 2, respectively. For the low, medium and high QC samples, no significant differences were found for the average calculated concentrations of six different analytical runs. For column 1, the accuracy and precision of low, medium and high QC ranged from 90.4 to 105.3%, 96.6 to 109.6%, 99.1 to 115.6% of nominal value and 2.89 to 10.2%, 1.72 to 5.03%, 1.92 to 6.95% (R.S.D.%), respectively. For column 2, the accuracy and precision of low, medium and high QC ranged from 95.7 to 102.3%, 99.8 to 110.8%, 95.4 to 113.4% of nominal value and 3.91 to 8.87%, 2.12 to 7.07%, 0.66 to 5.25% (R.S.D.%), respectively. This means that the performance of the extraction column from analytical runs 1 to 6 did not change significantly over the course of the clinical sample analyses.

In order to evaluate whether the difference between analytical runs 1 and 6 is significant for columns 1 and 2, a *t*-test (two-tail test) was also performed on the results of QC samples. The comparisons of the mean calculated concentrations of the low, medium and high QC samples of analytical runs 1 and 6 for columns 1 and 2 were performed respectively. For column 1, the experimental *t*-values for the low, medium and high QC are 0.525, 1.42 and 1.73, respectively, smaller than the critical *t*-value of 2.31 ($P = 0.05$). For column 2, the experimental *t*-values for the low, medium and high QC are 1.25, 1.91 and 0.345, respectively, again smaller than the critical t value of 2.36 ($P = 0.05$). This means that the mean calculated concentrations of the QC samples for the first and sixth analytical run in both extraction columns are not significantly different over the course of the analytical run for either columns; and the inter column data is not significantly different either.

4. Conclusions

A novel method was developed and assessed to extend the lifetime of extraction columns of high-throughput liquid chromatography for bioanalysis of human plasma samples. A 15% acetic acid aqueous solution was used as a mobile phase to remove proteins in the human plasma samples from the extraction column, and a 90% THF solution was used as a mobile phase to remove lipids from the extraction and analytical columns. The performance of the extraction column for the determination of compound I was evaluated based on the results of standards and QC samples in different analytical runs on the same extraction column and those on different extraction columns. No significant differences were found for the calculated concentrations in six different analytical runs on both columns. The slopes and intercepts of six different analytical runs on two different columns show no significant differences. And there was also no significant difference for the determination of compound I from the injection 1 through 2000 during clinical sample analyses.

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