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Direct quantification in bioanalytical LC–MS/MS using internal calibration via analyte/stable isotope ratio

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Abstract

The possibility to rationalize and simplify bioanalysis, without compromising the analytical quality, by omitting the calibration curves was studied. Using mass spectrometry (MS) and a stable isotope labeled internal standard it was possible to get equally good results by calculating the results directly from the analyte/internal standard area ratio and a predetermined response factor as by the traditional way, using a calibration curve run at the same occasion. To be able to use this simplified quantification method, that we call internal calibration, in its most simple form there are some prerequisites that must be considered: (1) The relative response should not be concentration dependent. (2) The relative response should be constant between batches/days. (3) The level of analyte in the internal standard should not be detectable. (4) There should be no influence from naturally occurring isotopes of the analyte on the internal standard peak area.

A bioanalytical LC–MS/MS method for a research compound was validated both with and without calibration curves and no significant differences were found regarding precision and accuracy. It was shown that all four prerequisites above were fulfilled. Validation data were very good for the whole concentration range, 0.010–30 μmol/L. Long-term data for QC samples showed excellent precision and accuracy.

Keywords: Stable isotope labeled internal standard; Calibration curve; LC–MS/MS

1. Introduction

To improve precision and accuracy in chromatographic bioanalytical methods an internal standard is usually added to the samples prior to the sample work-up. A good internal standard, usually a slight chemical modification of the analyte, should have physico-chemical properties similar to the analyte; similar recovery, similar detector response and similar retention but still be chromatographically well resolved. Using MS, the analyte and internal standard peaks do not have to be chromatographically resolved as long as they have different masses. That introduces the possibility to use a stable isotope labeled analyte as internal standard. This compound, where a sufficient number ($n = 3$ or more) of, e.g. ^{13}C or ^{15}N have been incorporated, will be virtually identical, chemically, physically and biologically, to the analyte and as close to an ideal internal standard as possible. This internal standard will have the same recovery, response and retention as the analyte. The stable isotope labeled internal standard will also compensate for matrix effects [1] and will have identical protein binding, a fact that have been utilized for the simultaneous determination of free and total concentrations in plasma [2]. Note also that deuterium labeling might introduce slight differences in the properties compared to the unlabeled compound [3] making deuterium labeled compounds less ideal as internal standards.

The ideal analytical system would be perfectly linear, the signal to concentration ratio would be constant regardless of concentration and then concentrations for unknown samples could be calculated by simply using a response factor. Unfortunately, the response from analytical systems are rarely constant and furthermore the response may vary from day to day due to, e.g. ageing and fouling of instruments, therefore a multi-point calibration curve is prepared and analyzed together with each batch of unknown samples. It has also been suggested that optimum precision and accuracy is obtained by using a minimum number of calibration points and perform multiple measurements on these [4]. Using analytical systems with known and proven linearity, e.g. LC-UV, this is a viable approach and it has been used at our laboratory for many years with excellent results.

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In bioanalysis, unknown samples with expected differences in concentration of a factor 1000 or more are not unusual, hence also the calibration curve has to cover this huge dynamic range. A common problem is that a small error at the higher concentrations might give rise to very high false intercepts, positive or negative, making the accuracy very low for the unknowns with low concentrations. In bioanalysis, the relative standard deviation is roughly the same for all data points; thus the absolute error, the variance, increases with increasing concentration (heteroscedastic data). Weighting is applied to transform data to a homoscedastic form, with constant variance, diminishing the influence of the high concentrations on the intercept when using calibration curves.

But calibration curves are not always linear and the mass spectrometer is a notoriously non-linear detector, mainly because the degree of ionization in the ion source decreases when the amount of ions increases. The quantification softwares of LC–MS systems contain several different algorithms for fitting the best line to non-linear calibration data. In LC–MS, the most used method for fitting a line to the points in the calibration curve is probably weighted ($1/X$ or $1/X^2$), quadratic non-linear regression. Still, the current US FDA guidelines for bioanalytical method validation [5] recommend: "The simplest model that adequately describes the concentration–response relationship should be used. Selection of weighting and the use of a complex regression equation should be justified". The application of different regression models to sets of bioanalytical data, trying to minimize the residual variance in the calibration curve, has resulted in suggestions of several sophisticated regression models [6–10]. In the present paper, instead of using statistical methods to transform data, a non-linear response is transformed to a linear calibration curve by using an internal standard that mimics the analyte.

In bioanalysis, according to our experience, roughly 20% of the total time for analysis is spent on preparation and analysis of calibration standards. To be able to omit these samples without sacrificing accuracy and precision would mean a significant step forward in productivity. When LC–MS/MS is used and a stable isotope labeled internal standard is available it is shown in this paper that the calibration samples can be omitted. The unknown concentrations in each sample can be calculated directly using internal calibration via the analyte/internal standard area ratio and a predetermined response factor. A bioanalytical method was validated using both the internal calibration method and traditional calibration curves. The results from the validations are compared and long-term data of the internal calibration method are presented.

2. Experimental

2.1. Model compounds

The analyte (*N*-[2-({(2*S*)-3-[(3*S*)-3-(4-chlorophenoxy) pyrrolidin-1-yl]-2-hydroxypropyl}oxy)-4-hydroxyphenyl]acetamide, hemi-fumarate salt) is a medium lipophilic compound, with an amine pK_a at 7.6 and a phenolic pK_a at 9.1. The internal standard was the analyte labeled with stable isotopes, two deuterium and three ^{13}C. Both compounds were synthesized at the Medicinal Chemistry Department, AstraZeneca R&D, Lund, Sweden. Stock solutions and dilutions were made in 0.025 M formic acid. The compounds were stable in solution for at least 3 months.

2.2. Procedures

Calibration samples and quality control (QC) samples were made by spiking of EDTA blank plasma. Calibration and QC samples were made from different weighings. The calibration samples ranged from 10 nmol/L to 30 μmol/L and calibration curves were prepared at six occasions. QC samples were prepared at four different concentrations, 0.025, 0.5, 5 and 25 μmol/L. To evaluate accuracy and precision, five QC samples at each of these four concentrations were analyzed at three different occasions. To evaluate the method in routine use, in total 112 QC samples collected at 14 occasions and during 2 months were compared.

All plasma samples were subjected to ultrafiltration prior to injection into the LC–MS/MS system. Using an eight-needle robot (Genesis RSP150, Tecan AG, Hombrechtikon, Switzerland) 120 μL plasma was transferred to a 96-well ultrafiltration plate with a collector plate (Multiscreen Ultracel PPB, Millipore Corp., Danvers, MA, USA) and 120 μL 500 nM internal standard in 0.05 M formic acid was added. The molecular weight cut-off of the ultrafiltration plate was 10 kDa. After mixing for 10 s, the plate was centrifuged at 2000 × *g* and 37 °C for 45 min. The collector plate with the ultrafiltrate, about 50 μL, was then placed in the cooled autosampler while awaiting injection.

2.3. LC–MS/MS

The chromatographic system consisted of a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) with cooled cabinets, two LC-10AD pumps and an SCL-10A controller (Shimadzu Corp., Kyoto, Japan). The column was an ACE 3 C18, 2.1 mm × 50 mm (ACT, Aberdeen, Scotland). The injection volume was 20 μL and the pumps were run in a binary gradient mode at a flow rate of 0.35 mL/min. Mobile phase A was 5% acetonitrile in 0.025 M formic acid and mobile phase B was 95% acetonitrile in 0.025 M formic acid. The gradient went from 0 to 75% B between 0.70 and 2.70 min and then rapidly back to 0% B, the effective time between injections was 3.5 min. Detection was performed using a Micromass Quattro Micro mass spectrometer with MassLynx 3.5 software (Micromass Ltd., Manchester, UK). The instrument was operated in the positive electrospray ionization (ESI) mode and responses were measured using multiple reaction monitoring (*m/z* 421 → 254 for the analyte and *m/z* 426 → 254 for the IS).

2.4. Calculations

The internal calibration results were calculated using

$$C_A = \frac{\text{area}_A}{\text{area}_{IS}} \frac{C_{IS}}{RF} \quad (1)$$