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With or Without Internal Standard in HPLC Bioanalysis. A Case Study

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Abstract

The mandatory strategy of using internal standard in HPLC is still controversial. Despite that the introduction of internal standard methodology in the early stage of development of HPLC technology was used to improve method accuracy and precision, there are still practical situations in which a simple external standard quantification is adequate. The aim of the study is to compare the determination of meloxicam (MXC) in human plasma by HPLC with or without using an internal standard according to some key points related to the reason of introducing the internal standardization such as the reducing of sample preparation errors or variability for low injection volumes. The HPLC analysis was performed on reversed phase with UV detection after protein precipitation. Piroxicam (PXC) was used as an internal standard. The two methods are compared in terms of accuracy and precision over the same concentration range. The stability of the analyte has been proved. According to the results, the quantitative determination of MXC in human plasma after simple protein precipitation by using PXC as an internal standard does not bring any significant improvement of accuracy and precision of the experimental measurements.

Introduction

Meloxicam [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1,2-benzothiazine-3-carboxamide-1,1-dioxide] is a non-steroidal anti-inflammatory drug. Unlike other oxicam derivatives, meloxicam (MXC) preferentially inhibits cyclooxygenase-2, but not selectively as coxibs. It inhibits prostaglandin synthesis, with analgesic, antipyretic, anti-inflammatory effects and without consequences on platelet aggregation (1–4).

The bioavailability of MXC after oral administration is 89%, but it has a high degree of plasma protein binding (99%) and low apparent distribution volume. MXC is metabolized to four biologically inactive metabolites and the main metabolite is 5-carboxymeloxicam. MXC is frequently prescribed for symptom control in rheumatoid arthritis, osteoarthritis in humans, and recent studies showed that this drug has antinociceptive and antiedematogenic effects in acute models of nociception (5, 6).

Several methods for the determination of MXC in human plasma were published. The most commonly used method was liquid chromatography: high-performance liquid chromatography with UV detection and LC–tandem mass spectroscopy (LC–MS-MS) (7–18). Sample preparation was performed mostly by protein precipitation, applying either the external standards (ESTDs) or the internal standard (ISTD) quantification method.

As it is well known, the ISTD quantification reduces the effect of interfering matrix components, minimizes the sample processing errors and the variability of detection. However, the ISTD method was often used even if it is not absolutely required and sometimes could be a source of variability (19).

In order to assess that was necessary to use an internal standard for quantitative determination of MXC from human plasma after protein precipitation, a drug with a high degree of binding to proteins (6), two quantitative HPLC-UV methods were developed, one

based on ESTDs method and another one in which the calibration was made by using piroxicam (PXC) as an ISTD.

Experimental

Reference substances, reagents, blank plasma

MXC working standard was provided by Polfa-Grodzisk (Poland) and PXC (internal standard) was purchased from Nantong Jinghua Pharmaceuticals CO LTD (China). Acetonitrile, methanol, potassium dihydrogen phosphate, perchloric acid, triethylamine and other chromatographic-grade chemicals used in the tests were purchased from Merck (Germany). HPLC-grade water was obtained using a Direct Q5 water purification system (Millipore, Milford, MA, USA). Blank human plasma was obtained from the Blood Center Tîrgu Mureş, Romania being used for the preparation of calibration standards in plasma (STD), quality control samples (QC) and those necessary for evaluating stability of MXC.

Chromatographic conditions

"ESTDs method". The HPLC system used for the determination was an Agilent Technologies 1100 Series and the analytical column was C8 Eclipse XDB (5 μm, 150 mm × 4.6 mm, Agilent), protected by a C8 precolumn (25 mm x 4 mm, Merck). The column temperature was set at 45°C. The mobile phase contained a mixture of 77% potassium dihydrogen phosphate 50 mM with triethylamine (0.3% *V/V*) and acetonitrile 23%, at it was delivered with a flow rate 1.7 mL/min. The detection wavelength was set at 364 nm. "ISTD method". The same equipment and the same chromatographic conditions were used as for external standard method, except the chromatographic column which was a C8 Solvent Saver Plus SB-C8 type (3.5 μm, 100 mm × 3 mm, Agilent), protected by an in line filter, and the phosphate concentration was 20 mM.

Preparation of standard solutions and quality control samples

Two stock solutions of MXC, one in methanol (300 μg/mL) and another in plasma (3 μg/mL) were used. Seven calibration standards of MXC (concentrations: 15, 30, 150, 900, 1,500, 2,100, 2,400 ng/mL) and three QC samples (concentrations: QCA 45 ng/mL, low level, QCB 1,200 ng/mL, medium level, and QCC 1,800 ng/mL, high level) were prepared independently by mixing for 5 s certain volumes of stock solution in plasma with different volumes of blank plasma. The standard plasma samples for stability studies were prepared at two levels of concentrations, 45 ng/mL (low level) and 1,800 ng/mL (high level), four times each, by mixing fixed volumes of stock solution in plasma (3 μg/mL) with blank plasma. The standard samples for dilution validation at a concentration of 7,200 ng/mL were prepared by diluting the stock solution (300 μg/mL) with blank plasma for 15 s, with a vortex mixer, then diluted further with plasma at 1,440 ng/mL. The recovery of MXC was measured at three concentration levels (45, 1,200, 1,800 ng/mL). For ISTD method, a stock solution of PXC in plasma (3 μg/mL) was added to STDs and QCs to provide a final concentration of 600 ng/mL PXC before samples processing.

Preparation of plasma standard samples before chromatographic analysis

All the plasma samples were prepared as follows: the proteins from a volume of 0.5 mL plasma sample were precipitated with 75 μL perchloric acid 20%; the mixture was stirred for 15 s with a vortex-mixer, let a minute to rest, then it was centrifuged at 10,000 rpm, for 10 min. The supernatant obtained was transferred into a vial and an appropriate aliquot was injected into the HPLC system.

The performances of the analytical methodology

The tested analytical performance parameters are described in standard guidelines (20, 21). "Specificity". The specificity was examined by screening six different batches of pooled human plasma. The tests were accomplished to confirm the absence of interfering compounds from endogenous plasma components. "Calibration curve". The linearity of the method was evaluated from the standard calibration curve obtained by using several concentration levels (15–2,400 ng/mL MXC), in at least five different series, by estimating appropriate accuracy for at least five calibration points, including the lowest and the highest calibration points. "Accuracy and precision". The intra-series accuracy and precision were determined by analyzing five different QC samples of MXC at three different concentrations. The accuracy was calculated as relative error of found values against theoretical concentration (Er%) and precision as the coefficient of variation (CV%). The acceptance criteria: the precision and accuracy values should be <15% of the actual values by considering at least three replicates, except the lower limit of quantification (LLOQ) where the parameters should not deviate by more than 20%. The inter-series accuracy and precision were determined by replicate analysis of a sample of each considered concentration of MXC in five different runs. "Lower Limit of Quantification (LLOQ)". The LLOQ was defined as a reproducible lowest concentration from the calibration curve (accuracy and precision had to be <20%). "Stability". The stability after preparation in the autosampler was tested as follows: the plasma standards were precipitated and the supernatants were analyzed immediately after preparation; the solutions were maintained in the autosampler to be reanalyzed after 24 h. The samples for the study of long-term stability were frozen below −20°C and the determinations were performed after one month. The samples for the study of freeze–thaw stability were frozen immediately after preparation at −20°C, thawed and then refrozen in three different days. In the day of analysis, the samples were processed in parallel with freshly prepared samples. "Recovery". In order to determine the retrieving efficiency (RE%), the samples prepared at three concentrations levels, five replicates for each concentration, were analyzed in the same day with the standard solutions obtained from stock solution in methanol and diluted with the supernatant from a protein precipitated blank plasma, at the concentration corresponding to plasma samples after preparation.

Results

Under the proposed chromatographic conditions, MXC is eluted in <5 min, for ESTD method, and PXC and MXC, in <3 min, for ISTD approach.

Analytical performance of the proposed chromatographic approaches

ESTD method

Specificity. Figures 1 and 2 show representative chromatograms of blank human plasma and a standard solution of MXC in plasma at LLOQ, respectively.

Linearity and LLOQ. The standard calibration curves on the MXC concentration ranges of 15–2,400 ng/mL in human plasma have had the mean mathematical model Area = 0.0172(±0.0078)c + 0.0142 (±0.065), with r > 0.997 and N = 7 calibration points. For the LLOQ of 15 ng/mL MXC, the accuracy was −3.8 and 2.3% for intra- and inter-series assays, respectively. The intra- and inter-series CV% for LLOQ were found to be 11.3 and 12.4%, respectively.

Accuracy and precision. The overall accuracy of the method, expressed as relative error (Er%) between found values and theoretical concentrations, was between −13.3% and 6.93%. The precision (CV%) had a variation within 0.9 ÷ 11.8%.

"Recovery". The mean recovery of MXC ranged between 59.6% and 65.6% for the three tested levels of concentration.

Dilution study. Dilution was performed with a CV lower than 5.3% and the accuracy ranged between 3.8% and 9.1%, for intra- and inter-series assays.

ISTD method

In the given chromatographic conditions, the analytes were separated in 3 min.

"Specificity". The chromatograms of plasma blank and plasma standards of MXC and ISTD are presented in Figure 3. The retention times were 2.7 min for MXC and 2.4 min for PXC, respectively.

Linearity and LLOQ. The plasma calibration curves were tested over the MXC concentration range of 15–2,400 ng/mL in human plasma with N = 7 calibration points. The mean calibration equation was Area ratio = 3.89(±0.02) concentration ratio +2.36(±0.01) and the correlation coefficient was >0.9980. The residuals for LLOQ of 15 ng/mL MXC were below 11%.

Accuracy and precision. The intra-series accuracy of ISTD method had values within −12.04 and 7.17% and the CV was between 6.99% and 12.29% for the selected three levels of concentrations.

Relative recovery of the analyte. The mean relative recovery was 96%, with a CV of 12.6% and it fitted within the limits of ±15%.

MXC stability

The stability was assessed under various conditions: (i) "Post-preparative stability". The percentage change in concentration was −3.4 and 0.02% at low and high concentration levels, respectively. (ii) "Stability in biological matrix at room temperature". The percentage change in concentration after keeping 4 h at the room temperature was −3.7 and 7.2% at low and high concentration levels, respectively. (iii) "Freeze–thaw stability". The percentage change in concentration of MXC was −14.3% at low concentration and −8.9% at high concentration. (iv) "Long-term stability". After 31 days at −20°C, MXC remained stable in human plasma, the concentration loss being −0.6% (at low concentration) and −9.04% (at high concentration).

Discussion

In order to develop a simple HPLC method for MXC determination in human plasma, with or without using internal standard, a reversed-phase mechanism was chosen. A C8 column was used as a compromise between the hydrophobicity of MXC and the versatility and stability of reversed stationary phase with alkane moieties.

The ISTD method was developed not only to solve specificity against plasma endogenous compounds for both analytes, but to propose a fast method suitable for high throughput analysis. Thus, the column's length, diameter and stationary phase particles size were selected adequately. The injection volume was reduced 2.5 times as a consequence of using an ISTD.

Analytical performance of the proposed chromatographic approaches

ESTD method

No endogenous interference was observed at the retention time of MXC (4.3 min), for different tested plasma batches proving method's specificity. The residuals of the calibration points have had a random variation, without having a tendency of increasing or decreasing with concentration. The method exhibited good linearity over the considered range of concentrations and the LLOQ had a variation of accuracy and precision within acceptable limits, both for intra- and inter-day assay. Both accuracy and precision parameters tested for the selected three levels of concentration in relation to the calibration curve were found to be within ±15% limits indicating a reasonable intra- and inter-series accuracy and precision for the proposed method. The mean recovery of MXC was proved to be consistent and reproducible at the same three levels of concentration. Under the proposed conditions, samples above highest limit of quantification could be diluted with accuracy and precision, for both intra- and inter-series assay.

ISTD method

In the given chromatographic conditions, the analytes were separated in 3 min with good resolution. The specificity of the analytical method was investigated by confirming the complete separation of

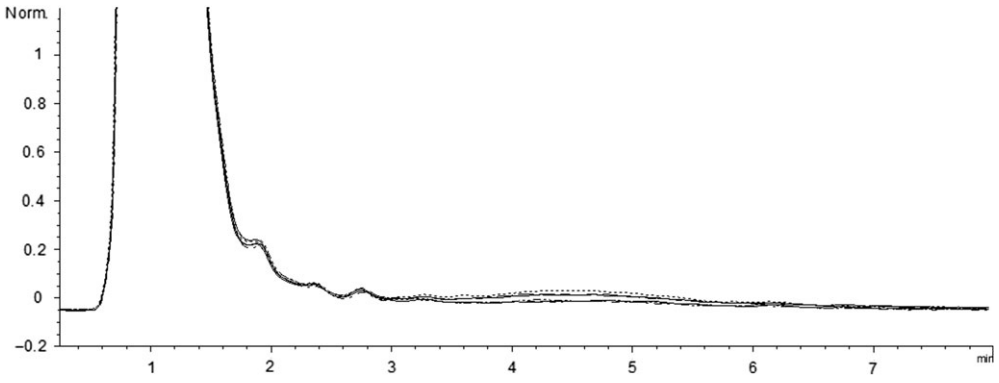

Figure 1. Chromatograms of different blank human plasma samples—ESTD method.

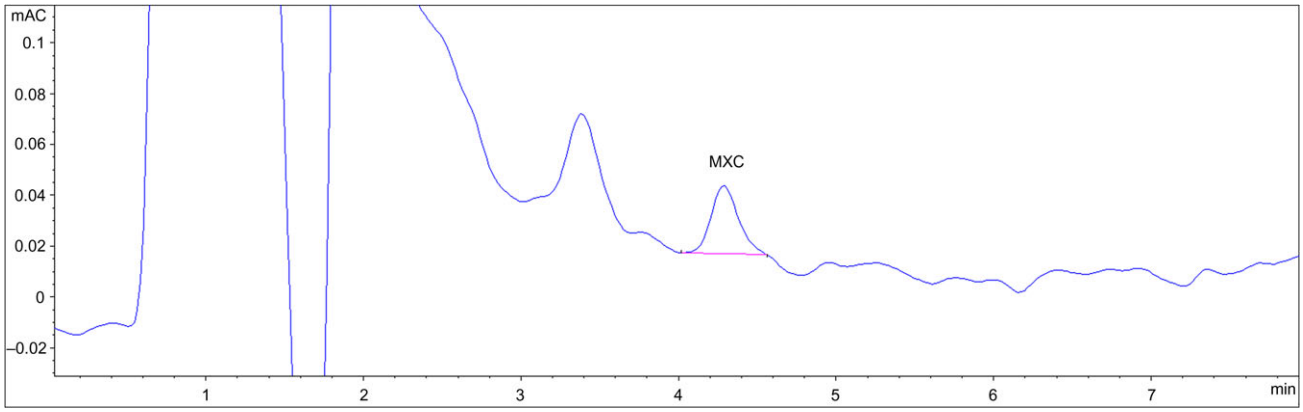

Figure 2. Chromatogram of a standard solution of MXC in plasma at lower limit of quantification—ESTD method. MXC, meloxicam.

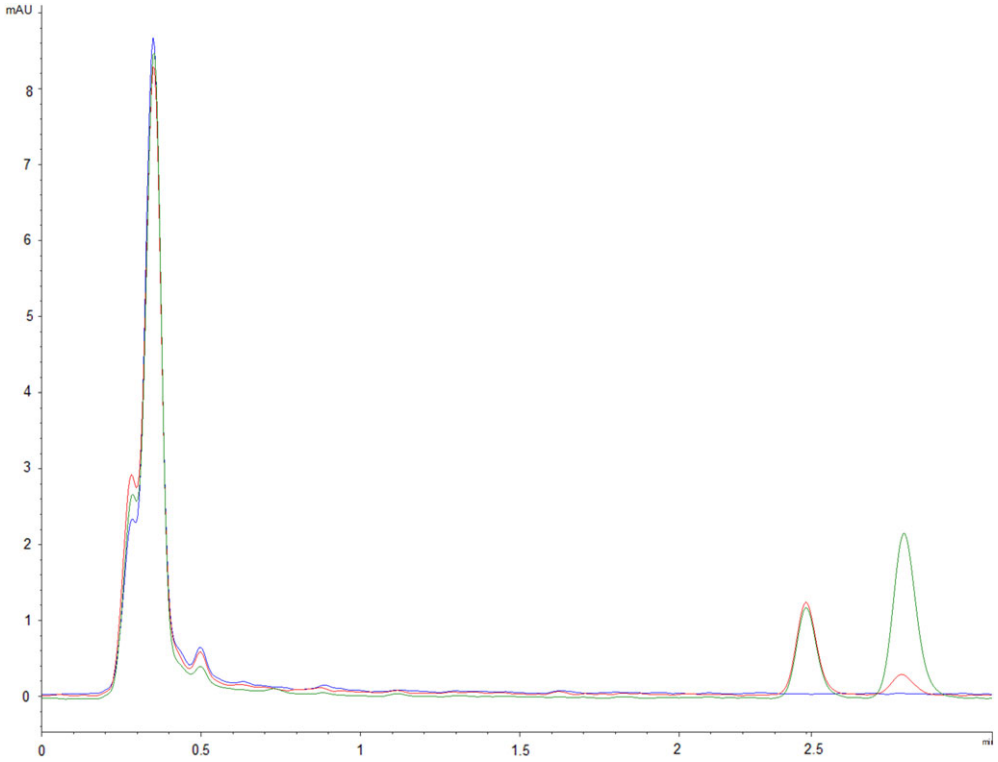

Figure 3. Overlaid chromatograms of blank plasma, lowest and higher calibration standards of MXC in plasma, MXC R_t = 2.7 min and internal standard PXC R_t = 2.4 min—ISTD method.

MXC and PXC peaks from endogenous compounds of human plasma samples obtained by spiking blank plasma with appropriate concentration of these compounds. No endogenous interference was found at the retention times of both substances. A linear calibration model was validated, residuals being inside the ±20% acceptance for LLOQ and within ±15% for the other levels of concentration. The intra-series accuracy and precision fitted between the acceptable limits of ±15% for the selected three levels of concentrations. The mean relative recovery was high and it had a variation that respected the limits between ±15%.

MXC stability

It has been demonstrated that MXC is stable in the selected conditions that are related to sampling, processing and analysis processes.

Methods comparison

It is a well-known fact that a quantitative chromatographic analysis by ISTD is recommended for determining analytes in biological samples when the sample preparation procedure has many steps or volumetric losses could occur, or the analytes reside in samples whose matrices have strong adsorption properties. It is a simple principle. The ISTD has to be added in the same concentration in samples and calibration standards at the beginning of sample preparation and the quantification is further performed by considering area ratio vs concentration ratio. Therefore, any volume loss during processes introduces the same error for analyte and ISTD and the area ratio remains practically unaffected.

Relatively recent, Dolan has described the ISTD concept in column chromatography with simulated data and important aspects

regarding the choice of internal standard (22, 23): the ISTD should not be a part of sample matrix; the mechanism of interaction with mobile and stationary phases has to be similar to analyte of interest but its peak has to be well resolved at baseline from other compounds (not absolutely necessary in liquid chromatography coupled with mass spectrometry, here the "resolution" being guaranteed through detection); it would be better if ISTD is eluted after analyte (in this case if the retention time of ISTD remains constant, that it means the chromatographic conditions until the retention time of ISTD work well); the ISTD has to be available in high degree of purity and it has to be stable under sample preparation and chromatographic conditions (otherwise the impurities and degradation products of ISTD could reduce the specificity by co-elution with analyte and the ISTD area will not resemble the right concentration); the ISTD is needless to have similar structure with the analyte (even if this condition guarantees similar extraction and chromatographic manner, it is possible that a different structural compound could convincible assure that all the previous conditions mentioned before and the analyte could be more affordable than a chemical related substance).

In our experiment, it is obvious that the internal standard, PXC, meet all the criteria necessary such as structural resemble, purity, it is not an endogenous compound in human plasma, it has the same protein-binding behavior as MXC (24, 25) and the chromatographic conditions allow specificity and baseline separation between neighbor peaks.

The methodology of internal standard quantification was introduced in the early stage of column chromatography in order to correct volume errors due to several factors such as poor precision of the old injection systems and errors during sample preparation such as liquid–liquid extraction or other complex methods with several steps of preparation (26, 27). The use of ISTD increases the costs, including time cost for method development and the analysis run time. On the other hand, the use of an internal standard will not correct data accuracy or it could be a source of variability: if the samples were not properly homogenized before adding the ISTD; the ISTD is added by weighting (in some cases) or as a solution (frequently), both methods being sources of variability due to manipulation errors during these processes. Usher *et al.* (28) analyzed scientific literature regarding the comparison of internal and external methods in HPLC in terms of precision. The authors demonstrated that in case of using low injection volumes, the precision is not improved by using an internal standard. As it was found in our work, the volume of injection for the second method was reduced for column protection reason but the internal standard addition did not improve the precision of the measurements. Concerning the optimum quantity of ISTD, references from the same authors indicate that the quantity of ISTD has to provide a peak with a similar area as for analyte (26) or should be as much as possible similar to the highest calibration point (29) but their results did not confirm it. The best approach should involve an experimental design in order to determine the optimal concentration of ISTD (30). In our work, the concentration of ISTD was selected within the lower half of the quantification domain taking into consideration a high variability for lower concentrations of the analyte.

By comparing the proposed two methods in terms of accuracy and precision for inter-series analysis, it has been found that the presence of internal standard improves moderately the accuracy of measurements for lower concentrations (−0.7% compared to 6.9%), but not for the high ones (−12.04% compared to 3.04%). Precision was not improved (6.99 ÷ 12.29% vs 7.94 ÷ 11.8%). The comparison of the accuracy and precision between ESTD and ISTD methods showed no statistical differences between pair values ($P > 0.05$, non-parametric test—Wilcoxon matched pairs). A possible explanation regarding the lack of accuracy and precision improvement is related to the protein-binding properties of the two substances. MXC and PXC are tightly bound (over 98%) and interact by strong electrostatic forces with high-affinity site located in subdomain IIA of human serum albumin, warfarin site I (6, 25, 31). Therefore their competition for the same site lead to displacement interaction in plasma mixtures and this phenomenon is concentration ratio dependent. It is obvious that in our proposed ISTD method the ratio between MXC and PXC (ISTD) is changed over the calibration range. More, the variability could be also generated by the relatively weak recovery for drugs having higher plasma protein-binding ability during sample preparation (32).

Conclusion

In conclusion, the quantitative determination of MXC in human plasma after simple protein precipitation by using PXC as an internal standard does not bring a significant improvement of the accuracy and precision of measurements. Our results confirm the statements of Usher *et al.* (28) and Dolan (22), according to whom it is first necessary to check that the instrument works suitably, including the injection system, then it is better to make a kind of pre-validation with or without internal standard and decide, according to obtained data, that the internal standard is requested or not.

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Previous presentation

Partial results were communicated as poster presentation at XIIIth Romanian National Congress of Pharmacy.

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