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Quantitative analysis of urinary C-peptide by liquid chromatography–tandem mass spectrometry with a stable isotopically labelled internal standard

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Abstract

We describe the first results of a quantitative LC–tandem mass spectrometry method for urinary C-peptide with the use of [$^2H_{14}$]C-peptide as internal standard. LC was based on gradient elution of a Hypersil PEP C_{18} column. Mass spectrometry was performed in the negative electrospray ionization mode and by monitoring of the transitions at m/z 1514/1334 ([$^2H_{14}$]C-peptide) and 1507/1320 (C-peptide). For sample preparation, we applied ultrafiltration. The analytical performance of the method in terms of measurement precision gave an RSD of <2% (n=10). The overall imprecision was investigated from independent analysis of two urine samples in six-fold and resulted in an RSD<5%. The limit of detection, expressed as signal-to-noise ratio 3, was ~0.15 ng C-peptide injected. Analysis of 10 random urine samples from laboratory volunteers showed interference-free ion chromatograms at a signal-to-noise ratio of ~75 on average. The C-peptide concentrations calculated from quantification by the bracketing calibration technique ranged from 32 to 165 ng/ml.

Keywords: C-Peptide; Peptides; Insulin

1. Introduction

C-Peptide is the connecting peptide that joins the A- and B-chains of insulin in the proinsulin molecule. It is a polypeptide (31 amino acids) with a molecular mass (M_r) of 3017 and is considered biologically inert. Most C-peptide is extracted via the kidneys into the urine where it remains stable and can be quantified. Urinary C-peptide has a concentration in the range of 40–150 ng/ml. For the routine analysis of C-peptide, a variety of commercial immunoassays are available. However, because C-peptide is relatively small and its antigenicity is low, development of immunoassays for C-peptide is rather challenging [1]. Thus, the establishment of a reference method that allows evaluation and standardisation of these assays would be useful [2].

Recent advances in liquid chromatography–mass spectrometry (LC–MS) techniques made LC–MS

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enormously useful in many aspects of polypeptide and protein analysis (e.g., determination of molecular mass; kinetic studies; sequence analysis). Thus, LC–MS seemed the preferable technique for a reference method for C-peptide. However, reports on the application of LC–MS for quantitative analysis of diagnostically important polypeptides/proteins in human body fluids are scarce [3–8]. Quantitation strategies apply selective enzymatic digestion and measurement of unique polypeptide fragments [3,4] as well as measurements of the intact proteins [5–8]. To the best of our knowledge, only two groups reported methods that used isotope dilution (ID) [3,7,8]. One of them described an off-line ID–LC–MS assay for serum C-peptide [7]. The other described an ID–LC–MS method, after enzymatic digestion, for apolipoprotein A-1 [3]. Our interest in the LC–MS analysis of C-peptide came from the wish to expand our reference method panel for analytes that are important for the management of diabetes, among them glucose [9,10] and glycohaemoglobin [11,12]. We started our method development for urinary C-peptide some years ago [13], however, we postponed further development until we could obtain stable isotopically labelled C-peptide [7].

Here, we report on first results of a quantitative method for urinary C-peptide that uses [$^2H_{14}$]C-peptide as internal standard, ultrafiltration (UF) for sample preparation, and LC–tandem mass spectrometry (MS–MS) for detection.

2. Experimental

2.1. Materials

Proinsulin C-peptide fragment 33–63 (M_r 3017) was obtained from ICN Biomedicals (Costa Mesa, CA, USA). Isotopically labelled [$^2H_{14}$]C-peptide (label: [$^2H_2$-Gly]$_7$) was obtained from the University Medical Centre of Geneva [7]. Stock standard solutions (concentration 250 ng/μl) were made by dissolving non-labelled or labelled C-peptide in a 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) solution. These solutions were further diluted in 0.1% BSA to working standards containing 1 ng/μl.

UF was done with Centrex-UF2 devices with a nominal molecular mass cut-off value of 3000 and a membrane of regenerated cellulose (Schleicher & Schuell, Dassel, Germany).

All chemicals were super-quality grade and purchased from Romil (Cambridge, UK).

2.2. Instrumentation

The LC–MS–MS instrument used was a VG Quattro II double stage MS from Micromass (Altrincham, UK). It was coupled to a HPLC system Model 325 from Kontron Instruments (Milan, Italy), equipped with an autosampler 465. LC was performed on a Hypersil PEP C_{18} column (150×2.1 mm, 5 μm bead size, 300 Å pore size) from Alltech (Deerfield, IL, USA).

UF was done with a Biofuge primo R from Heraeus Instruments (Hanau, Germany).

2.3. Sample pretreatment

All plastic and glassware that came into contact with C-peptide was washed with a 1% BSA solution before use. Urine (1 ml) was spiked with [$^2H_{14}$]C-peptide and fortified with BSA to reach a final concentration of 0.04%. Then, UF was performed for 45 min at 7000 *g* to obtain ~100 μl retentate. Afterwards, 1 ml water was added, mixed, and UF was repeated to obtain a final retentate volume of ~200 μl (~25 min).

2.4. LC–MS–MS conditions

From the final retentate, 30 μl was directly injected into the LC system. Gradient chromatography (cycle time 10 min) was performed with water–acetonitrile–*n*-propanol–formic acid–trifluoroacetic acid (TFA) as mobile phase (eluent A: 80:20:1:0.06:0.01, v/v; eluent B: 60:40:1:0.06:0.01, v/v) at a flow-rate of 170 μl/min. The programme started with 100% eluent A, then eluent B was increased from 0 to 100% within 4 min and held constant during the next 2 min. The system was returned to eluent A during the next 0.1 min, to re-equilibrate for 3.9 min until the next injection. Under these chromatographic conditions, C-peptide eluted after ~8.6 min.

LC–MS measurements were performed in the negative electrospray tandem MS mode, i.e., monitoring the transitions at m/z 1514/1334 ($[^2H_{14}]$C-peptide) and 1507/1320 (C-peptide). Dwell times were 1.5 s. The MS settings were: collision gas argon at $3 \cdot 10^{-3}$ mbar, collision energy 40 V, cone 40 V, capillary 4.0 kV, and source temperature 175°C.

2.5. Analytical performance

Pure measurement imprecision ($n=10$) was investigated for standards (1 ng/μl, 5 μl injected) spiked with $[^2H_{14}]$C-peptide as internal standard (MS ratios ~1). Overall method imprecision was determined from quadruplicate analysis of two urine samples (containing ~40 and ~110 ng C-peptide per ml), i.e., of each sample four aliquots were weighed in, spiked with internal standard (to obtain a MS ratio ~1), processed by UF and analysed.

The recovery of the UF procedure was determined with the same two samples. For this experiment, the internal standard was added before and after UF.

The limit of detection (LOD) defined as signal-to-noise (S/N) ratio 3 was determined by dilution of a processed urine sample. Finally, the method was used for quantification of 10 random urine samples from laboratory volunteers (male and female, age 23–58 years).

3. Results and discussion

We opted for UF as preparation technique because it allows convenient sample purification (with respect to substances with low M_r) and concentration (substances with high M_r). For sufficient UF recovery, cut-off values of at least one-half or one-third the M_r of the analyte are recommended. As the relative M_r of C-peptide is ~3000, UF devices with M_r 1000 nominal cut-off would be the best choice. However, in our experience these devices were impractical because they required extremely long filtration times (>2 h for 1 ml). Thus, we investigated devices with higher cut-off values. With the Centrex-UF2 devices (nominal cut-off M_r 3000), UF of 1 ml urine was finished within 45 min at 7000 *g*. Including the washing step, filtration took about 70 min, which we considered acceptable for our application. It resulted in concentration of the urine sample by a factor of ~5 and purification by a factor of ~10. The recovery of the UF process with the Centrex-UF2 devices was 60% on average. For LC–MS analysis, we injected 30 μl of the retentate onto the LC column. For a urine sample in the normal range (~50 ng/ml) and processed as described, this would correspond with ~4.5 ng C-peptide injected.

The pure measurement imprecision, expressed as relative standard deviation (RSD) was <2% ($n=10$) (5 ng C-peptide injected, ratio C-peptide/internal standard ~1) (see also Table 1).

The overall imprecision from independent analysis of two urine samples in six-fold was, respectively ~3–5% (MS ratios ~1) (see also Table 1).

The LOD expressed as S/N ratio 3 was calculated to be ~0.15 ng C-peptide injected. On quantification of urine samples from apparently healthy laboratory personnel, we obtained in average a S/N ratio of ~75 (see, e.g., Fig. 1 below), proving that the aforementioned LOD was sufficient for our purpose.

From the interference-free chromatograms obtained for all urine samples (in Fig. 1, the ion chromatograms obtained from analysis of sample 4 are shown), it is obvious that the method using negative electrospray LC in combination with tandem MS detection of the transitions at m/z 1507 $[M-2H]^{2-} \rightarrow 1320$ (C-peptide) and 1514→1334 ($[^2H_{14}]$C-peptide) allows specific analysis of C-peptide.

Last but not least, we proved the usefulness of the developed method by duplicate analysis of 10 random urine samples from laboratory volunteers. The concentrations found ranged from 32 to 165 ng/ml (see Table 2). Until now, analysis was done by

Table 1
Intra-day imprecision for standards and two representative urine samples

Sample[a]	RSD (%)
Standard (1 ng/μl)	1.6[b]
Urine A (~40 ng/ml)	4.3[c]
Urine B (~110 ng/ml)	3.6[c]

[a] The amount of $[^2H_{14}]$C-peptide added to all samples was adjusted to obtain a MS ratio of ~1.

[b] Pure measurement imprecision ($n=10$).

[c] Imprecision including measurement and independent sample preparation ($n=6$).

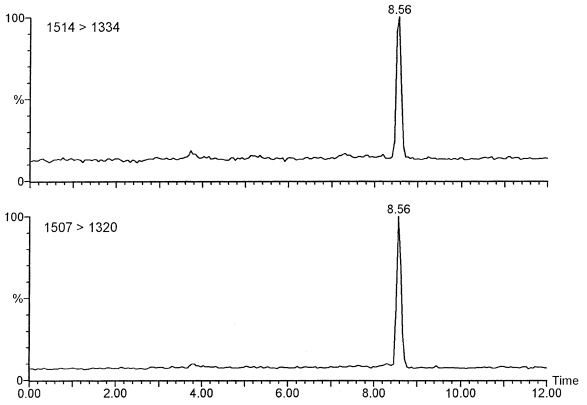

Fig. 1. Representative ion chromatograms of urine sample 4 (containing ~50 ng/ml C-peptide) processed according to the conditions described in the text. Time scale in min.

sampling a constant volume of urine (1 ml) and adding the same amount of internal standard to each sample aliquot, which resulted in MS ratios ranging from 0.3 to 2.5. For quantification, standard mixtures containing C-peptide and internal standard in ratios of 0.25, 1, 2 and 3 were used and the unknown concentrations were calculated by bracketing. However, for future work, we will investigate whether the bracketing technique, a full calibration curve or single-point calibration at the 1:1 ratio is the most appropriate calibration method for our purposes. Note that for the latter calibration technique the sample volume would need to be adapted, dependent on the concentration of the analysed urine sample.

Table 2
C-Peptide concentrations found in random urine samples from 10 different persons

Sample	C-Peptide (ng/ml)[a]
1	32
2	35
3	48
4	50
5	70
6	73
7	83
8	89
9	162
10	165

[a] Each result is the mean of a duplicate analysis.

Note that in the meantime, we found a commercial source of labelled C-peptide, namely [$^2H_{16}$]C-peptide (label: [$^2H_8$-Val]$_2$) from Bachem (Bubendorf, Switzerland) at a costprice only twice the one for C-peptide itself. This indicates that quantitative analysis of peptides by isotope dilution is a realistic analytical option in the future.

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