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Utilizing ELISA-plate based immunopurification and liquid chromatography-tandem mass spectrometry for the urinary detection of short- and long acting human insulin analogues

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ABSTRACT

The measurement of human insulin and its synthetic analogues in biological matrices has become increasingly important not only in clinical fields but also in doping control. The use of insulin and its analogues have been included in the list of prohibited substances published by the World Anti-Doping Agency (WADA). This study describes a qualitative method for detection of insulin analogues (lispro, aspart, glulisine, glargine, degludec, detemir) in human urine. The sample preparation consists of a preconcentration step using ultrafiltration followed by an immunoaffinity extraction with an antibody precoated ELISA plate. The obtained extracts are analyzed by conventional high-performance liquid chromatography–electrospray tandem mass spectrometry (LC-ESI–MS/MS). The limits of detection range between 10 pg/ml and 150 pg/ml. The applicability of the method was proven by the analysis of real urine samples obtained from diabetic patients treated with synthetic insulin analogues.

1. Introduction

Insulin is a double-chain peptide with a molecular weight of ~6 kDa. Apart from the therapeutic importance of its artificially designed analogues (Lispro, Gluisine, Aspart, Detemir, Glargine, Degludec), their potential performance enhancement effects for athletes have also been discussed [1]. Therefore all types of insulins are included in the list of prohibited substances by the World Anti-Doping Agency (WADA) [2].

Several mass spectrometry based assays have been reported for the determination of insulins in urine [3–7] and serum/plasma matrices [7–16]. Because of their low urinary and plasma concentrations (<1 ng/ml) a variety of sample preparation techniques have been described to reach these low concentration-levels being immunoaffinity purification (IAP) using IAP-columns [3–5,17], IAP using magnetic beads [6,9,11,12,18] or solid phase extraction (SPE) [10,14,19,20]. Prior to IAP, a sample volume reduction step is often included and can either consist of SPE or [3–6] or molecular weight (MW) based centrifugal filtration [7,11].

The aim of this work is to expand the possibilities to isolate and sensitively measure synthetic insulins in urine. Human insulin selective ELISA assays are also commercially available and used for clinical applications [21,22], however to the best of our knowledge their use in combination with mass spectrometry for synthetic urinary insulin determination has not been described yet.

The present study introduces a new strategy which consists of sample preparation using ELISA plate based IAP in combination with MW based centrifugal filtration and subsequent mass spectrometric detection.

2. Experimental

2.1. Chemicals and materials

Insulin Lispro was purchased from Eli Lilly (Indianapolis, USA), Insulin Aspart, Insulin Glulisine, Insulin Glargine, Insulin Detemir, Insulin Degludec, Insulin Bovine, Bovine Serum Albumin (BSA) and Betamethasone were purchased from Sigma Aldrich (Bornem, Belgium). Insulin Glargine Metabolite was a kind gift of the Cologne anti-doping laboratory. Mercodia isoInsulin kit, including Mercodia wash buffer was purchased from Bio-Connect Diagnostics (Huissen,Nederland), Amicon Ultra 3 kDa 0.5/15 ml centrifugal filters were purchased from Merck (Darmstadt, Germany). LC–MS grade acetonitrile (ACN) and water were obtained from J.T. Baker (Deventer, Netherlands), formic acid (HCOOH) from Fisher Chemical (Madrid, Spain). Stock and working solutions were prepared

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and stored in polypropylene microcentrifugal vials, type Eppendorf (Eppendorf, Hamburg, Germany). For performing the dilution steps polypropylene pipette tips (Eppendorf, Germany) were used.

2.2. Instrumentation

The HPLC system consisted of a Dionex Ultimate 3000 LC (Thermo Scientific, Bremen, Germany) equipped with a degasser, Dionex Ultimate 3000 UPLC pump, an autosampler thermostated at 15 °C and a thermostated (40 °C) column compartment. A Waters UPLC Peptide BEH C18 (2.1*50 mm) 1.7 μm particle size column and a Waters Acquity Protein BEH C18 (2.1*5 mm) precolumn were used for LC separation. Mobile phases were A: H_2O (containing 0.2 v/v% formic acid) and B: acetonitrile (containing 0.2 v/v% formic acid). For the separation the following gradient elution was used: 1% B hold for 0.5 min and increased to 15% in 1 min, then further increased to 35% in 4 min and held there for 1.5 min, increased to 45% in 8.5 min, increased to 95% in 8.6 min and held there for 0.5 min, decreased to 1% B in 9.2 min and equilibrated for 2.9 min, giving a total runtime of 12.1 min. The flow rate was 0.3 ml/min.

The LC-system was coupled with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Scientific, Bremen, Germany). The mass spectrometer was equipped with an electrospray ionization source. The parameters of the ion source were the following: spray voltage 3.5 kV, capillary temperature 350 °C; sheath gas 50, auxiliary gas 50, collision gas pressure 3mTorr. The different collision energies and S-lens settings are summarized in Table 1.

2.3. Preparation of solutions used for sample preparation and validation

All peptide stock standard solutions were prepared at 0.1 mg/ml using a mix of A-water, B-acetonitrile, C-formic acid (a:b:c,60/40/0.02 v/v%) and were stored at −20 °C. Working solutions (100 and 50 ng/ml) were prepared using the same mixture and also contained BSA at a concentration of 5 μg/ml. Bovine insulin was used as internal standard. A solution was prepared at the concentration of 1 μg/ml using the mix of A-water, B-acetonitrile, C-formic acid (a:b:c, 60/40/0.02 v/v%) and was stored at −20 °C. A mixture of A-water, B-acetonitrile, C-formic acid (a:b:c,65/35/0.02 v/v%) containing 5 μg/ml BSA and 1 μg/ml Betamethasone was prepared as elution buffer. Betamethasone was included in the elution buffer to control the precision of the injection volumes of the liquid chromatographic autosampler.

2.4. Administration study

As a proof of concept, urine samples from seven diabetes patients who were treated with different forms of insulin medication were obtained from the Department of Internal Medicine (Endocrinology) and Hematology of the Ghent University Hospital, with the approval of the Ethical Committee of the Ghent University (reference: 2010/314). Samples were stored at −80 °C awaiting analysis. Two excretion urine samples that were distributed among doping control laboratories by WADA were also analyzed.

2.5. Sample preparation

To 15 ml urine 2 μl of ISTD Bovine insulin solution was added. The pH of the sample was adjusted to ~7 by adding 1 ml of 3.75 M Tris/HCl buffer. The sample was vortexed and centrifuged for 20 min at 4000 rcf. The supernatant was then transferred in an Amicon Ultra 15 filter and centrifuged at 4000 rcf for 90 min. The retentate was transferred in an Amicon Ultra 0.5 filter and centrifuged for 40 min at 10000 rcf. Finally the retentate (~50 μl) was transferred into an ELISA well. The ELISA-plate was incubated for 1.5 h at room temperature with gentle shaking. The wells were washed 5 times with the diluted Mercodia wash buffer solution (prepared according to the specification of the manufacturer) after incubation. By the addition of 60 μl of elution buffer the analytes were eluted and transferred to a polypropylene tube for LC–MS analysis.

2.6. Validation

Validation was carried out according to Eurachem Guidelines [23]. Ten different blank urine samples were collected for the validation of the method in a representative way covering a pH range of 5.1–6.9 and density of 1.011–1.0255 g/cm^3.

Each urine sample was spiked at 0.15, 0.075, 0.05, 0.025, 0.01 ng/ml to determine the LOD of the method, which was considered the lowest concentration at which the targeted analyte could be detected in all ten urine samples using two diagnostic ion transitions with a signal-to-noise ratio greater than 3 and a retention time difference of less than 0.2 min to the reference. To obtain concentrations of 0.15, 0.075, 0.05, 0.025 ng/ml, 15 ml of blank urine was spiked from the 100 ng/ml working solution by the addition of 22.5, 11.25, 7.5, 3.75 μl respectively, to obtain peptide concentrations of 0.01 ng/ml 15 ml of blank urine was spiked from the 50 ng/ml working solution by adding 3 μl.

Retention time stability was assessed by comparing the retention time of the target analytes after extraction and without extraction in neat standard solutions.

Table 1
Molecular formulas, monitored precursor and product ions, and mass spectrometric parameters (collision energies, and S-lens settings) of the investigated insulin analogues.

	Molecular Formula	Molecular weight (Da)	Precursor Ion	Product Ion (Collision E(eV), S-Lens)
Lispro	$C_{257}H_{383}N_{65}O_{77}S_6$	5807	1162,6	216,8 (36;150)
			968,9	216,8 (34;150)
Aspart	$C_{256}H_{381}N_{65}O_{79}S_6$	5826	1165,5	135,9 (36;150)
			972,2	135,9 (39;150)
Glulisine	$C_{258}H_{384}N_{64}O_{78}S_6$	5823	1165,88	328(39;150)346(39;150)
Glargine	$C_{267}H_{404}N_{72}O_{78}S_6$	6063	867,2	135,7(33;150)
Glargine Metabolite	$C_{255}H_{380}N_{64}O_{76}S_6$	5750	959,5	225,8(35;150)135,9(40;150)
			1151,4	225,8(44;150)
Detemir	$C_{267}H_{402}N_{64}O_{76}S_6$	5917	1184,7	357(41;180)454,3(35;150)
Degludec	$C_{274}H_{411}N_{65}O_{81}S_6$	6104	1222,12	310(39;150)641,6(42;150)
Human	$C_{257}H_{383}N_{65}O_{77}S_6$	5807	968,9	226 (36;150)
			1162,6	226 (44;150)
Bovine	$C_{254}H_{377}N_{65}O_{75}S_6$	5733	956,8	135,8 (30;150)
			956,8	226 (35;150)

Table 2
LODs and recoveries of the validated method.

Insulins	Lispro	Aspart	Glulisine	Glargine	Glargine Metabolite	Detemir	Degludec
LOD (pg/ml)	*<10*	*50*	*50*	*75*	*50*	*50*	*150*
Recovery (%)	*28*	*22*	*21*	*20*	*23*	*10*	*11*

Selectivity was tested during the validation procedure to probe for interference with the MSMS transitions of the product ions at the expected retention times of the different insulin analogues. For this reason, during the validation a mixture prepared from other doping classes (in total 327 substances) was processed. Also all 10 blank urines spiked only with the internal standard were checked. To check if transitions of the insulin analogues interfere with each other, standard solutions of each analogue (50 ng/ml) were injected.

Recoveries were estimated by comparing the peak area of the target compounds in 10 urines spiked before the extraction of the samples with the peak area of samples spiked after the extraction. These samples were spiked at the concentration of 50 pg/ml.

Carry-over was assessed by injecting blank samples (solvent used for elution) after each urine sample during the validation process.

3. Results and discussion

3.1. Method development

3.1.1. Sample preparation

Because of their low urinary concentrations (<1 ng/ml) insulins are often extracted starting from a large volume of urine. Generally between 25 ml [3,4] and 5 ml [5,6,18] of urine is reduced to ~1 ml to facilitate incubation with antibodies. To reduce these volumes, SPE is often applied [3–6]. Because in our approach 15 ml of urine was concentrated, SPE often resulted in blocked cartridges and low recoveries (<50%/data not presented). Another useful approach to preconcentrate peptides is the employment of MW filtration (MWF). This technique has already been found successful for the analysis of erythropoietins (EPO) [24] and for the preconcentration of insulins from blood [7,11] and urine matrices [7]. Comparing the SPE with the MWF approach showed that the latter was more robust regarding recovery and blockage, despite the fact that the low MW cut-off (3 kDa) filters made the centrifugation time relatively long (~2 h). An additional benefit of using the MWF is that after preconcentration the retentate (approximately 50 μl) can be directly loaded into the wells of the ELISA-plates whereas an SPE step requires an additional evaporation and redissolution step.

Antibody-extraction of insulins can be performed in different ways. Although better recoveries have been achieved by using immuno affinity chromatography (IAC) (~80% for the short acting analogues) [4], the batch mode use of magnetic beads coated with antibodies (~30% for the short acting analogues) has been favored due to its simple protocol. [6,9,11,12,18] Next to the above described techniques, immunoassays where antibodies are immobilized in wells of a plate are also commercially available. This approach has already been successfully applied in the field of doping control for the purification of erythropoietin from urine [25]. The use of plates with human insulin-specific antibodies is more widespread in clinical applications to investigate hypoglycemia and their limitations and ability to detect synthetic insulin analogues have been evaluated in previous studies [21,22]. These results show high variations and reveal that many of these assays fail to detect the analogues. In this study Mercodia isoInsulin kit was used and in agreement with earlier results showed sufficient cross reactivity to all the investigated insulin analogues [22]. Therefore it is an appropriate alternative of the magnetic bead approach. Reproducible results with acceptable recovery were achieved (Table 2), allow-

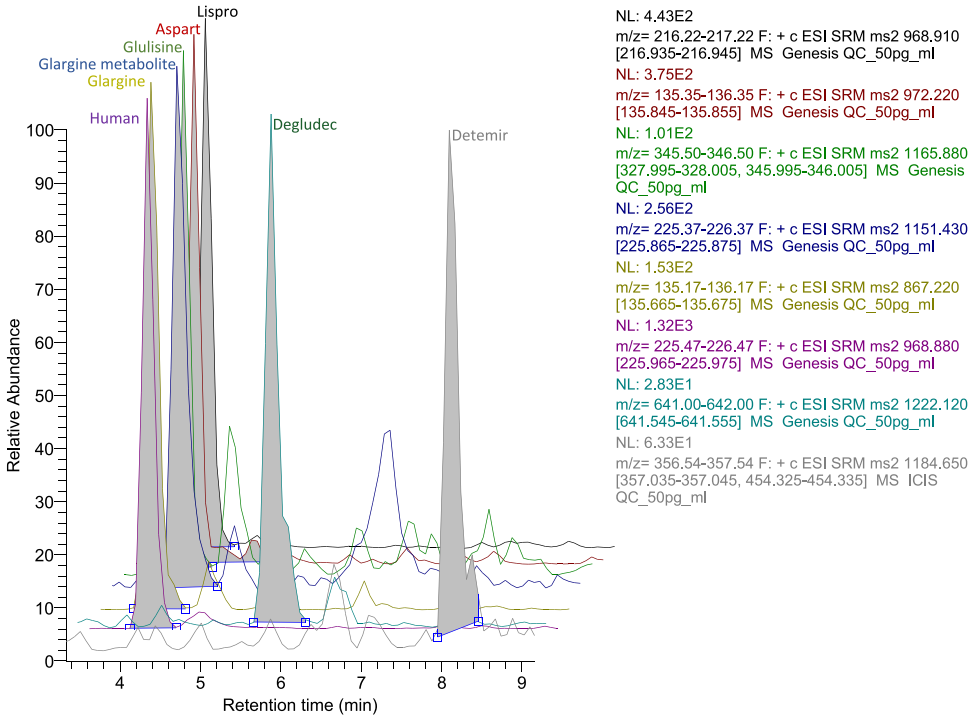

Fig. 1. Chromatographic separation of Human insulin and its analogues.

Table 3
The collected excretion samples with indication of the administered insulin analogue, dose, elapsed time between administration and sample collection and the results of the analysis. (*the detection of Glargine was covered by the metabolite of Glargine).

Sample	Administered Analogue	Dose	ΔT (hours)	Detection
WADA 1	Lispro	12IU/0,42mg-	–	√
WADA 2	Lispro	12IU/0,42mg-	–	√
Patient 1	Aspart	6 IU/0,21 mg	4	√
Patient 2	Glulisine	12,4 IU/0,44 mg	3	√
Patient 3	Glulisine	16 IU/0,56 mg	3	√
	Glargine*	–	–	√
Patient 4	Aspart	3IU/0,11 mg	12	√
Patient 5	Glulisine	Continous pump	–	√
Patient 6	Aspart	10 IU/0,35 mg	4	√
	Glargine*	12IU/0,42 mg	12	√
Patient 7	Aspart	10 IU/0,35 mg	3	√
	Glargine*	12IU/0,42 mg	10	√

ing to reach the required detection limits (see validation section) in all analyzed samples.

One of the key aspects of the method development phase was the effort that has been made to avoid sample losses during sample preparation due to the adsorptive nature of the peptide molecules described by numerous researchers [11,20,26–30]. Several acidic elution buffers were tested with increasing percentage of organic solvents and with the inclusion of BSA as a carrier protein. Changing the character of the elution buffer prompted the analytes to reside preferentially in the buffer and not to stick to the walls of HPLC vials, resulting in significantly increased recoveries. Eventually the mixture of A-water, B-acetonitrile, C-formic acid (a:b:c, 65/35/0.02 v/v%), containing 5 μg/ml BSA was found to be optimal.

3.1.2. Liquid chromatography-Mass spectrometry

Besides the great differences in sample preparation (see Introduction), the mass spectrometric platforms used by several research groups differ as well. Detection can be accomplished using triple quadrupole [3–5,10,11,14,17,19,20], high resolution mass spectrometers [6,7,12,13] coupled to conventional LC [3,4,13,17,19] or nano-LC instruments [5–7,9,11,12,18]. Applications using the nano chromatographic systems enabled to reach the lowest detection limits in urinary matrix [6,7], however there are still challenges with their use, including increased costs, complicated troubleshooting and longer chromatographic runs. [31] Therefore in this work conventional LC–MS/MS was preferred for detection.

The applied gradient allowed chromatographic separation of insulin Detemir and Degludec from the other analytes due to their more apolar character, whereas Aspart, Glulisine, Lispro, Human insulin, Glargine and its metabolite showed very similar chromatographic behavior (Fig. 1). As reported earlier the analytical column had to be conditioned before the first use to deactivate remaining silanol groups, which can inhibit the recoveries of the insulins. [32]

In addition to the chromatographic retention times the differentiation of the analogues was accomplished based on their specific MS/MS spectra. The selected ion transitions were very similar to those of previous studies, however in most cases the differentiation of the analogues was not only based on one precursor-product ion combination. For example, to distinguish human insulin from insulin Lispro the diagnostic m/z 226 and m/z 217 ions were not only obtained by the fragmentation of the five-fold protonated precursor ion as in earlier studies [3–6,9,11,12,18], but also by fragmentation of the six-fold protonated precursor ion.

3.1.3. Method validation

The validated method, presented in this paper shows good sensitivity and is capable of detecting 5 out 7 of the insulins using two precursor-daughter ion transitions at the current minimum required performance limit (MPRL) of 50 pg/ml. Table 2 summarizes the results of the reached detection limits. These LODs were in the same order of magnitude of previous studies where 25 ml of urine was extracted and the short- and long acting analogues were not incorporated in the same method [3,4]. As already mentioned in the previous paragraph, lower LODs have been achieved using nano-liquid chromatography coupled to nano-scale mass spectrometry [6,7]. The current method allows the use of a more affordable and robust instrumentation and incorporates both the rapid- and the long acting insulin analogues in a single method. Only one precursor-product ion transition of insulin Degludec (932-641) could be measured at the required level of 50 pg/ml. This can be explained by the absence of a second ion transition that can be measured sensitively and by the low recovery of this compound which was also reported by Thomas et al. [7]. Probably, the more complex structure of this analogue; the presence of fatty acid groups, prevent efficient binding to the antibodies. This limitation does not hinder the screening of this analogue, however for confirmation purposes the search for possible metabolites is needed to obtain more identification points. The measurement of only one ion transition (867-135) of insulin Glargine resulted in a detection limit below 50 pg/ml. However, targeting one of its earlier characterized metabolites [3] provided ion transitions that could be sensitively measured and cover for the detection of insulin Glargine misuse.

Assessing the selectivity of the method showed no interfering peaks at the expected retention times of the analytes. However, it has to be noted that a small interference with the ion transition of insulin Aspart (~5%) was observed when insulin Glulisine at concentrations higher than 50 ng/ml was injected. It was due to the fact that the fragmented precursor ions of Aspart and Glulisine have masses with a difference smaller than ~0.5 Da and the low resolution mass spectrometer with the used settings only allowed unit resolution. Nevertheless, for confirmation purposes the analysis of more selective ions or the reduced forms (B-chain) are recommended [4,18].

The results of the recovery test are listed in Table 2. Analyte recoveries concerning the centrifugal filtering followed by the immunoaffinity purification varied between 10 and 28%. These values were lower than the ones obtained by other researchers varying between (16–109%) [3–7,18], however were still satisfactory to reach the required detection limits.

No carryover was present in the blank samples injected after the urine samples. Evaluating the retention time criteria showed that all analogues met the set requirement.

3.1.4. Results of the excretion study

The proof of concept was shown by applying the method to urine samples obtained from diabetic patients who were treated with insulin Lispro, Glulisine, Aspart and Glargine analogues. Table 3 summarizes the type and dose (IU stands for international unit) of the administered insulin, the elapsed time between injection and

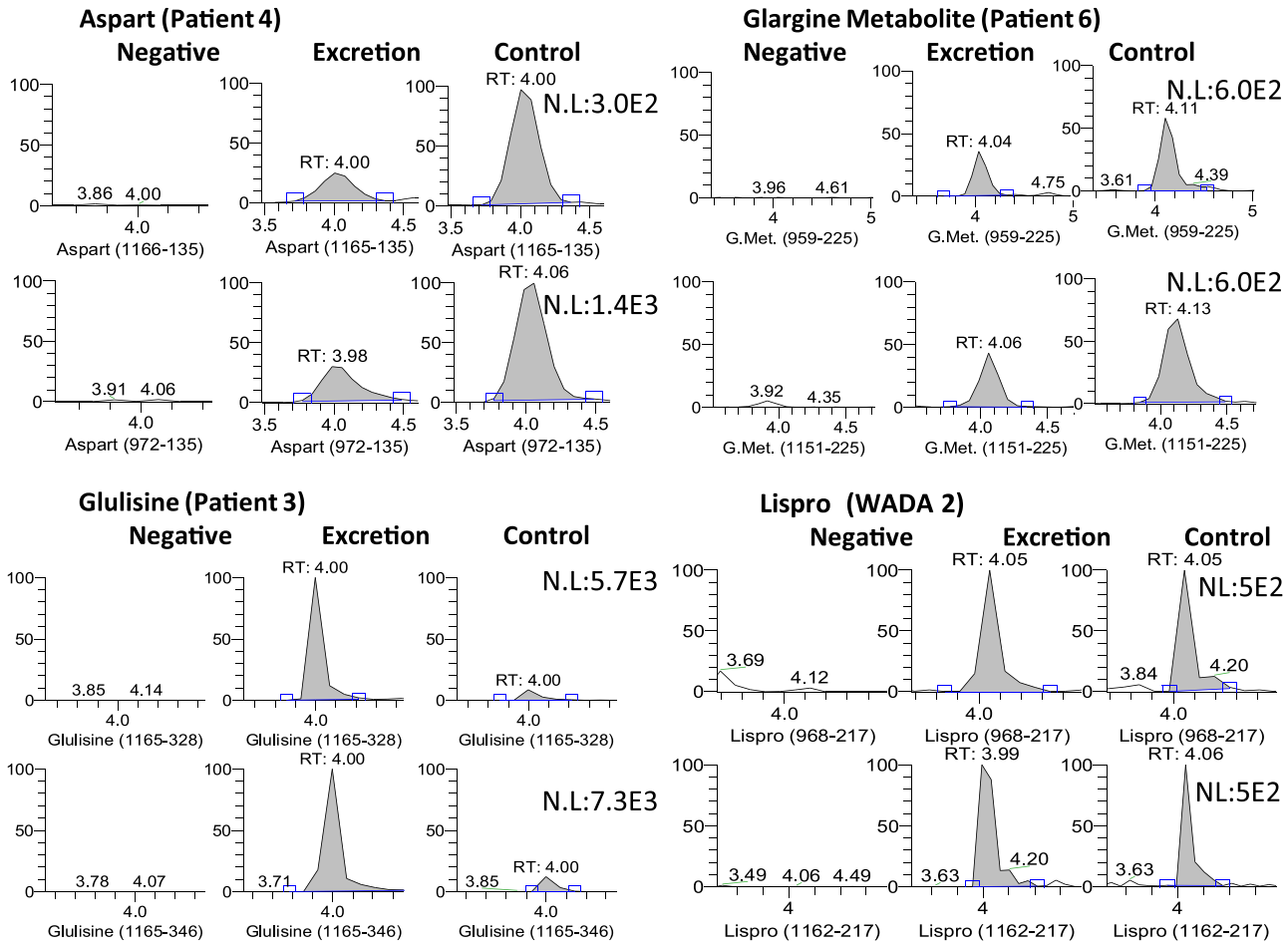

Fig. 2. Examples of chromatograms of the analyzed excretion samples, showing the presence of insulin Aspart, Glargine, Glulisine and Lispro in the excretion and spiked control samples (spiked at 50 pg/ml).

sample collection and the outcome of measurements. The developed approach enabled us to detect all analogues in the collected samples. Insulin Lispro could also be detected in the external quality assessment samples (EQAS) sent by WADA to several doping laboratories to monitor the capacities to correctly identify and report samples containing insulin Lispro. Later, the estimated concentration of Lispro in the samples (50 and 30 pg/ml) has also been revealed by WADA, which was a good indicator of the required sensitivity and was met by our developed strategy.

In accordance with earlier reported studies the long-acting analogue Glargine in its intact form could not be detected [3]. In Fig. 2 examples of detected analogues are presented. For each analogue at least two SRM transitions were used to provide unequivocal proof for the presence of a particular insulin.

4. Conclusion

A simple, qualitative assay has been successfully developed and validated for the determination of insulin analogues. Employing the combination of MW filtration and IAP on an ELISA plate resulted in very clean extracts and enabled the detection of the insulin analogues in all of the collected excretion samples. The main benefits of this strategy lie in the reproducibility of the sample preparation step and in the use of conventional chromatographic and mass spectrometric systems offering a promising tool to the arsenal of available insulin assays and easy method transfer to other laboratories.

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