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Quantitative determination of peptide drug in human plasma samples at low pg/ml levels using coupled column liquid chromatography–tandem mass spectrometry

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ABSTRACT

Plasma concentrations after administration of peptide drugs are often low due to the high potency often seen with this class of compounds. In this work a bioanalytical method based on coupled column liquid chromatography–tandem mass spectrometry (LC–MS/MS) is presented for quantification of a peptide drug, FE 202158, under clinical development. A volume of 0.5 ml human plasma is solid phase extracted on a weak cationic exchanger. After evaporation of the solvent to dryness, the reconstituted sample is injected into a coupled column liquid chromatography system. A heart-cut from the initial column, a cyano column, is trapped on a C_4 column and thereafter injected into a microbore C_{18} column. For the detection a triple quadrupole mass spectrometer, equipped with a TurboIonSpray interface working in positive ion mode, is used. The design of the system is described and the gain in sensitivity and selectivity, compared to a conventional system, is discussed. Data from validation of the bioanalytical method are presented. For human plasma samples a lower limit of quantification (LLOQ) of 5.00 pg/ml (=4.77 pmol/l) was achieved. The inter-assay precision was less than 11% and bias was within ±4% over the whole validated range of 5.00–860 pg/ml.

1. Introduction

Peptide drugs are often highly potent compounds and are known to be a challenge to monitor in blood plasma samples at therapeutic doses due to the low plasma concentration often seen for this class of compounds. Peptides have historically often been quantified in biological fluids by means of immunoassay due to its high sensitivity. Analysis of low molecular weight peptides has normally been restricted to the use of competitive binding assays with the inherent limitations in specificity, i.e. cross-reactions to endogenous peptides or metabolites. Mass spectrometric detection increases the specificity considerably but the sensitivity is not optimal for this class of compounds due to the formation of multiple-charged ions and non-optimal fragmentation pattern. Beside that, peptides often show adsorption to surfaces, limited stability and do not seldom induce chromatographic issues [1].

The development of the LC–MS/MS technology has made it possible to develop and validate bioanalytical methods with lower limit of quantification (LLOQ) levels in the pg/ml range without the limitations in specificity seen with immunoassay. Protein precipitation combined with single-column LC–MS/MS permits quantification of peptides down to approximately 500 pg/ml in plasma samples [2], which is often sufficient for toxicokinetic investigations and for clinical studies of low and mid-potent peptides. For highly potent peptides different designs of sample preparation procedures as well as liquid chromatographic systems needs to be considered in order to meet the high requirements on assay sensitivity. Quantification limits in the low pg/ml range, or even lower levels, must be achievable. Current generation of mass spectrometric equipment has the potential sensitivity for reaching such concentration levels provided that a relatively large volume of plasma sample is efficiently purified before the detection in the mass spectrometer, performed under optimal conditions.

Various two-dimensional column liquid chromatography mass spectrometric detection approaches for determination of peptides in biofluids have been published, including both on-line and off-line applications. Most publications are focused on increasing the peak resolving capacity compared to a single-column system for use in qualitative determination of peptides formed after tryptic digestion of proteins, e.g. for proteomics work [3,4,5,6,7] but also quantification of proteins by means of the tryptic digestion approach [8]. Rather few coupled column applications for quantitative determination of peptide drugs in biofluids have been published and they have often had different objectives with the design of the system, e.g. automation of the sample pretreatment procedure and thereby shorten analysis time [9,10], increase separation speed

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[11], increase selectivity [12] or sensitivity [13]. In the latter work, a coupled column nano-liquid chromatography–tandem mass spectrometry system was used for quantification of peptides down to 50 pmol/l.

Different approaches for designing coupled column liquid chromatography systems have been described and discussed by Bedani et al. [14,15] and Guiochon et al. [16], from both a practical and theoretical perspective. When the whole sample is subject to two-dimensional separation the system is considered to be comprehensive and is indicated with LC × LC, but if just a fraction is transferred the term heart-cutting is often used, normally indicated with 2D-LC. Combinations of different separation mechanisms/stationary phases for the different columns have been evaluated for their peak resolving capacity by Gilar et al. [17]. Examples of the investigated stationary phases are different combinations of RP, SCX, SEC and HILIC. The outcome of the investigation was that no separation mode in 2D-LC offered a complete orthogonality of the separations but the combination of RP–RP used at different pH in the both dimensions offered a practical approach with an orthogonality comparable to the other combinations, which is further evaluated in a subsequent work [18].

The aim of this work was to develop and validate a bioanalytical method with the capability of quantifying a peptide drug candidate down to low pg/ml plasma concentrations, applicable to routine analysis in clinical studies. The intention was to use an increased sample volume in the solid phase extraction and a coupled column microbore liquid chromatographic system connected to a highly sensitive tandem mass spectrometer for the detection. The peak resolving capacity is not considered as the goal in this work but other aspects as obtaining a chromatogram free from interfering peaks and getting high capability of removing phospholipids, known to cause ion suppression effects, from the chromatographic peak window is considered as important in order to obtain a highly sensitive assay.

2. Materials and methods

2.1. Materials

FE 202158, a synthetic V1a agonist under drug development (cysteine-phenylalanine-isoleucine-homoglutamine-asparagine-cysteine-proline-ornithine(isopropyl)-glycine-NH_2) with a molecular weight of 1048 Da was used as model peptide throughout this work. The substance was synthesised at PolyPeptide Laboratories Inc. (Torrance, CA). The standard stock solution was prepared in ammonium acetate (pH 4.0; 10 mM) at a concentration of 1.0 mg/ml. It is stable for at least 4 months when stored at −20 °C. As internal standard a stable isotopic analogue to FE 202158 was used, FE 202561, where ten hydrogen atoms are replaced with deuterium atoms and one nitrogen atom is replaced with a ^{15}N atom. It was synthesised by Ferring Research Institute (San Diego, CA). Potassium EDTA human plasma was from Richmond Pharmacology, Atkinson Morley's Hospital (London, UK). All other chemicals were of p.a. quality or equivalent.

2.2. Sample pretreatment and solid phase extraction

A Tecan Freedom robotic sample processor (Tecan Group Ltd., Männedorf, Switzerland) was used for all pipetting of plasma samples, including preparation of calibration samples. A Gilson ASPEC XL4 sample processor (Middleton, WI) equipped with a Gilson 404 syringe pump and Gilson 735 ASPEC software, version 4.04, was used for all solid phase extraction. Teflon coated probe needles of 183 mm was used together with PTFE tubings of 1/8″ × ID2.40 mm for all sample handling. Waters Oasis WCX® 30 mg (Waters Corporation, Milford, MA) was used as SPE column, in tab-less design being compatible to Gilson 96-well column holder. A Savant Speedvac Concentrator (GMI, Inc., Ramsey, MN) was used for drying the extracts.

2.3. Coupled column liquid chromatography system

A coupled column liquid chromatography system consisting of a HTS PAL autosampler (CTC Analytics AG, Zwingen, Zwitzerland) and three Shimadzu LC-20AD LC pumps (Shimadzu Corporation, Kyoto, Japan) plus a Knauer K 501 pump (Knauer Wissenschaftliche Gerätebau, Berlin, Germany), used for column rinsing, was designed as shown in Fig. 1. A Reprosil-Pur CN, (2.0 mm × 50 mm, 5 μm) column from Dr. Maisch HPLC GmbH (Ammerbuch-Entringen, Germany) was used as initial column. The trap column was a Kromasil C_4 (1.0 mm × 50 mm, 10 μm) from AkzoNobel (Amsterdam, The Netherlands) and a Kromasil C_{18} (1.0 mm × 50 mm, 3.5 μm) was used as analytical column. The switching valves were of 6-port type from VICI Valco Instruments Co., Inc. (Houston, TX). The software used for controlling the mass spectrometer was also used for controlling LC pumps and switching valves. The flow rate for the gradient pump system (pumps A and B) was 0.07 ml/min. The flow rate for both pumps C and D was 0.40 ml/min.

2.4. Mass spectrometric detection

An Applied Biosystems/MDS Sciex API 5000 Triple Quadrupole mass spectrometer (Applied Biosystems, Inc., Foster City, CA), equipped with a TurboIonSpray interface working in positive ion mode, was used for the detection. For FE 202158 the m/z 525 → 328 transition was monitored. Corresponding setting for detection of the internal standard (FE 202561) was m/z 530 → 328.

2.5. Bioanalytical method

Plasma samples stored at −70 °C in Sarstedt 2 ml cryo-vials were thawed and vortex mixed. Calibration samples in human plasma were freshly prepared from a standard stock solution of 1.00 mg/ml FE 202158 in ammonium acetate (pH 4.0; 10 mM), using an automated procedure programmed on a Tecan robotic sample processor. The standard stock solution was diluted in ammonium acetate (pH 4.0; 10 mM), with 0.1% human serum albumin (HSA) and 0.01% Triton X-100. Seven calibration samples in the range 5.00 to 1010 pg/ml were prepared in human plasma. All plasma samples were provided with 50 μl internal standard (FE 202561), dissolved in ammonium acetate (pH 4.0; 10 mM), with 0.1% HSA and 0.01% Triton X-100, yielding a plasma concentration of 415 pg/ml. To all samples, a volume of 600 μl phosphoric acid (0.07 M) was added to 500 μl plasma sample. All these steps were performed with the Tecan robotic sample processor.

The subsequent solid phase extraction was performed in 96-well format on a Gilson ASPEC XL4 sample processor working in sequential mode of operation. The columns were conditioned with 1 ml methanol:acetonitrile (70:30, v/v) followed by 1 ml of phosphoric acid (0.07 M). In the sample-loading step, 1 ml of the acidified plasma sample was added to the column. The column was rinsed in three subsequent steps; 1 ml methanol:water (10:90, v/v) (=system liquid), 2 × 1 ml ammonium acetate (pH 7.3, 0.27 M) and 1 ml methanol:acetonitrile (70:30, v/v). The analyte was eluted from the column with 1 ml acetonitrile:formic acid (1 M) (50:50, v/v) and collected in a 1 ml round-well deep-well plate. All samples were evaporated to dryness using a Savant SpeedVac Concentrator. After the evaporation, the samples were reconstituted in 150 μl methanol–triflouroacetic acid (pH 2.3; 7 mM) (10:90, v/v).

A sample volume of 50 μl was injected into the coupled column liquid chromatography system using methanol–triflouroacetic acid

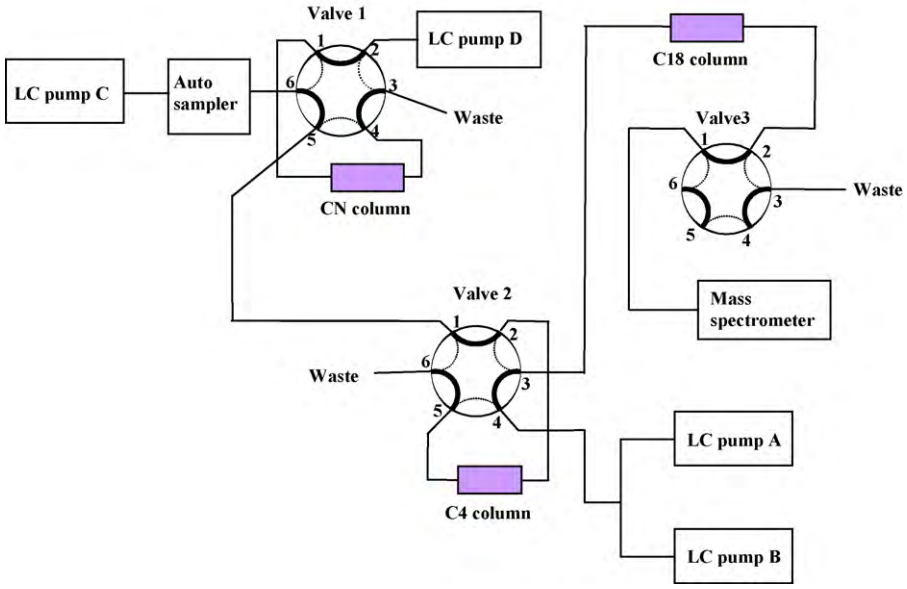

Fig. 1. Schematic view of the coupled column liquid chromatographic system. The sample is injected into the cyano column, a heart-cut from the first chromatogram is trapped on the C_4 column, followed by injection into the C_{18} column and detection in the mass spectrometer. The total flow rate for the gradient LC pumps (pumps A and B) was 0.07 ml/min. Mobile phase A consisted of ammonium acetate (pH 4.0; 10 mM) and mobile phase B consisted of 100% acetonitrile. Time setting for valve switching and the linear gradient is shown in Table 1. The flow rate for both pumps C and D was 0.40 ml/min (isocratic). The mobile phase composition in pump C was methanol:triflouroacetic acid (pH 2.3; 7 mM) (15:85, v/v) and in pump D, i.e. the rinsing pump, acetonitrile:formic acid (1 M) (50:50, v/v).

(pH 2.3; 7 mM) (15:85, v/v) as mobile phase C. Ammonium acetate (pH 4.0; 10 mM) was used as mobile phase A and acetonitrile as mobile phase B. The mobile phase D in the rinsing pump was acetonitrile:formic acid (1 M) (50:50, v/v). The time setting for valve switching and gradient is shown in Table 1. For all samples the peak area was determined for both the analyte and the internal standard. The calibration equation was determined by linear regression, weighted by $1/conc.^2$, of the area ratio obtained from the calibration sample versus the spiked concentration of FE 202158 in the calibration samples.

3. Results and discussions

3.1. Sample pretreatment and solid phase extraction

Since FE 202158 and its internal standard are highly adsorptive to most plastic and glass surfaces, special caution must be taken when the compound is present in pure water solutions without any protection by the biological matrix. This is of special importance when the concentration is low. Throughout this work, 0.1% HSA and 0.01% Triton X-100 has been used as additives when organic solvents were not possible to use due to its poor compatibility with plasma.

A polymer-based weak cationic exchanger was chosen as stationary phase for the solid phase extraction due to its inherent potential of producing relatively clean extracts although a large sample volume is applied on the column. Since the column is of mixed-mode sorbent type with both weak ion exchange and reversed phase properties, it is possible to tune its retention characteristic by changing pH of the solutions used in the SPE procedure. The plasma sample was loaded on the column under acidic conditions due to the need for decreasing the protein binding of the peptide before loading. Although the stationary phase is almost uncharged at such low pH there are still sufficient interactions, probably mainly of reversed phase mechanism type, for retaining the analyte on the column. Strong basic conditions had to be avoided due to the limited stability of the peptide at high pH. In the next step the column was rinsed with buffer solution under neutral conditions making the stationary phase negatively charged. Thereby it was possible to wash the column with a high content of organic solvent in the subsequent step, which efficiently elutes hydrophobic matrix components but with the analyte still retained in the column. In the final step the peptide was eluted from the column by decreasing the pH in the eluent and thereby making the stationary phase uncharged.

3.2. Coupled column liquid chromatography system

The liquid chromatographic system was designed in respect to highest possible sensitivity for analysis of human plasma samples

Table 1
Overview of valve switching position and chromatographic gradient versus time after sample injection in the coupled column liquid chromatographic system.

Time (min)	Switch pos. valve 1	Switch pos. valve 2	Switch pos. valve 3	Gradient setting, % mobile phase A	Gradient setting, % mobile phase B
0	A	A	A	90	10
2.0	A	B	A	90	10
4.5	B	A	A	90	10
9.0	B	B	A	90	10
10.0	B	B	B	90	10
12.0	A	B	B	↓	↓
13.0	A	A	A	40	60
13.1	A	A	A	90	10

Valve position A is defined as connections between tubing connector 1–6, 2–3 and 4–5. Corresponding connections for position B are 1–2, 3–4 and 5–6.

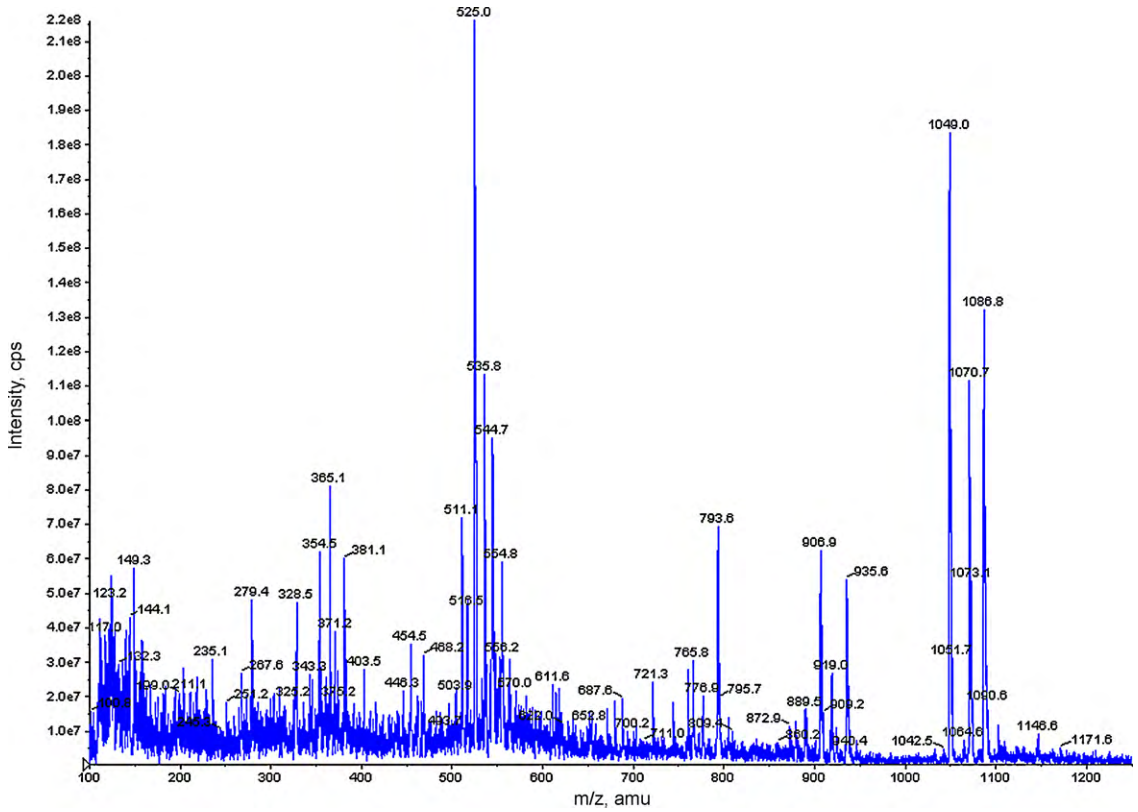

Fig. 2. Q1 scan of a 3 μM solution of FE 202158 in ammonium acetate (pH 4.0; 10 mM): acetonitrile (80:20, v/v) infused into the mass spectrometer.

using tandem mass spectrometry as detection principle. A prerequisite for that is a system with high inherent selectivity being capable of producing narrow peaks under conditions compatible with a highly sensitive and selective mass spectrometric detection system. Influence from ion suppressing agents in the matrix and interfering peaks in the chromatogram needs to be minimised.

The choice of stationary phases in the different columns, and its dimensions, were decided out of the strategy of placing the column with strongest retention in reversed phase mode next to the mass spectrometer. Therefore, an analytical column with small inner diameter packed with 3.5 μm particles of C_{18} material was chosen. The dimension of the column, 1.0 mm × 50 mm, is favourable for the detectability both due to the concentrating effect and the possibility of using a shorter spray distance setting, when working under appropriate flow rate.

Since a relatively robust column used next to the autosampler was considered necessary, it was decided that it should have an inner diameter of at least 2.1 mm. As a consequence, a relatively large peak volume from the initial column was inevitable. Therefore, a third column working as a trap column could not be avoided in order to keep the injection volume on the analytical column low. The trap column was not intended to contribute significantly to any increase in selectivity. A column (1.0 mm × 50 mm) packed with end-capped C_4 material, with the relatively high coverage of 3.8 μmol/m^2, was chosen as trap column due to its moderate retention and for being relatively free from undesirable secondary retention mechanisms with the peptide. Due to the need for obtaining compatibility with the other columns, a column with even lower retention characteristics was chosen as initial column. A cyano column with the dimension of 2.1 mm × 50 mm working in reversed phase mode fulfilled that requirement.

Different mobile phase buffer compositions were investigated in order to obtain both high sensitivity and good chromatographic performance. Ammonium acetate (pH 4.0; 10 mM) as buffer component in the mobile phase for the separation on the analytical column was chosen although 5.2 times higher peak area was achievable using 0.1% acetic acid and 3.6 times higher peak area with 0.1% formic acid. However, in both these mobile phase compositions FE 202158 was eluted as a broad front peak.

For peptides with a molecular weight comparable to FE 202158 both single-charged and double-charged ions should be considered as potential precursor ions. A full scan between *m*/*z* 100 and 1250 was recorded and it can be seen in Fig. 2 that high signal was obtained for both the double-charged and the single-charged ion, and when the mass spectrometric detection settings were separately optimised for each precursor ion it was revealed that 2.2 times higher signal was obtained for the double-charged ion. One major product ion of *m*/*z* 328 could be seen both for the single-charged precursor ion and for the double-charged precursor ion. This product ion was chosen for detection of both FE 202158 and its internal standard.

Use of other types of stationary phases might have increased the selectivity potential further by making the two separation mechanisms orthogonal to each other, e.g. by combining ion exchange or HILIC with the reversed phase separation. However, the use of an ion exchanger column requires relatively high ion strength in the mobile phase which might have a negative effect on the detectability of the peptide in the mass spectrometer. The combination of reversed phase chromatography in the first dimension and HILIC in the second dimension has potential advantages related to assay selectivity and sensitivity. The latter fact is due to the higher signal normally obtained from mobile phases containing high content of organic solvent when detection is performed with ESI-MS. On the other hand, on-line coupling of these two separation modes are known to be problematic and the first successful approach, to our best knowledge, for quantitative analysis was not published until 2009 [12]. The reversed phase-HILIC approach was also tried

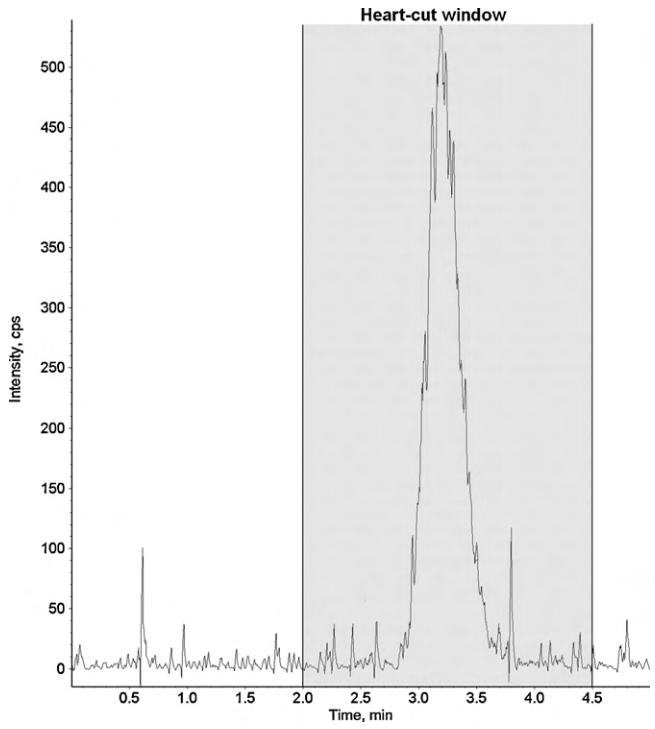

Fig. 3. A chromatogram from injection of FE 202158 on the Reprosil-Pur CN, 2.0 mm × 50 mm, 5 μm, column. A retention time window between 2.0 and 4.5 min is heart-cut and trapped on a 10 μm Kromasil C_4, 1.0 mm × 50 mm, column for further separation in the coupled column liquid chromatography system.

in our laboratory, but the obtained peak shape did not meet the requirements for our method.

During the first 2 min after sample injection the peptide is retained on the cyano column. Fig. 3 shows the chromatography on the cyano column. A heart-cut between 2.0 and 4.5 min from the cyano column is then trapped on the C_4 column. Between 4.5 and 9.0 min the peptide is back-flush eluted from the C_4 column and compressed on the analytical column, where after the gradient elution starts. The retention time was 11.6 min on the analytical column, expressed as time after sample injection into the initial column. The over-all cycle time was 13.1 min for the system, which can be almost halved in the case time over-lapping processes are used, i.e. injection of a new sample on the initial column before finalization of the chromatographic separation on the analytical column for the former sample. However, that requires appropriate software for controlling the different processes, such as valve switching, gradient control, data acquisition, etc., in a more complex way and has not been utilized in this work.

3.3. Assay sensitivity

A chromatogram obtained after injection of a human plasma sample spiked to 5.0 pg/ml is shown in Fig. 4, which also was the concentration level defined as the LLOQ in the method validation. The signal to noise ratio was determined to 13. The peptide could be determined at that level with a precision of 11% and with an accuracy of 96%. A comparison between injection of extracted plasma sample into the coupled column system and into a single-column system (Kromasil C_{18}, 3.5 μm 2.1 mm × 50 mm, 0.40 ml/min), with same mobile phases as the coupled column system, in respect to peak height and signal to noise is illustrated in Fig. 5. The peak height was 2.7 times higher and the signal to noise was 4.1 times higher in the coupled column system.

3.4. Removal of interfering matrix components

The presence of five phospholipids giving rise to product ions of m/z 184, known to be present in plasma and induce ion suppression effects in electrospray MS quantification [19,20], were monitored. Following transitions were monitored: m/z 496 → 184, 520 → 184, 524 → 184, 704 → 184 and 758 → 184. In Fig. 6, chromatograms

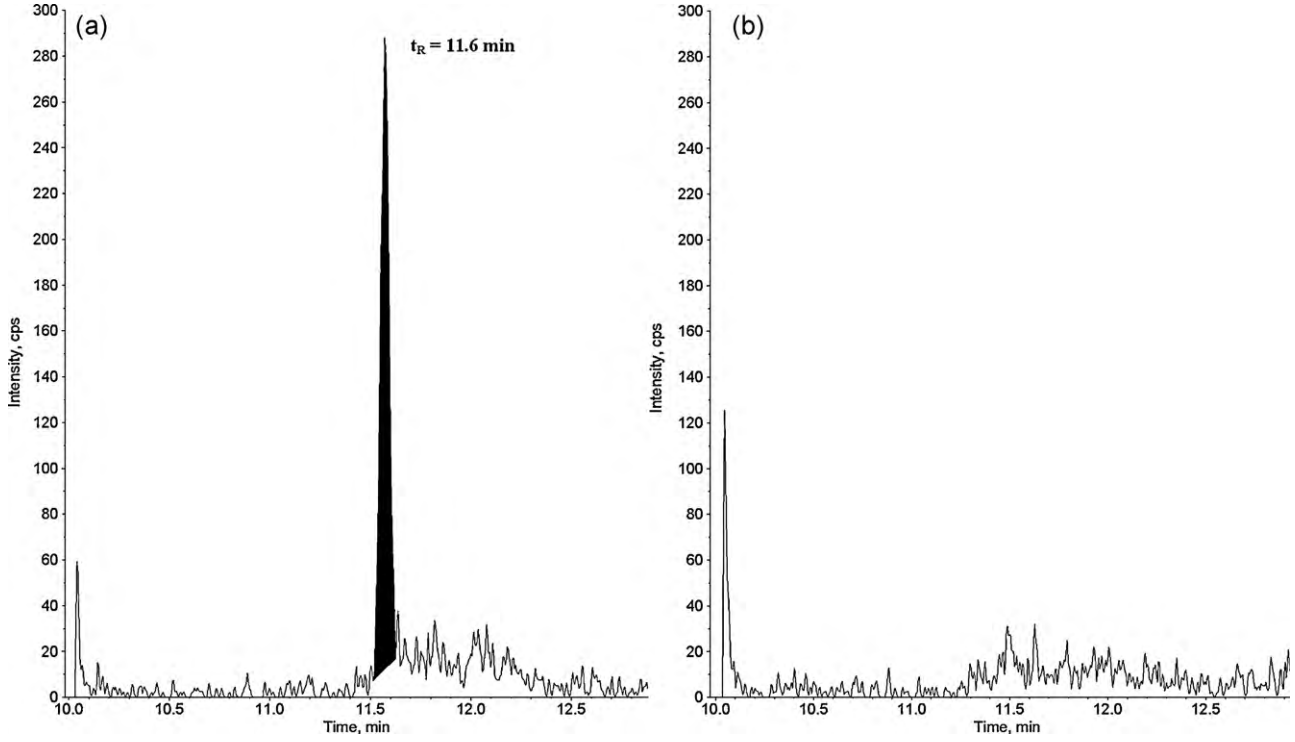

Fig. 4. Chromatogram from injection of a solid phase extracted plasma sample (a) spiked to 5.0 pg/ml and (b) blank plasma injection.

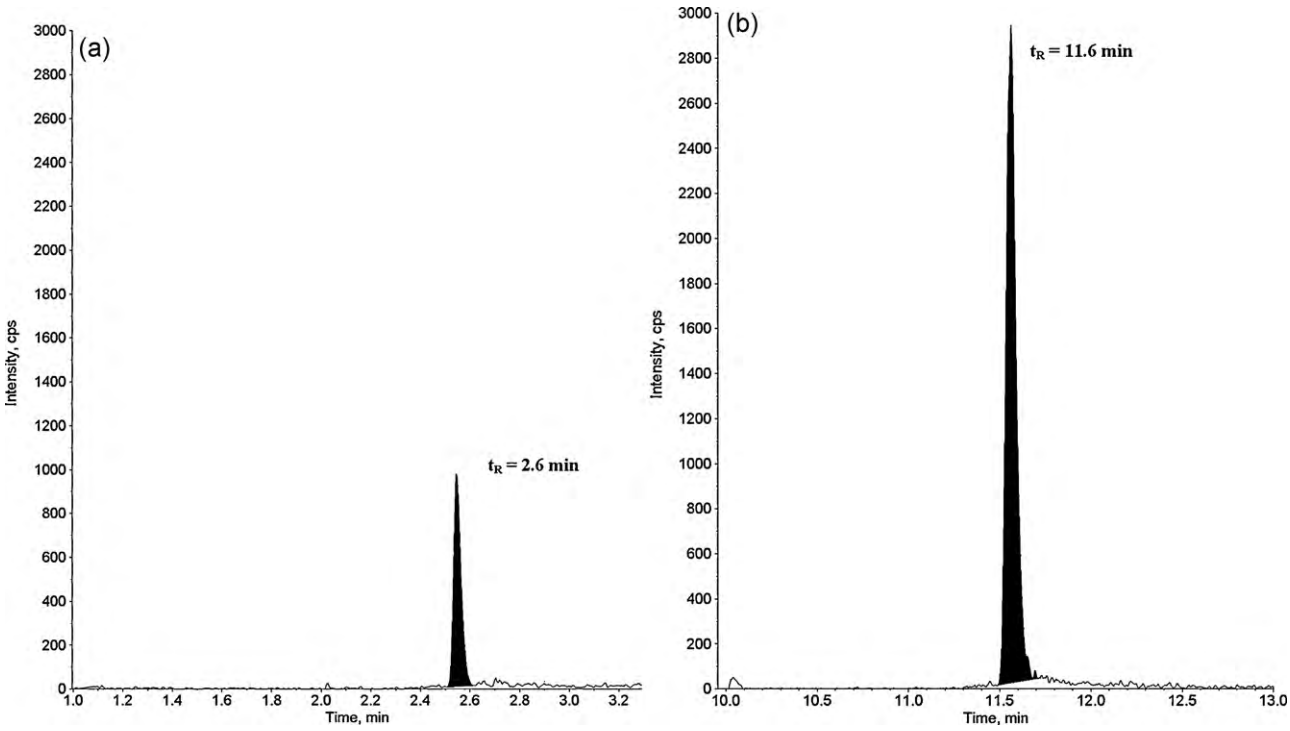

Fig. 5. Comparison of chromatograms obtained from injection of a 71.0 pg/ml sample on (a) a single-column system consisting of a Kromasil C_{18}, 3.5 μm 2.1 mm × 50 mm, 0.40 ml/min, (b) the coupled column liquid chromatography system. The mobile phase composition and gradient setting was equivalent in the systems. The peak height was 2.7 times higher in the coupled column system and the signal to noise ratio was 146 for the coupled column system but only 36 in the single-column system, which makes a four-fold increase in signal to noise.

show the presence of phospholipids, expressed as TIC, after injection of a plasma sample into a single-column system, described in Section 3.3, and injection into the coupled column system. It can be seen that the presence of phospholipids has been significantly reduced in the coupled column system. An alternative way to illustrate the low presence of ion suppressing agents is to compare the peak height obtained from injection of a plasma sample with the peak height from a neat solution of comparable concentration. The chromatographic peak from injection of a neat solution, corresponding to a plasma concentration of 7 pg/ml, was of the same height and area as from injection of a plasma sample, see Fig. 7, indicating low presence of ion suppression/enhancement effects.

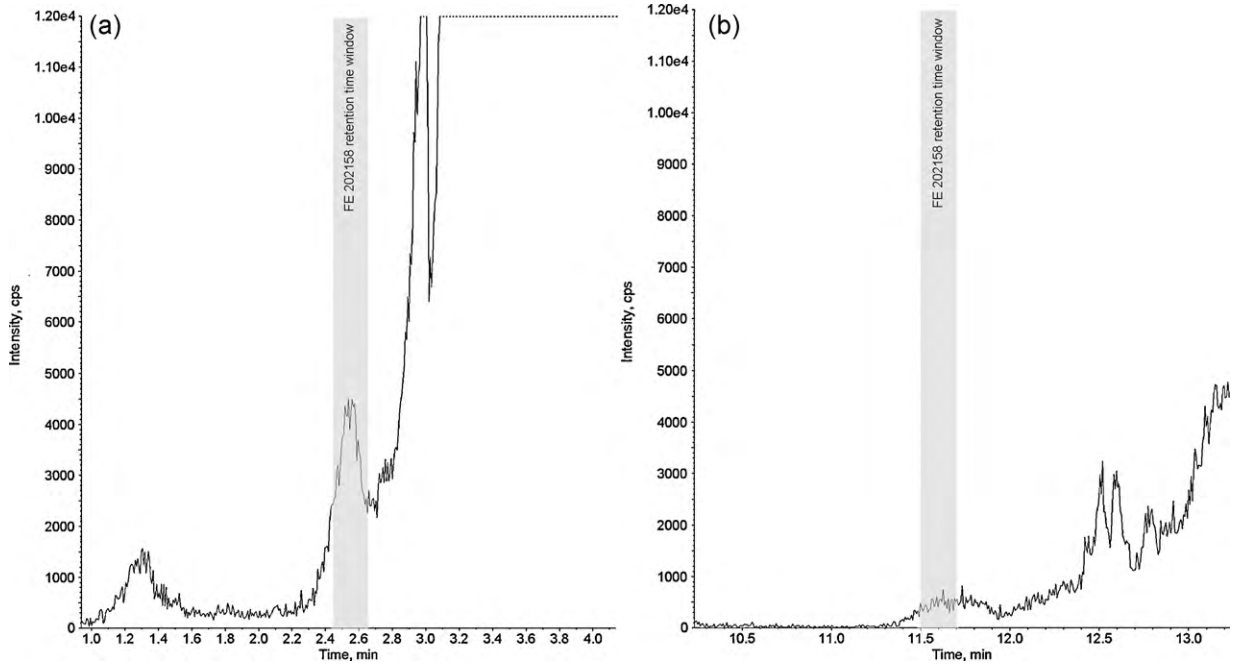

Fig. 6. Chromatograms showing the presence of phospholipids after injection of a solid phase extracted plasma sample into (a) a single-column system consisting of a 3.5 μm Kromasil C_{18}, 2.1 mm × 50 mm, 0.40 ml/min and (b) the coupled column system. The chromatograms are presented as TIC of following transitions: m/z 496 → 184, 520 → 184, 524 → 184, 704 → 184 and 758 → 184.

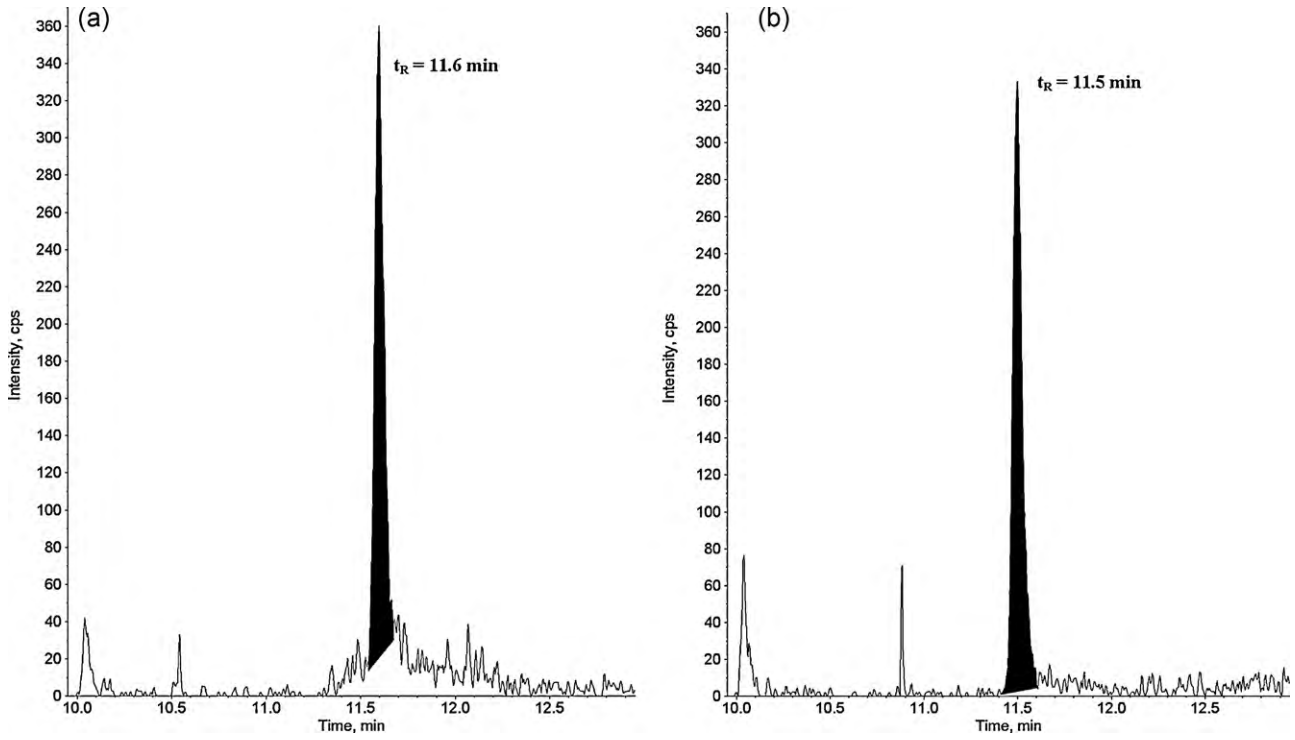

Fig. 7. Comparison of chromatograms obtained from injection (a) of a solid phase extracted blank plasma sample spiked to 7 pg/ml and injection of a (b) neat solution corresponding to a 7 pg/ml sample. The absence in peak height differences indicates low presence of ion suppressing/enhancement effects.

3.5. Assay validation

The developed analytical method was validated according to the principles stated in the FDA Guidance for Industry, Bioanalytical Method Validation [21]. Validation parameters as accuracy, precision, limit of quantification, calibration characteristics, dilution linearity, specificity/selectivity, carry-over effects, recovery and stability were investigated. Incurred sample repro-

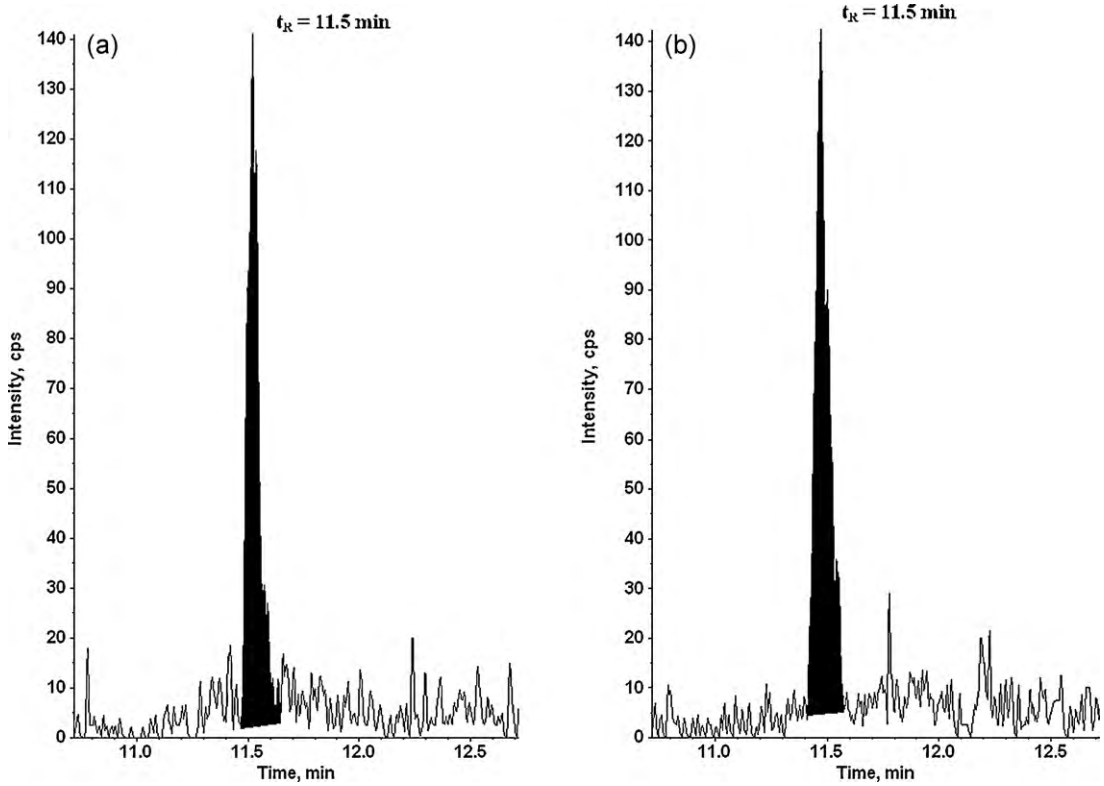

Fig. 8. Comparison of chromatograms obtained from injection (a) of a spiked plasma sample (calibration standard of 5.00 pg/ml) and (b) an authentic plasma sample, determined to 5.55 pg/ml, obtained from a clinical trial on FE 202158.

Table 2
Overview of data from intra- and inter-assay precision and accuracy assessment.

	Spiked conc. (pg/ml)			
	LLOQ 5.00	LOW 15.0	MID 71.0	HIGH 860
Occasion 1	4.12	14.1	69.6	830
	4.89	13.6	71.1	861
	4.40	14.1	70.5	833
	4.64	14.2	68.6	890
	5.00	13.0	75.8	848
	4.15	13.5	70.7	864
Occasion 2	4.57	13.3	75.5	870
	3.92	14.8	74.3	907
	5.05	14.0	67.9	828
	5.21	14.1	73.2	868
	4.92	15.7	73.5	866
	5.19	15.0	74.2	888
Occasion 3	5.79	15.0	76.5	926
	4.21	15.6	75.4	924
	4.80	15.7	68.1	969
	5.04	14.2	72.8	941
	4.78	15.4	74.0	928
	5.64	15.7	76.4	894
Intra-assay precision and accuracy Occasion 1				
Mean	4.53	13.8	71.1	854
% CV	8	3	4	3
Mean bias (%)	−9	−8	0	−1
n	6	6	6	6
Intra-assay precision and accuracy Occasion 2				
Mean	4.81	14.5	73.1	871
% CV	10	6	4	3
Mean bias (%)	−4	−3	3	1
n	6	6	6	6
Intra-assay precision and accuracy Occasion 3				
Mean	5.04	15.3	73.9	930
% CV	12	4	4	3
Mean bias (%)	1	2	4	8
n	6	6	6	6
Inter-assay precision and accuracy Occasions 1–3				
Mean	4.80	14.5	72.7	885
SD	0.511	0.890	2.89	40.6
% CV	11	6	4	5
Mean bias (%)	−4	−3	2	3
n	18	18	18	18

ducibility (ISR) was investigated in samples from a clinical study.

The inter-assay precision, expressed as CV, was between 4% and 11% over the entire range, see Table 2. Corresponding intra-assay variation was less than 12% for all levels and occasions. The accuracy, expressed as bias, was in the range of −9% to +8% for all concentration levels and at all occasions.

Different curve fitting models were investigated in the assessment of calibration characteristics. Linear regression with weighting $1/conc.^2$ described the investigated concentration-response relationship in an adequate way and gave both good accuracy and precision over the entire analytical range. Back-calculated concentration was within ±2% over the calibrated range.

Assessment of dilution linearity revealed that it was possible to dilute the plasma sample at least 12 times. The obtained bias was within ±2% from nominal concentration on all three occasions.

In the specificity/selectivity investigation blank plasma from six individuals were analysed in duplicate. No interfering peaks could be observed in any of the chromatograms. For the investigation of possible matrix related modifications of the ionization process in the mass spectrometer, the same six aliquots of blank plasma were also spiked with FE 202158 to 71.0 pg/ml and were analysed in six replicates of each. The obtained bias was between 2% and 10% and the precision, expressed as CV, was between 3% and 6%, see Table 3.

The extraction recovery, including losses in the evaporation/re-dissolution step, was between 55% and 62% over the entire analytical range. The extraction recovery over the solid phase extraction column only was approximately 80%. Addition of appropriate component, known to stabilize peptides in dry state, minimise adsorption effects and/or facilitate re-dissolution, to the solution used for eluting the analyte from the solid phase extraction column would possibly have increased the recovery somewhat. However, such components may sometimes have a negative influence on the chromatographic separation and/or the mass spectrometric detection and was not tested in this work. Influence of solvent evaporation technique, vial material and solution additive on adsorption of peptides in the sample-drying step has been comprehensively investigated by Pezeshki et al. [22].

The carry-over effect was assessed by determining the peak area at the retention time for FE 202158 in a chromatogram from a blank human plasma sample injected directly after the calibration sample with the highest concentration (1010 pg/ml). The carry-over effect was assessed in three analytical runs without observing any peak in the blank plasma injections.

The short-term stability of FE 202158 in human plasma at room temperature was monitored during 5 h. No instability could be observed. Freeze and thaw stability assessment showed that the human plasma samples were stable for at least three cycles. Long-term freezer stability assessment indicated stability for at least 9 months.

Incurred sample reproducibility (ISR) [23,24,25] assessment of samples from a clinical trial on FE 202158 showed that 90% of the reanalysed samples were within the acceptance range of ±20% from the originally reported value. Chromatograms obtained from analysis of plasma samples originated from dosed subjects were

Table 3
An overview of the results from injection of six different individual plasma batches spiked with FE 202158 to 71.0 pg/ml for the investigation of possible matrix related modifications of the ionization process in the mass spectrometer. Each individual plasma batch was analysed in six replicates.

	Individual 1	Individual 2	Individual 3	Individual 4	Individual 5	Individual 6
Observed concentration (pg/ml)	77.4	72.3	73.6	70.7	73.4	76.2
	77.5	74.1	75.3	75.7	78.9	76.7
	74.3	71.9	67.7	73.0	71.9	71.3
	78.3	71.3	66.9	73.8	83.4	78.0
	80.2	76.4	72.5	73.2	77.4	72.0
	78.9	72.7	78.3	71.0	81.2	72.0
Mean	77.8	73.1	72.4	72.9	77.7	74.4
SD	1.99	1.86	4.40	1.85	4.44	2.92
% CV	3	3	6	3	6	4
Mean bias (%)	10	3	2	3	9	5
n	6	6	6	6	6	6

virtually identical with those from spiked plasma samples, as seen in Fig. 8.

3.6. Ruggedness of assay in routine analysis of clinical samples

The validated bioanalytical method was used for analysis of 730 plasma samples from a clinical trial on FE 202158, divided in 24 runs, including the ISR assessment. Four runs were rejected, which makes a rejection rate of 16.7%. The reasons to the rejected runs were in one occasion failure in meeting QC acceptance criteria, in one occasion due to failure in calibration curve preparation explained by malfunction of a Tecan robotic sample processor and in two occasions due to failure in meeting carry-over acceptance criteria. The likely explanation behind the latter two occasions was precipitation or dirt from the samples in the autosampler injection valve. The carry-over problem was solved by rinsing the valve.

The assay is technically more complex than an ordinary single-column LC–MS/MS based assay and requires longer training sessions before a new operator can start using the system but the assay seems not to require longer time for trouble-shooting and re-runs. This can probably be explained by the fact that the system purifies the plasma sample very efficiently and reduces problems associated with biological samples to a minimum, e.g. removal of matrix effects which otherwise often give rise to failure in meeting run acceptance criteria.

4. Conclusions

The presented coupled column liquid chromatography–tandem mass spectrometric system permits quantification of a peptide drug candidate down to 5.00 pg/ml levels in plasma samples. The method passed all acceptance criteria defined in relevant guidance documents for method validations. Although the system is more complex than an ordinary single-column system, the robustness of the system was satisfactory for using it in routine analysis of samples from clinical studies without any need for extensive trouble-shooting.

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