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Application of a C-peptide electrospray ionization-isotope dilution–liquid chromatography–tandem mass spectrometry measurement procedure for the evaluation of five C-peptide immunoassays for urine

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Abstract

This study applied electrospray ionization-isotope dilution–liquid chromatography–tandem mass spectrometry for the evaluation of five urinary C-peptide immunoassays via split-sample measurements. The immunoassays measured in duplicate in the same run, the comparison method in triplicate over different runs. From the data, the within-run imprecision and the method comparison total RSDs were calculated. Regression analysis revealed on the one hand systematic differences, on the other, an excellent correlation between the test and comparison methods. From the spread of the data around the regression line in comparison with the 95% prediction intervals from the total RSD, sample-related effects and/or specificity problems were apparent and investigated.

Keywords: Immunoassays; Split sample measurements; C-Peptides

1. Introduction

C-Peptide is a by-product in the synthesis of insulin by the pancreatic beta cells [1,2]. It consists of 31 amino acids, has a molecular mass of 3020 and is the connecting peptide in proinsulin. From this position in the proinsulin molecule, it is thought to play an important role in the synthesis of insulin by ensuring the correct pairing of the cysteine residues during the formation of the interchain disulfide bonds [1]. While C-peptide is produced in equimolar concentrations with insulin, it undergoes little metabolism by the liver [3] and has a high urinary clearance. Therefore, contrarily to insulin, urinary C-peptide values are high in healthy subjects.

For analysis of C-peptide, a variety of commercial

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immunoassays are available. They all are designed to measure both serum and urinary C-peptide (see Refs. [4,5] for reviews about measurement techniques and clinical relevance of serum and urine applications). In most circumstances C-peptide is determined in serum, nevertheless, urinary C-peptide measurements are used for assessing the β-cell function in particular in insulin-treated diabetes mellitus [6,7]. The development of immunoassays for C-peptide is a challenge because of its low immunogenicity [4]. Also the choice of the standard material and the production of labelled C-peptide analogues are not straightforward. For example, the standard material not only requires purity and stability control but also confirmation of immunological identity to the natural molecule [8], whereas labelling, e.g., with ^{125}I, might be complicated due to the absence of tyrosine residues [5,9]. Moreover, correct calibration and sufficient specificity of an immunoassay is difficult to prove because of the absence of a reference measurement procedure [10,11], such as isotope dilution–liquid chromatography–tandem mass spectrometry (ID–LC–MS–MS). However, in virtue of continuous progress in technological development in the field of LC–MS, this technique now appears to gradually become a common tool in peptide and protein analysis [12–22]. Nevertheless, it is still a fact that reports on the application of LC–MS for quantitative analysis of diagnostically important polypeptides/proteins in human body fluids are scarce and do by far not all use isotope dilution. Indeed, because of the relative difficulty and hence high cost for synthesis of stable-isotopically labelled analogues of polypeptides, the measurement procedures rather make use of a homologue [22] or a diastereomer [20] as internal standard. To the best of our knowledge, only two groups reported a quantitative measurement procedure using isotope dilution [12,14,18], one of them for serum C-peptide [12].

In this study, we started from an earlier developed electrospray ionization (ESI) ID–LC–MS–MS quantitative measurement procedure for urinary C-peptide that uses [$^{2}H_{16}$]C-peptide as internal standard and ultrafiltration (UF) for sample preparation [23]. However, with the aim to use it for evaluation of the performance (e.g., correctness of calibration and specificity) of immunoassays, we optimized the measurement procedure and thoroughly evaluated its analytical performance characteristics in order to judge whether it could serve as comparison measurement procedure. Based on the positive outcome of the latter investigations, we evaluated five frequently used immunoassays for determination of urinary C-peptide utilizing for the split-sample measurements a panel of 45 urine samples from apparently healthy subjects.

2. Experimental

2.1. Materials

Proinsulin C-peptide fragment 33–63 (M_r 3020.3) was obtained from ICN Biomedicals (Costa Mesa, CA, USA). It was delivered in a vial containing 250 μg of freeze-dried material and had a peptide content of 89% and a purity by HPLC of >99% (according to the manufacturer's information). The purity was taken into account for calculation of the C-peptide content in the calibrators. Isotopically labelled [$^{2}H_{16}$]C-peptide (label: [$^{2}H_{8}$–Val^{7,10}] was obtained from Bachem (Bubendorf, Switzerland). Stock standard solutions (concentration 250 ng/μl) were prepared by carefully weighing the added volume (~1 ml) of 1% protease-free bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) solution to the vial taking the density into account to know the exact volume used for dissolution of the vial content. From this stock solution a series of 1/5 gravimetrically diluted solutions in the same BSA solution were prepared to obtain working solutions of, respectively, 50 and 10 ng/μl. Immediately after preparation, the working solutions were divided in 100-μl portions in polypropylene vials and frozen at −20 °C until the day of analysis. Each day of analysis, an aliquot was thawed and gravimetrically 1/10 diluted with the 1% BSA solution to obtain working solutions of ~1 ng/μl. Once diluted, the aliquots were never reused. UF was done with Centricon YM-3K devices with a nominal molecular mass cut-off value of 3000 and a membrane of regenerated cellulose (Millipore, Bedford, MA, USA).

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

Random urine specimens were sampled from apparently healthy male and female volunteers be-

tween 15 and 65 years old. They were chosen in such a way to cover a wide concentration range. The samples were treated in accordance with the standards of the Committee for Medical Ethics of the Ghent University. Immediately after donation, the bulk urine samples were stored at −20 °C (between 4 to 8 weeks) until the start of the actual method comparison. At that moment, the urine donations were thawed, aliquoted in 1-ml portions and restored at −20 °C until the day of analysis. All sample aliquots were shipped frozen on dry ice to the companies or laboratory participating in this study. They were thawed on the day of analysis and never reused. The C-peptide concentrations of the samples ranged from 2.95 to 151.3 ng/ml with a mean value of 40.5 ng/ml as measured by ID–LC–MS–MS.

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 Hamilton PRP-3 column (50×2.1 mm, 3 μm bead size, 300 Å pore size) from Hamilton (Rena, NV, USA).

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

All immunoassays used in the evaluation study were applicable for the quantitation of serum and urinary C-peptide. The following automatic test systems were evaluated: the C-peptide immunoassay from Byk-Sangtec Diagnostica (Dietzenbach, Germany) (art. No. 316.171), performed on a Liaison analyzer (Diasorin, Saluggia, Italy), the assay from DPC (art. No. L2KEP2) (Los Angeles, CA, USA) performed on an Immulite 2000 analyzer and the Tosoh (Minato-ku Tokyo, Japan) AIA-Pack C-peptide (art. No. 020284) performed on an AIA-21 test system. The assays were all performed in the application laboratories of the respective manufacturers/distributors for Belgium, i.e., in the laboratories of Diasorin (Brussels, Belgium), DPC (Breda, The Netherlands) and Eurogenetics Headquarters (Tessenderlo, Belgium). Further, two manual radioimmunoassay (RIA) kits were included in the study, i.e., the C-PEPsp-RIA (art. No. 30 040 80) and C-PEP-RIA New (art. No. KIP0411) from Biosource (Nivelles, Belgium). The measurements were performed in the routine clinical laboratory of one of the authors (AZ Middelheim, Antwerpen, Belgium) and the application laboratory of Biosource Europe (Nivelles, Belgium), respectively. For calibration, all immunoassays made use of the World Health Organization (WHO) 84/510 international reference preparation (IRP) [24].

2.3. Sample pretreatement

For the ID–LC–MS–MS measurement procedure sample preparation consisted of UF. Depending on the concentration of the sample, an appropriate volume of urine (max. 2 ml) was gravimetrically weighed in so that 15 or 40 ng C-peptide was taken through analysis. To the sampled urine, the stable isotopically labelled internal standard (I.S.) was added in a 1:1 ratio. After an equilibration time of 30 min, ultrafiltration was performed for 45 min at 7000 *g* to obtain ~100 μl retentate. Then, twice, 650 μl of water was added, mixed, and UF was repeated to obtain a final retentate volume of ~30 μl to which 120 μl of water was added.

For the immunoassays, no sample pretreatement was required.

2.4. LC–MS–MS conditions

From the final volume (~150 μl) 30 μl was directly injected into the LC system. Gradient chromatography (cycle time 16 min) was performed with water–acetonitrile–trifluoroacetic acid (TFA) as mobile phase (eluent A: 100:0:0.02, v/v; eluent B: 50:50:0.02, v/v) at a flow-rate of 0.2 ml/min. The gradient started with 90% eluent A, then eluent B was increased from 10 to 70% (within 10 min) and held for 2 min. After returning to eluent A, the system was re-equilibrated for 3.9 min until the next injection. Under these chromatographic conditions, C-peptide eluted after ~10.5 min.

LC–MS measurements were performed in the negative electrospray tandem MS mode following the transitions at m/z 1508>1499 (c_{15}-fragment) and m/z 1516>1507 for, respectively, C-peptide and its labelled analogue. Dwell times were 1.5 s. The MS settings were: collision gas argon at $3 \cdot 10^{-3}$ mbar,

collision energy 30 eV, cone 60 V, capillary 3.5 kV, and source temperature 175 °C.

2.5. Internal quality control (IQC)

Internal quality control (IQC) for the ID–LC–MS–MS measurement procedure was done with the Lyphochek Quantitative Urine Control, Normal Level 1 from Bio-Rad (Irvine, CA, USA). IQC of the immunoassays was performed with materials recommended by the manufacturers and using the test-system-specific target values, i.e., the Bio-Rad Lyphochek Immunoassay Plus Control materials for Diasorin, the DPC C-peptide Control Module, the Eurogenetics Multi-analyte controls and the Biosource kit controls. With the Biosource C-PEPsp-RIA kit, two additional Bio-Rad Lyphochek Immunoassay Plus Control samples were used.

2.6. Specificity assessment

For the assessment of potential interferences in the ID–LC–MS–MS measurement procedure, the MS instrument was set to additionally monitor the ion transition at m/z 1508 >1327 (c_{12}-fragment). Further, HPLC was performed with a different gradient elution (from 10% eluent B to 90% eluent B in 14 min) with, respectively, a Hamilton polymer (150× 2.1 mm, 3 μm, 300 Å) and a silica-based Hypersil PEP C_{18} column (150×2.1 mm, 5 μm, 300 Å) from Alltech (Deerfield, IL, USA).

For the assessment of potential interferences of the immunoassays, we collected (according to the basic LC protocol) 16 different fractions of each 40 μl (12 s) in 300 μl of Immulite 2000 C-peptide diluent, starting 1.5 min before until 1.5 min after the retention time of C-peptide. The measurements of the immunoreactivity of the LC–MS collections were done with the DPC test on the same day.

2.7. Split-sample measurement protocol

Single measurement of the 45 urine samples with ID–LC–MS–MS required 2 entire days of analysis. The measurement protocol consisted of injection of two calibrators, eight samples, two calibrators, etc., until measurement of all samples processed on that day. IQC samples were randomly measured in the series as duplicate. The measurements were repeated to obtain in the end three results for each sample, taking care that the injection sequence of the samples was modified. Completing the measurements lasted 2 weeks in total. For a run to be taken into account, the limit for the deviation of the mean IQC value from the target value was set to 3%. For the method comparison, the mean of the three results for each sample was taken into account.

With the immunoassays, all samples were measured in one run. At the beginning of the run, each IQC sample was analyzed in duplicate. When the preset criteria of the respective manufacturer were fulfilled, the measurements were started in the following way: sample 1 A and B until sample 8 A and B; each IQC level in duplicate; etc., until analysis of all samples; the run was finished with a duplicate measurement of each IQC level.

The split-sample measurements were organized in such a way that the maximum storage time of the aliquoted urine was 2 weeks. To this end, the measurements by the laboratories applying the immunoassays were done within 1 week, namely in the second week of the ID–LC–MS–MS measurements.

2.8. Statistical methods

The within-run imprecision for the ID–LC–MS–MS measurement procedure was calculated from the pooled SDs from the triplicate determination of the individual samples using the formula:

$$\mathrm{SD} = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2 + \ldots (n_k - 1)s_k^2}{n_1 + n_2 + \ldots n_k - k}}$$

with 1, 2, ... k referring to the different series of measurements and n the number of measurements in a series.

For the immunoassays, the within-run imprecision was calculated from the data of the duplicate measurements of the urine samples using the formula:

$$\mathrm{SD} = \sqrt{\sum d^2/(2{*}n)}$$

The method comparison total relative standard deviation ($\mathrm{RSD_{tot}}$) was calculated by taking the square root of the sum of the squares of the RSDs of

the immunoassay and the comparison measurement procedure (ID–LC–MS–MS). Regression analysis was performed using the weighted Deming regression method [25] as calculated with the CBStat program of K. Linnet (www.cbstat.com).

3. Results and discussion

3.1. Optimization and evaluation of ID–LC–MS–MS

To obtain a more robust measurement procedure, several parameters of our earlier developed ID–LC–MS–MS measurement procedure for quantitation of urinary C-peptide [23] were optimized. With respect to the LC conditions, we preferred to use a shorter (50 instead of 150 mm) column made of polymer material, i.e., Hamilton PRP-3. Indeed, this material is especially designed for robustness upon chromatographic purification and isolation of peptides and proteins. Further, the composition of the original mobile phase (i.e., water–acetonitrile–*n*-propanol–formic acid–TFA) was simplified. Experience showed that *n*-propanol was not indispensable, thus it was omitted. With respect to the addition of formic acid, which was necessary for the ionization process, we preferred to do it post-column and made use of the principle of gas phase addition in the nebulizing gas line. Although our instrument was not equipped with a triaxial probe, we could do gas phase addition via the bubble trap as described elsewhere [26]. To minimize the influence of ionization suppression caused by co-elution of other compounds, gradient elution was performed in 16 min instead of 10 min.

Mass spectrometric detection was still performed in the negative electrospray ionization mode, but other ion transitions were monitored. We optimized the measurement procedure to obtain maximum ion intensity and monitored the most intense daughter ion of the C-peptide spectrum nl. c15-fragment at ion transition m/z 1508>1499 for C-peptide and m/z 1516>1507 for [$^{2}H_{16}$]C-peptide instead of the earlier monitored c12-fragment at ion transition m/z 1508>1327 for C-peptide.

Under these conditions, we obtained always interference-free chromatograms. The limit of detection at a signal-to-noise ratio 3 improved from 150 to 90 pg C-peptide with the optimized measurement procedure.

3.2. Study design

We applied our earlier developed [23] and optimized ID–LC–MS–MS measurement procedure for the evaluation of five immunoassays for the quantitation of urinary C-peptide. Table 1 gives an overview of the main characteristics of the respective immunoassays. The kits were all developed for the determination of C-peptide in serum as well as in urine. However, to the best of our knowledge, none of the

Table 1
Technical characteristics of the C-peptide immunoassays (applicable for measurement in serum and urine)

Company (analyzer or kit)	Immunoassay principle	Detection principle	Sample volume (μl) (dilution)
Diasorin (Liaison)	Sandwich	Luminescence	50 (1:10)*
DPC (Immulite 2000)	Competition	Luminescence	75 (1:20[a])*
Eurogenetics (AIA-21)	Sandwich	Fluorescence	20 (1:10)*
Biosource (C-PEPsp-RIA)	Competition	Radioactive	100 (1:21)
Biosource (C-PEP-RIA New)	Competition	Radioactive	100 (1:21)

Note: All analyzers were calibrated with the WHO IRP 84/510.
* Dilutions were made automatically by the analyzers.
[a] Some highly concentrated samples were 1:40 diluted.

assays had been evaluated before for its application in urine.

The split-sample measurements were done with a panel of 45 urine specimens. Ideally, the latter should have been analyzed freshly after collection. For logistic reasons, this requirement could not be fulfilled in our study and we chose fresh/frozen samples in bulk (max. 4–8 weeks). We aliquoted them shortly before the start of the measurements and organized the study in such a way that the measurements were all performed at the different sites within 2 weeks. From these precautions, we assumed that all the laboratories worked with ''identical'' aliquots.

3.3. Standard solutions

We opted for 1% protease-free BSA solutions for stabilizing the ID–LC–MS–MS C-peptide standard stock solutions. Our experience with LC–MS gave indication that polypeptide/protein solutions were more stable in a 1% BSA solution. The stock solution was gravimetrically prepared by careful weighing the added amount of the 1% BSA solution. We realized that, even by working in this way, the accuracy of our calibrators depends on the accuracy of the 250 μg filling by the manufacturer. However, we were informed by the latter that the amount of material dispensed in the vials was verified in such a way that a mass of 250±5 μg could be guaranteed.

The stock solution was gravimetrically 1/5 diluted to obtain working solutions of 50 and 10 ng/μl. These solutions were aliquoted in 100 μl aliquots in plastic vials and stored at −20 °C. Of each dilution series, one aliquot was stored to test, on regular basis, the others (see Fig. 1). The difference between the tested aliquots was always <1.5%, proving that the working solutions remained stable. Each day of analysis, a new vial was thawed and gravimetrically diluted to obtain working solutions of ~1 ng/μl. Once thawed and diluted, a solution was never reused.

The immunoassays were all calibrated with the same WHO IRP 84/510 [24]. Indeed, the latter was used to assign values to the manufacturers' secondary calibrator.

3.4. Internal quality control

For accuracy assessment of the ID–LC–MS–MS measurement procedure, no certified materials were available. Therefore, we selected a stable, lyophilized IQC urine material that we assigned ourselves for its content of C-peptide (15.4 ng/ml). This was done by measuring a sample aliquot in duplicate during 7 consecutive days. Each day of analysis a new IQC vial was reconstituted and an independent calibration was performed. This material was subsequently included for IQC in the split-sample

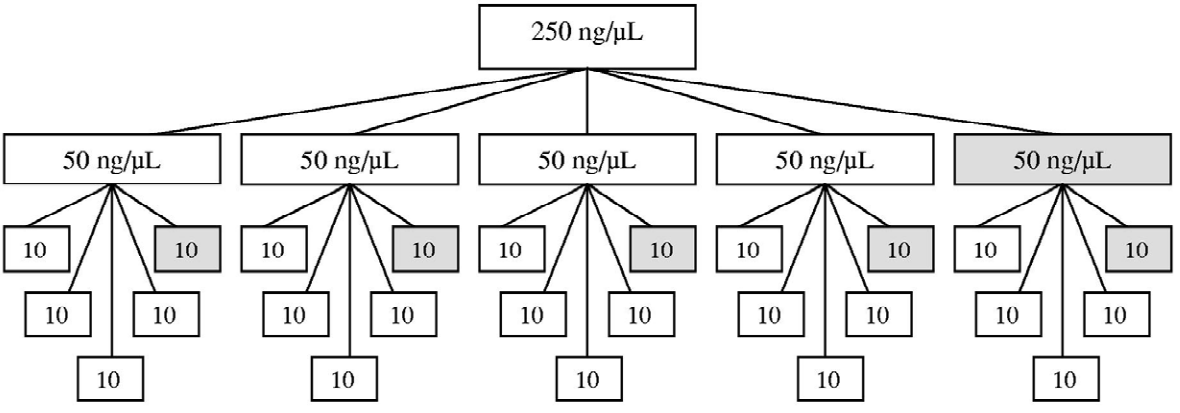

Fig. 1. Overview of the dilution series of the C-peptide standard solutions. The aliquots marked in grey are the aliquots which were tested against each other to control the stability of the standard solution. Note: concentrations of the solutions in ng/ml.

measurements. The measurements with the immunoassays were performed in one laboratory in one run. To ensure that the results were representative for the respective test systems, the manufacturers were asked to take special care of IQC. In particular, deviations from the target values should be <5% (at concentration levels >6 ng/ml), or <0.3 ng (≤6 ng/ml). Note that all IQC materials (see Table 2) had a serum matrix, hence contained C-peptide concentrations much lower that those covered by the urine samples. The respective manufacturers did consider that this was not a hindrance to adequate IQC, because all the tested immunoassays can be performed on both serum and urine, and because the urine samples are diluted to approximately the serum concentration range.

Table 2 shows that for all immunoassays the mean deviations from the IQC target values were <0.3 ng or 5%, from which we conclude that the measurement values for the samples of the study were representative for the inherent accuracy of the respective immunoassays. For the ID–LC–MS–MS it demonstrates that the mean deviation from the assigned target value (15.4 ng/ml) amounted to 2.7%.

3.5. Method comparison—systematic error

The results of the method comparison are presented in Fig. 2A–E and Table 3 (weighted Deming regression and second-order correlation). For reasons explained below, we additionally present the comparison between Eurogenetics and Diasorin (Fig. 2F). Besides the data points, Fig. 2 shows the line of identity (dotted), the weighted Deming regression line (full), and the dispersion of the points around the regression line (dot and dash) predicted by the RSD_{tot} of the respective measurement procedure pairs (note: the latter will be discussed below).

The data reveal considerable systematic differences between the immunoassays and ID–LC–MS–MS and, more important, between the immunoassays themselves. Indeed, the slopes ranged between 0.802 and 2.438. The latter is striking as all immunoassays apply the same calibrator (IRP 84/510) [24]. Note however, that systematic differences for patient samples were already described in the certification report of IRP 84/510, irrespective whether the local standard or the IRP was used for calibration. After visual inspection, some measurement procedures (e.g., Eurogenetics and Biosource C-PEP-RIA New)

Table 2
Overview of the deviations from the IQC target values by the different immunoassays and the ID–LC–MS–MS measurement procedure

Measurement procedure (analyzer or kit)		Target value (*A*) (ng/ml)	Measured value (*B*) (ng/ml)	Delta (*B* − *A*)*
Diasorin	IQC 1	2.00	1.91	<0.3 ng
(Liaison)	IQC 2	14.70	14.10	5.0%
DPC	IQC 1	0.70	0.77	<0.3 ng
(Immulite 2000)	IQC 2	3.00	2.94	<0.3 ng
	IQC 3	5.30	5.15	<0.3 ng
Eurogenetics	IQC 1	1.00	1.07	<0.3 ng
(A1A-21)	IQC 2	5.80	5.68	<0.3 ng
	IQC 3	18.20	17.44	4.2%
Biosource	IQC 1	0.72	1.00	<0.3 ng
(C-PEPsp-RIA)	IQC 2	3.56	3.69	<0.3 ng
	IQC 3	0.72	0.57	<0.3 ng
	IQC 4	3.02	2.78	<0.3 ng
Biosource (C-PEP-RIA New)	IQC 1	0.88	0.82	<0.3 ng
	IQC 2	3.29	3.29	<0.3 ng
ID–LC–MS–MS	IQC 1	15.41	15.82	2.7%

* Delta expressed in absolute value (ng) for targets with concentrations ≤6 ng/ml, in percent for those >6 ng/ml.

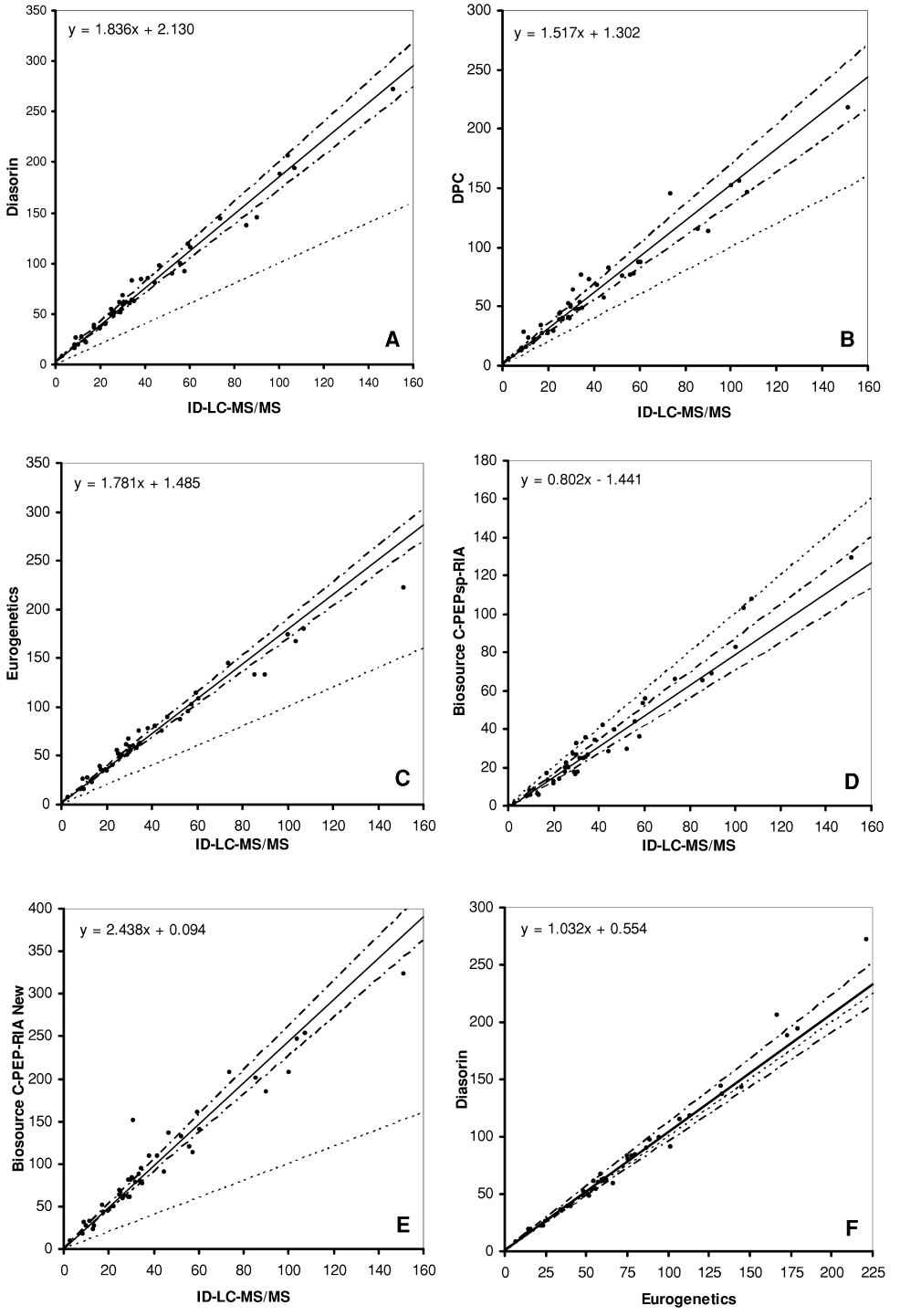

Fig. 2. Measurement results (ng/ml) for urinary C-peptide obtained by five immunoassays compared to those by an ID–LC–MS–MS measurement procedure. (A) Diasorin; (B) DPC; (C) Eurogenetics; (D) Biosource C-PEPsp-RIA; (E) Biosource C-PEP-RIA New. (–): Weighted Deming regression; (– –): 95% prediction interval based on RSD_{tot}; (· · ·): $X = Y$.

Table 3
Weighted Deming regression and second-order correlation data characterizing the comparison of the C-peptide immunoassays with ID–LC–MS–MS

Assay	Slope ±95% CI	Intercept ±95% CI	r (second order)
Diasorin	1.836±0.09	2.130±1.969	0.991
DPC	1.517±0.084	1.302±0.819	0.985[a]
Eurogenetics	1.781±0.099	1.485±2.086	0.992
BioSource C-PEPsp-RIA	0.802±0.142	−1.441±3.488	0.980
BioSource C-PEP-RIA New	2.438±0.289	0.094±7.095	0.986[b]

[a] Sample 4 (74 ng/ml) omitted (visually) as outlier.
[b] Sample 45 (31 ng/ml) omitted (visually) as outlier.

seem not to respond in a linear way and regression was recalculated with a second-order function. As shown in Table 3, all immunoassays had an excellent second order correlation with the ID–LC–MS–MS measurement procedure ($0.980 < r < 0.992$).

3.6. Method comparison—robustness/specificity

The imprecision of the different immunoassays was calculated from the duplicate analysis of the samples and was independent of the C-peptide concentration. The RSDs ranged from 3.1% (Eurogenetics) to 7.5% (DPC) (see Table 4). The imprecision of the ID–LC–MS–MS measurement procedure was also independent of the C-peptide concentration and amounted to 3.0%. For the method comparison study, the mean of duplicates of the immunoassays was compared with the mean of triplicates for ID–LC–MS–MS. Thus, for calculation of the RSD_{tot}, the RSDs given in Table 4 for, respectively, the immunoassays and the ID–LC–MS–MS measurement procedure were divided by $\sqrt{2}$ and $\sqrt{3}$. As a result, the ID–LC–MS–MS measurement procedure was slightly less imprecise than the best immunoassay (RSDs, respectively, 1.7 and 2.2%). The RSD_{tot} was used to calculate the 95% prediction interval ($=1.96 \cdot RSD_{tot}$) for the data pairs along the regression line in Fig. 1. By comparison of the 95% prediction intervals with the actual spread of the data around the regression line, the overall sample matrix robustness and specificity of the immunoassays can be estimated. Taking into account the non-linearity of certain comparisons, visual inspection learns that the actual observed spread is about twice as high as expected from the RSD_{tot}. This indicates sample-related effects and/or specificity problems (note: some of the variability may originate from the storing and thawing procedure at the different locations). However, whether the observed spread was due to the immunoassays or to the ID–LC–MS–MS measurement procedure could not be predicted beforehand. Interestingly, when we correlated the immunoassays with each other, we found that the pair Eurogenetics/Diasorin gave a higher correlation coefficient than with the ID–LC–MS–MS measurement procedure ($r = 0.995$ versus 0.992). The latter may be indicative for a better specificity of the immunoassays or for the fact that they are more similar with each other, for example, because both have similar cross-reactivities to C-peptide-like molecules that are not detected by the LC–MS–MS measurement procedure. Possible interference with proinsulin could be excluded, because it does not appear in human urine [27]. However, it has been described that, due to incomplete cleavage of the proinsulin molecule, C-peptide with two additional amino acids at the N and/or

Table 4
Imprecision data of the measurement procedures and RSD_{tot} of the method comparisons

Measurement procedure	RSD (%)	RSD_{tot} (%)
ID–LC–MS–MS	3.0	
Diasorin	4.7	3.7
DPC	7.5	5.6
Eurogenetics	3.1	2.8
Biosource C-PEPsp-RIA	7.0	5.2
Biosource C-PEP-RIA New	4.5	3.6
Eurogenetics vs. Diasorin		4.0

Note: For calculation of RSD_{tot} the RSD of ID–LC–MS–MS was divided by $\sqrt{3}$, the RSDs of the immunoassays by $\sqrt{2}$.

C-terminus (Arg–Arg–C-peptide or C-peptide–Lys–Arg, respectively) may appear in the blood stream or urine [28]. Kippen et al. used MS to investigate the amount of C-peptide–Lys–Arg in serum and found that the concentration of C-peptide–Lys–Arg can amount up to 4 to 10% of that of C-peptide [12]. Moreover, immunoassays cannot distinguish C-peptide from those various fragments [12]. Unfortunately, these substances could not be tested because they were not commercially available. Thus, we had to investigate the specificity by indirect means.

Although theoretically high specificity of the ID–LC–MS–MS measurement procedure can be assumed because of detection based on tandem MS, it is still advocated to investigate potential interferences at the m/z value monitored for both C-peptide and internal standard. The latter could be easily done by processing all samples without addition of internal standard and subsequent monitoring of m/z 1516>1507. Potential interferences at the m/z value typical for the ion transition of C-peptide (m/z 1508>1499) were investigated by improving the chromatographic resolution or monitoring different ion transitions. The former was performed by use of a longer column with the same polymer packing material and by use of a second column that was silica-based instead of polymer-based (see Experimental). With both type of columns, no underlying interferences were observed. For monitoring of a different ion transition, the second most intensive daughter ion in the collision activated dissociation (CAD) spectrum of C-peptide, i.e., m/z 1508>1327 (c_{12}-fragment) was chosen. None of these experiments gave an indication for the presence of interferences. Nevertheless, we cannot definitively exclude small sample related effects in our ID–LC–MS–MS measurement procedure in its current state of development. The way to do this is participation in round-robin trials with other laboratories performing ID–LC–MS–MS measurements of urinary C-peptide. To the best of our knowledge, this is currently not possible.

Since no interference was found in the ID–LC–MS–MS measurement procedure, we finally investigated the specificity of the immunoassays exemplary with the DPC assay. We collected, therefore, different LC fractions eluting from 1.5 min before until 1.5 min after C-peptide and submitted them to measurement with the DPC Immulite 2000 system. In Fig. 3 the resulting immunochromatogram is superseded to the LC–MS–MS chromatogram. Indeed, Fig. 3 shows immunoreactivity in some fractions eluting before and after the C-peptide peak, indicating that cross-reactive substances may be present in urine. So, the immunoreactivity in the fractions eluting before C-peptide can confirm our suspicion that C-peptide–Lys–Arg and/or other interferences are present in some samples. From these specificity experiments, it might be explained why some immunoassays correlate better with each other than with ID–LC–MS–MS. Of course, unequivocal confirmation of these speculations would require elucidation of the extent and identity of the non-specific binding. This could be done by adding the antibodies used in the different immunoassays to the complex urine matrix and by using subsequently full-scan LC–MS to elucidate the identity of the non-specifically bound molecules.

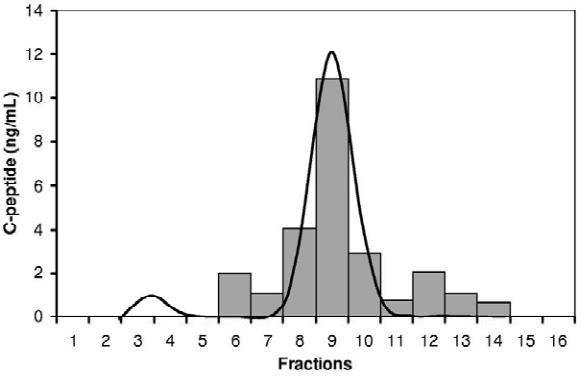

Fig. 3. Immunochromatogram (measured with the DPC test) of a processed urine sample with indication of the elution profile of C-peptide obtained with the ID–LC–MS–MS measurement procedure.

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