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Target analyte quantification by isotope dilution LC-MS/MS directly referring to internal standard concentrations – validation for serum cortisol measurement

Abstract

Background: Isotope dilution LC-MS/MS methods used in the clinical laboratory typically involve multi-point external calibration in each analytical series. Our aim was to test the hypothesis that determination of target analyte concentrations directly derived from the relation of the target analyte peak area to the peak area of a corresponding stable isotope labelled internal standard compound [direct isotope dilution analysis (DIDA)] may be not inferior to conventional external calibration with respect to accuracy and reproducibility.

Methods: Quality control samples and human serum pools were analysed in a comparative validation protocol for cortisol as an exemplary analyte by LC-MS/MS. Accuracy and reproducibility were compared between quantification either involving a six-point external calibration function, or a result calculation merely based on peak area ratios of unlabelled and labelled analyte.

Results: Both quantification approaches resulted in similar accuracy and reproducibility.

Conclusions: For specified analytes, reliable analyte quantification directly derived from the ratio of peak areas of labelled and unlabelled analyte without the need for a time consuming multi-point calibration series is possible. This DIDA approach is of considerable practical importance for the application of LC-MS/MS in the clinical laboratory where short turnaround times often have high priority.

Keywords: cortisol; direct isotope dilution analysis (DIDA); liquid chromatography-tandem mass spectrometry (LC-MS/MS); stable isotope labelled internal standard.

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Introduction

Today liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods employed in the clinical laboratory are routinely performed as batch analyses, involving a multi-level calibrator series followed by "unknowns". This batch mode of analysis is applicable for analytes like 25-hydroxyvitamin D which do not require reporting within short time frames. However, it is likely that LC-MS/MS will be increasingly used in fields, such as clinical toxicology where often single samples have to be analysed instead of batches and it is desirable to obtain results rapidly. This may also apply to measuring serum cortisol at a high level of accuracy in the context of diagnosing adrenocortical dysfunction in septic shock [1]. Thus, analysing a calibration series in the conventional and time consuming operation mode of LC-MS/MS analysis is a substantial drawback in clinical mass spectrometry, which indeed makes a more widespread application of LC-MS/MS questionable in this setting.

The current practice to perform multi-point calibration for clinical LC-MS/MS analyses is adopted, on the one hand, from standard biomedical method validation protocols and, on the other hand, from traditional, non-automated ligand binding assays. In these latter tests, typically complex and non-linear calibration functions are observed which definitely require in most cases at least five calibration points for the quantification of unknowns. In LC-MS/MS, in contrast, in the majority of assays, linear response is observed over a wide range of concentrations and the need for multi-point calibration is indeed questionable. Notably, the relation between the number of calibration samples and the analytical reliability of routine LC-MS/MS methods or the "stability" of a calibration is hardly studied systematically in the methodological literature of clinical mass spectrometry so far.

Direct isotope dilution analysis (DIDA) is an alternative quantification method. The principle of DIDA is based on the assumption (and precondition) that the

physico-chemical behaviour of the native analyte molecule is very similar to that of its isotope labelled counterpart used as the internal standard. For these labelled compounds very similar ionisation yields and consequently LC-MS/MS-peak areas are observed as with native (unlabelled) analyte molecules. Therefore, when adding the stable isotope labelled internal standard compound solution in a strictly quantitative manner to a biological sample as the first step of sample preparation, it is possible to determine the analyte concentration in the sample from the LC-MS/MS-peak area of the native, unlabelled analyte molecule in relation to the peak area of the labelled internal standard compound, by simple calculation of an individual sample.

The aim of our study was to investigate the feasibility of this DIDA principle in the field of routine clinical laboratory application of LC-MS/MS where DIDA is not used so far. We tested the hypothesis that applying DIDA is not inferior to the standard approach of multi-calibrator isotope dilution LC-MS/MS quantification with respect to accuracy and reproducibility in the quantification of serum cortisol as an exemplary analytical system.

Materials and methods

A comparative validation protocol was applied including four analytical series: In these series cortisol was determined as described previously [2] in spiked quality control (QC) samples, in human serum pools and in 25 patients' samples using LC-MS/MS involving a six-point set of calibration samples and three-fold deuterated ([9,12,12-^{2}H]cortisol) as the internal standard in a conventional approach, using a standard chromatography software program (MassLynx, Waters). Unlabelled cortisol was purchased from Sigma Aldrich (Steinheim, Germany; purity 98.7%) and labelled cortisol was purchased from Cambridge Isotope Laboratories (Andover, USA; purity: 98%). According to the MS-scan investigation experiments, no three-fold deuterated cortisol was detectable in the native cortisol formulation, nor was unlabelled cortisol found in the formulation of labelled cortisol. Serum pools were prepared from leftover samples sent for clinical chemistry investigations to our laboratory. Twenty-five leftover individual patients' samples sent for cortisol testing to our laboratory were included in the series after anonymisation. Calibrators were prepared by spiking a solution of bovine serum albumin (70 g/L) in phosphate buffered saline to the following cortisol concentrations: 12.3, 24.7, 49.4, 98.7, 197 and 395 µg/L. QC samples were prepared in the same way (49.4, 148 and 296 µg/L).

After this conventional analysis and quantification, the peak areas (of analyte and labelled internal standard) recorded for QC samples, pool samples and patients' samples were re-assessed separately for each sample. Based on their raw peak areas (Figure 1) – as a second and independent quantification process – cortisol concentrations were determined using an Excel sheet according to the above described DIDA principle, not taking into account the calibration samples analysed in the respective series at all. In this way for any of the four analytical series two sets of quantitative data were obtained for each validation material and individual patient's sample (conventional quantification vs. DIDA). Accuracy (% deviation from target concentration for spiked samples) and reproducibility (% relative standard deviation) were comparatively assessed for the two different quantification processes. The FDA criteria for bioanalytical method validation were applied [3, 4]. Individual patient's sample results were plotted for comparison.

Results

The data of the validation experiment are summarised in Table 1. For the three spiked QC samples, analytical accuracy realised using the DIDA quantification was judged to be not inferior when compared to the conventional approach of calibration with an external series of calibrants. Regarding reproducibility, a total of seven samples (spiked QC samples and pool samples) were assessed, and in all samples lower CVs were observed for DIDA when compared to conventional calibration. Applying a t-test for paired observations, CVs of DIDA were significantly lower compared to conventional calibration ($p<0.05$). Very close agreement was found for paired results of patients' samples ($r>0.999$; Figure 2).

Both methods met the requirements of the FDA guidance for bioanalytical method validation (CV and bias <15%; see Table 1) [3].

Discussion

Our study demonstrates that isotope dilution-LC-MS/MS can allow – besides traditional batch analysis – reliable "ad-hoc" analyses of single samples without the need to perform a multi-point calibration when the DIDA approach is used. In the comparative imprecision study, DIDA even showed superior reproducibility when compared to conventional calibration. Potentially multi-point calibration introduces a somewhat higher degree of "noise" into the analytical system, resulting from the unavoidable variation in preparing and analysing six individual calibration samples. Regarding the degree of analytical accuracy, however, both principles were judged equal (Table 1). The reliability of DIDA quantification was further confirmed by the analysis of a set of individual patients' samples, displaying fully commutable results.

In newborn screening for inborn metabolic diseases by LC-MS/MS, the principle of estimating analyte concentrations derived from the concentration of the added internal standard is already used. This use, however, is in

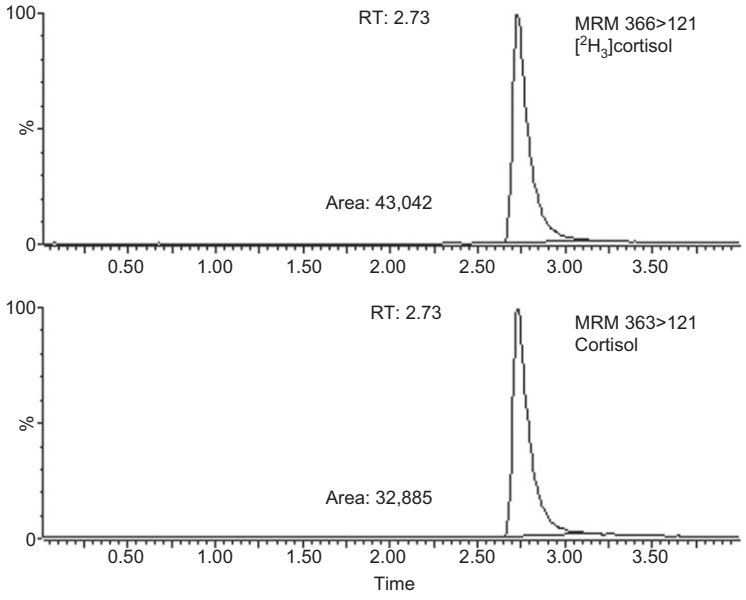

Figure 1 Example for the quantification of a target analyte concentration based on the LC-MS/MS peak area of a stable isotope labelled internal standard compound.

Peak area of the target analyte cortisol, 32,885 counts, and of the internal standard [$^2H_3$]cortisol, 43,042 counts. Since the ionisation behaviour of both cortisol molecules is assumed to be very similar and since the volume of the serum aliquot and the internal standard solution applied during sample preparation are identical, the concentration of native cortisol is calculated as follows: Concentration of cortisol = area of cortisol * (concentration of [$^2H_3$]cortisol/area of [$^2H_3$]cortisol). For the serum sample with the shown LC-MS/MS chromatogram, a cortisol concentration of 150 µg/L is calculated (concentration of [$^2H_3$]cortisol in the internal standard solution, 196 µg/L; 98% isotopic purity).

a semi-quantitative approach and in a very high analyte concentration range, using flow injection instead of HPLC separation [5]. A similar approach is also described for the quantification of inorganic elements [6]. Here, we demonstrate the applicability of this approach also for the quantification of small molecule analytes in a low concentration range and applying chromatographic separation.

We demonstrated for one exemplary analyte that DIDA is feasible for clinical chemistry application of LC-MS/MS. It must be noted, however, that the reliability of DIDA is determined by the accuracy of the declared concentration of the internal standard compound. The content and entire isotopic purity of the internal standard preparation employed is crucial and should be specified on the level of a reference material if DIDA is used in clinical diagnostic – similar to the specification of calibrator samples in standard tests of laboratory medicine. Similar to the conventional application of stable isotope labelled compounds for internal standardisation, compounds should be labelled in at least three positions of the molecule in order to avoid spectral overlap with naturally occurring molecules. Furthermore, labelling has to be chemically stable under storage and mass spectrometric conditions with no Deuterium-Hydrogen exchange.

	Target concentration, µg/L	Mean concentration, µg/L		Accuracy, %		Imprecision, CV%	
		CC	DIDA	CC	DIDA	CC	DIDA
QC 1	49.4	50.4	50.3	102	102	3.9	3.6
QC 2	148	145	149	98.0	101	5.7	2.8
QC 3	296	284	293	95.9	99.0	4.7	1.8
Pool 1		28.3	27.3			4.1	2.6
Pool 2		283	292			5.0	2.9
Pool 3		37.9	37.3			4.6	4.5
Pool 4		202	207			6.8	3.1

Table 1 Quantification of serum cortisol concentrations by isotope dilution LC-MS/MS using conventional six-point calibration (conventional calibration, CC) and using direct isotope dilution analysis (DIDA): Results of a validation study including 20-fold determination of quality control (QC) samples and human serum pool samples analysed in four independent series over a period of 14 days.

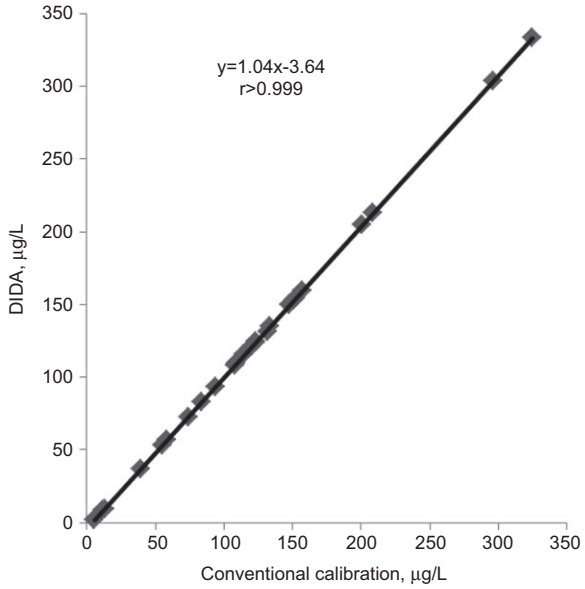

Figure 2 Comparison of serum cortisol results in 25 clinical samples obtained applying conventional calibration and DIDA, respectively.

DIDA – by principle – only can lead to reliable results if the ionisation behaviour of target analyte and stable isotope-labelled internal standard compound is very similar within the relevant concentration range (corresponding to linearity in the conventional calibration approach). We studied serum cortisol measurement for a proof of concept of DIDA-LC-MS/MS, considering the relevance of reliable quantification of this marker for intensive care medicine [1]. When considering applying this principle for other analytes in different sample matrices, of course a careful analyte-individual validation of this approach of quantification is required, in order to exclude differential isotope effects and non-linearity in the ionisation behaviour or chromatographic characteristics of stable isotope labelled molecules compared to the respective unlabelled counterparts [7, 8]. In this context, for DIDA methods the limits of minimum and maximum isotope ratios have to be established, thereby describing the measuring range. In our present study on cortisol, reliable and consistent results were observed for isotope ratios (native cortisol/labelled cortisol) from 0.14 to 1.5.

The widespread application of LC-MS/MS in clinical laboratories offers substantial potential advantages over standard technologies, such as photometry and immunoassays: flexible and straightforward method development for innovative small molecule analytes; highest analytical specificity; the potential to record metabolites patterns; no interferences from heterophilic antibodies; low running costs for analytical consumables instead of antibody-based reagents; and reliable standardisation and traceability realising assay-independent reference ranges. Thus, LC-MS/MS can address many of the unmet needs of clinical diagnostics and enables substantial innovation in clinical chemistry [9]. Given the workflow of today's typical clinical laboratories, comprehensive realisation of these potentials requires the development of fully automated random-access MS/MS-based analysers offering identical practicability as standard clinical chemistry analysers do [10]. We believe that the implementation of such an instrument concept can be facilitated by the DIDA principle. Our results also suggest the short-term implementation of convenient DIDA functionalities to standard chromatography software. Furthermore, the availability of stable isotope labelled materials specified on the level of reference materials is desirable.

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Conflict of interest statement

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References

1. Briegel J, Sprung CL, Annane D, Singer M, Keh D, Moreno R, et al. Multicenter comparison of cortisol as measured by different methods in samples of patients with septic shock. Intensive Care Med 2009;35:2151–6.
2. Kirchhoff F, Briegel J, Vogeser M. Quantification of free serum cortisol based on equilibrium dialysis and isotope dilution-liquid chromatography-tandem mass spectrometry. Clin Biochem 2011;44:894–9.
3. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Guidance for Industry – Bioanalytical Method Validation, May 2001. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf. Accessed 28 August, 2012.

4. Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, et al. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. J Pharm Sci 1992;81:309–12.
5. Rashed MS, Bucknall MP, Little D, Awad A, Jacob M, Alamoudi M, et al. Screening blood spots for inborn errors of metabolism by electrospray tandem mass spectrometry with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles. Clin Chem 1997;43:1129–41.
6. Dombovári J, Becker JS, Dietze HJ. Isotope ratio measurements of magnesium and determination of magnesium concentration by reverse isotope dilution technique on small amounts of ^{26}Mg-spiked nutrient solutions with inductively coupled plasma mass spectrometry. Int J Mass Spectrom 2000;202:231–40.
7. Stokvis E, Rosing H, Beijnen JH. Stable isotopically labeled internal standards in quantitative bioanalysis using liquid chromatography/mass spectrometry: necessity or not? Rapid Commun Mass Spectrom 2005;19:401–7.
8. Wang S, Cyronak M, Yang E. Does a stable isotopically labeled internal standard always correct analyte response? A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. J Pharm Biomed Anal 2007;43:701–7.
9. Vogeser M, Seger C. A decade of HPLC-MS/MS in the routine clinical laboratory – goals for further developments. Clin Biochem 2008;41:649–62.
10. Vogeser M, Kirchhoff F. Progress in automation of LC-MS in laboratory medicine. Clin Biochem 2011;44:4–13.