Fast LC-MS-MS Determination of Metformin in Human Plasma with Simultaneous Liquid-Liquid Extraction and Derivatization

E. Fedorov; J.-F. Larocque; A. Pimenov; M. Mancini

WARNEX Bioanalytical Services, Laval, QC, Canada

Overview

- *Purpose:* to develop a versatile, rapid and sensitive LC-MS-MS method for the determination of metformin in bioequivalence studies on combination drugs and extended release formulations.
- *Methods:* liquid-liquid extraction with simultaneous derivatization followed by a reverse phase LC-MS-MS determination.
- *Results:* the MS behavior of obtained derivatives is investigated, an analytical method with targeted parameters is developed and validated. The possibility to meet even more demanding requirements without any substantial modification of this method is demonstrated.

Introduction

Metformin is a well-known oral antihyperglycaemic agent used in treatment of type 2 diabetes. To address the pharmocokinetics of new extended release combination formulations of metformin, a simple, robust and sensitive analytical method for an accurate measurement of low concentrations of metformin in human plasma is required.

Recently, two rapid and sensitive liquid chromatography – tandem mass spectrometry (LC-MS-MS) methods for the determination of metformin in human plasma were published [1,2], using protein precipitation without derivatization. Since the retention of metformin by reversed phase HPLC mechanism is poor, both methods are using hydrophilic interaction chromatography (HILIC) conditions, the first one on cyano HPLC column [1], the other using secondary interactions with residual silanols of a C8 sorbent [2]. This approach requires mobile phases with high acetonitrile content, thereby enhancing the atmospheric pressure ionization. The most important disadvantage of the HILIC conditions in this particular case is that it would not be suitable for simultaneous determination of multiple analytes from a combination drug treatment in one chromatographic run, where the other analytes having lower polarity would not be retained in hydrophilic interaction chromatography, thereby requiring a separate method.

This LC-MS-MS method was developed to allow a versatile, rapid and sensitive determination of metformin in bioequivalence studies on combination drugs and extended release formulations using liquid-liquid extraction and simultaneous derivatization followed by a reverse phase chromatographic separation.

Methods

Metformin, phenformin and *p*-nitrobenzoyl chloride were obtained from Sigma-Aldrich Co. All solvents used were HPLC grade from Fisher Co., all other reagents were ACS grade.

A Perkin-Elmer series 200 pump and autosampler were used for injection and chromatographic separation. An API-III+ triple quadrupole mass spectrometer (PE Sciex) with a Heated Nebulizer (APCI) source was used as an MS-MS detector.

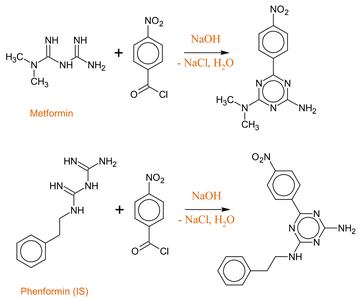
The plasma samples were allowed to thaw and reach room temperature. An aliquot of 200µl was taken and transferred to a screw-cap tube and 25µl of a solution of internal standard phenformin (500 ng/ml in purified water) was added. The extraction/derivatization step was performed with 0.5 ml of a solution of 4-nitrobenzoyl chloride in dichloromethane (10mg/ml) after addition of 0.5 ml of 10% aqueous sodium hydroxide. After 1 hour on a reciprocating shaker at room temperature, the phases were inversed with 4ml of ethyl acetate and separated by flash-freezing in an ethanol-dry ice bath. The organic extract was evaporated at 40°C under a gentle stream of nitrogen and the sample was reconstituted with 0.25 ml of mobile phase.

The chromatographic separation was achieved using isocratic conditions on a Luna-C18 (2) 3µ 50×3mm HPLC column (Phenomenex) with a corresponding SecurityGuard 4×2mm pre-column and a 0.5µ pre-filter (Upchurch) at room temperature. The mobile phase consisted of methanol, acetonitrile and water (6:1:3, v/v) with 10mM of ammonium bicarbonate at a flow rate of 0.8 ml/min. The injection volume was 20µl.

The mass spectrometer was operated in positive unit resolution mode, with nebulizer temperature set at 550°C and interface temperature set at 60°C. UHP nitrogen was used as curtain gas (1 l/min), nebulizer gas (80 psi) and auxiliary gas (2 l/min). The UHP argon was used as collision gas (CGT=300). For quantitative purposes, the data were collected in MRM mode with a dwell time of 150 msec, monitoring the transition with mass to charge ratio (*m/z*) 261→96 for metformin and 337→105 for phenformin.

Results

As shown elsewhere [3,4], metformin and phenformin both form *s*-triazine derivatives with *p*-nitrobenzoyl chloride in presence of a strong alkali:



This derivatization reaction is easily combined with a liquid-liquid extraction of the reaction products, both the reagent and the derivatives being more soluble in organic solvent than in water. The excess reagent is hydrolyzed to sodium *p*-nitrobenzoate which is extracted to the aqueous layer. The optimal reaction/extraction conditions giving quantitative yield and recovery have been determined by Tache *et al.* [4]. Since the best solvent for this was dichloromethane, a further modification of the method was performed to simplify the phase separation step by reversing the aqueous and organic layers with ethyl acetate after completion of the reaction. This allowed the use of flash-freeze technique to separate the organic extract.

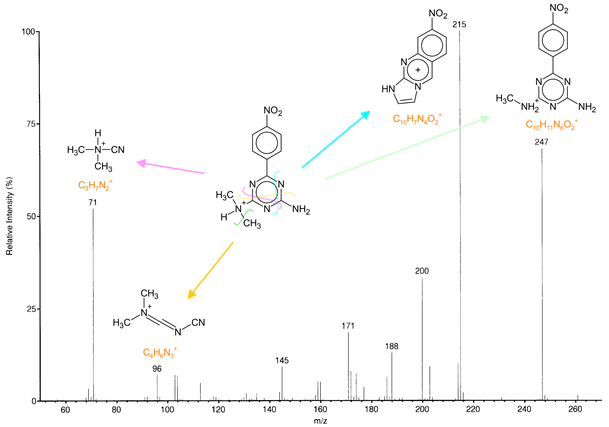


Figure 1. Product ion mass spectrum of protonated molecular ion of metformin derivative at low collision energy.

The *s*-triazine product formed in this reaction is much less polar than the original metformin molecule and exhibits good retention in reverse phase HPLC conditions, similar to that observed for other antihyperglycaemic agents used in combination drugs, making it possible to perform the analysis in the same chromatographic run.

Both derivatized metformin and phenformin produce intense protonated molecular ions in the APCI source. These ions undergo a collision-induced fragmentation in the collision cell giving intense fragments. Depending on the collision energy, the most intense fragments for the metformin derivative have *m/z* 71 or 215 (*Figure 1*). For the phenformin derivative these are with *m/z* 105 and 291 (*Figure 2*).

It is assumed that during the fragmentation these triazine cations undergo skeletal rearrangement to give the condensed aromatic structures that can be tentatively assigned to fragments with *m/z* 215 obtained from protonated molecular ion of the metformin derivative and the corresponding fragment with *m/z* 291 from the phenformin derivative.

Regarding the low molecular weight fragment with *m/z* 71 from the metformin derivative, the same fragment is reported to be one of the most abundant from the underivatized metformin molecular ion fragmentation [1,2]. For the fragment with *m/z* 96 there is a similarity with a well known class of triazine pesticides, giving a fragment with the same *m/z* in their MS-MS spectra [5].

Unlike these fragments which are most probably produced from the parent ion through the breakage of the triazole ring, the fragment with *m/z* 105 from phenformin derivative seems to be a product of further fragmentation of the daughter ion with *m/z* 291. It should be noted, however, that it is isobaric to the phenyldiazonium ion, but it is difficult to elucidate what the mechanism is for the formation of such a fragment from this triazine structure.

To better understand the fragmentation pathways of these s-triazine compounds, ion-trap multiple fragmentation experiments should be performed, which is beyond the scope of the present work.

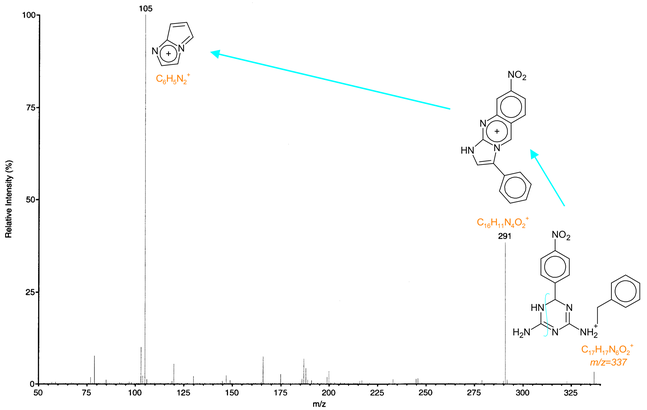


Figure 2. Product ion mass spectrum of protonated molecular ion of phenformin derivative at low collision energy.

The best sensitivity could be obtained with *m/z* 261→71 transition, but the signal was attenuated by using the transition with *m/z* 261→96 which is less intense by more than one order of magnitude. This was dictated by the fact that the required range for the method was 25 to 3000 ng/ml and the linearity range of the API-III+ mass spectrometer is narrow. Even with this transition, as is shown in the chromatogram at the LLOQ level 25 ng/ml (*Figure 3*), the limit of detection is about 2 ng/ml. It is evident that using the most abundant transition, the range of this method can be easily lowered to an LLOQ of less than 1 ng/ml without any change in the sample preparation or chromatography.

The calibration was linear in the required analytical range. No interferences from plasma or other matrix effects were observed. The triazine derivatives show no sign of degradation during several days at room temperature in the injection vials. The method was validated in our laboratory according to the FDA guidelines and was used in routine bioanalytical production.

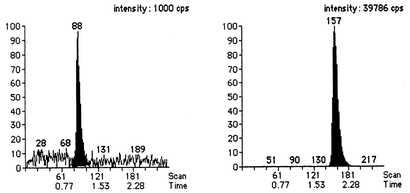


Figure 3. Representative chromatogram of extracted plasma LLOQ sample (25 ng/ml).
Left: metformin derivative, *m/z* 261→96, right: phenformin derivative, *m/z* 337→105

Conclusions

This LC-MS-MS method for the determination of metformin in human plasma with simultaneous liquid-liquid extraction and derivatization with *p*-nitrobenzoyl chloride is relatively simple, sensitive, fast and robust. It holds a potential to dramatically increase the sensitivity simply by choosing the most abundant transition without any change in sample preparation or HPLC conditions. Moreover, just a small change in sample preparation procedure is required to make this method suitable for the quantification of multiple analytes from a combination metformin drug in a single LC-MS-MS run. Further research efforts would be required to better understand the fragmentation pathways of the *s*-triazine derivatives of metformin and phenformin.

References

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