Altasciences CLINICAL RESEARCH ALGORITHME PHARMA

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A Strategy for Maintaining Chromatographic Peak Shape Due to Incompatible Extract and Mobile Phase by LC-MS/MS

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OVERVIEW

NOVEL ASPECT

An injection protocol eliminating incompatibility between extract and mobile phase solvent composition was developed in order to avoid peak shape distortion.

METHOD

Pomalidomide was extracted from human plasma by simple protein precipitation and extracts injected using a custom program designed to inject an aqueous plug along with the sample. The "effective" composition injected was compatible with initial chromatographic mobile phase composition.

RESULTS

While protein precipitation of pomalidomide with acetonitrile (ACN) in human plasma resulted in high recovery, it was only possible to dilute the supernatant 3-fold without compromising the LLOQ. The final composition of 25% ACN created poor peak shape when using an initial gradient of 5% ACN.

Introducing an aqueous plug in the autosampler needle during injection allowed for solvent compatibility between the extract and the initial separation conditions. It was demonstrated that the aspiration of the aqueous plug after sampling extract, or sandwiching the extract, furnished the desired symmetrical peak shape. Under these conditions, the targeted LLOQ of 500 pg/mL for pomalidomide was reproducible.

INTRODUCTION

Organic-based protein precipitation is one of the most common high-throughput techniques used for the extraction of analytes from biological matrices. However, the high percentage of organic solvent in the extract is often incompatible with reversed-phase chromatography. While the dilution of sample extracts with aqueous solvent can circumvent the distorted chromatographic peak shape, in many cases, challenging LC-MS/MS detection limits prevent this approach. Therefore, a cost-effective autosampler program was designed to eliminate incompatibility between extracts and the mobile phase solvent.

METHOD

SAMPLE PROCESSING

- Calibrants and QCs for pomalidomide were prepared in human plasma (K_2ETDA) for an analytical range of 0.500 to 150 ng/mL
- Pomalidomide-^{15}N-$^{13}C_5$ was used as the internal standard
- Samples were extracted by protein precipitation using ACN
- Supernatant was then diluted 3-fold with type 1 water prior to injection

CHROMATOGRAPHY

- Agilent Technologies Series 1100 pumps and autosampler
- Reversed-phase chromatography with gradient elution

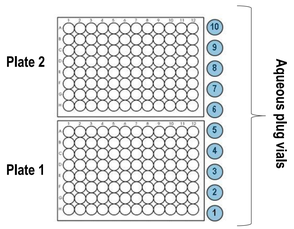

Figure 1. Representative sample tray in Agilent 1100 autosampler

- Custom Injection Program:

(**red** : aqueous plug before sample; **green**: aqueous plug after sample Red and green: sandwich aqueous plug)

  - **1: Draw 30 µL from vial 1, Draw 1 µL of air**
  - 2: Draw 20 µL from sample
  - 3: Wash needle in flush port for 10 seconds.
  - **4: Draw 1 µL of air, Draw 30 µL from vial 1**
  - 5: Inject onto column

DETECTION

- SCIEX API 5500, MRM acquisition in negative ion ESI
  - Pomalidomide: *m/z* 272.1 > 161.0
  - Pomalidomide-^{15}N-$^{13}C_5$: *m/z* 278.0 > 162.0

RESULTS

The primary objective of the study was to develop a cost-effective LC-MS/MS method for the quantitation of pomalidomide in human plasma with a targeted LLOQ of 500 pg/mL. Optimal mass spectrometric conditions were achieved in MRM mode for sensitivity and selectivity. A gradient chromatography with high aqueous content was necessary to allow the detection of the LLOQ. A simple protein precipitation extraction was developed for high-throughput.

While protein precipitation with ACN provided a high recovery of the analyte, it was only possible to dilute the organic supernatant 3-fold in order to achieve the LLOQ. However, the final content of 25% ACN caused peak shape distortion (fronting) when using the initial gradient conditions of 5% ACN.

The impact of the extract solvent strength on chromatography is demonstrated in Figure 2A, where an LLOQ prepared in pure solution at 25% ACN displays poor peak shape. On the other hand, the same LLOQ prepared in 5% ACN clearly shows a symmetrical peak shape (Figure 2B), thus indicating the necessity to inject the sample with a solvent composition similar to the initial mobile phase composition.

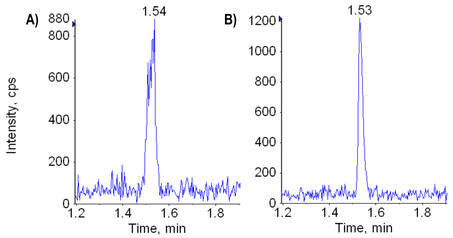

Figure 2. Impact of sample solvent composition on peak shape for an LLOQ reference solution (A) ACN:H_2O 25:75% v/v (B) ACN:H_2O 5:95% v/v

To allow solvent compatibility between extract and initial separation conditions, the introduction of an aqueous plug (type 1 H_2O) in the autosampler needle was evaluated via a custom injection program. The aqueous plug was either aspirated before or after the extracted sample or the extract was sandwiched between two aqueous plugs (Figure 3).

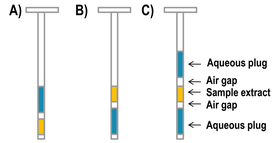

Figure 3. Representation of the aqueous plug and extract in the injection needle (A) plug aspirated before extract (B) plug aspirated after extract (C) extract sandwiched by plugs

Aspirating the aqueous plug prior to the extract failed to improve the peak shape when compared to an extract injected without any custom program (Figure 4A and 4B). However, aspiration of the aqueous plug after the extract or, sandwiching the extract with two aqueous plugs, provided the desired symmetrical peak shape with no major differences in retention time or response (Figure 4C and 4D).

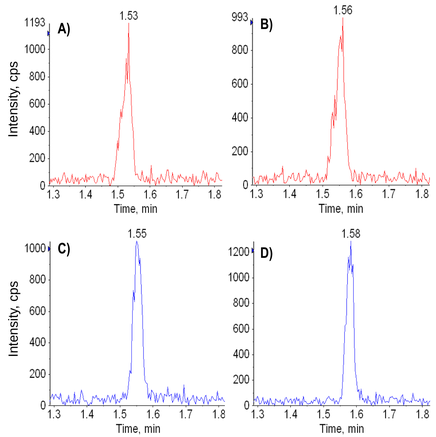

Figure 4. Chromatograms of an extracted LLOQ plasma sample (500 pg/mL in ACN:H_2O 25:75 v/v) injected (A) without aqueous plug (B) plug aspirated before extract (C) plug aspirated after extract (D) extract sandwiched by aqueous plugs

Using the custom injection program with the aqueous plug aspirated after the extract, a calibration curve was from 0.500 to 150 pg/mL (r^2 > 0.995, weighted 1/x), no carry-over was observed and all acceptance criteria were met (Table 1).

The configuration aspirating the aqueous plug after the extract rather than the sandwiched approach was preferred for higher throughput purposes. Notably, to ensure proper function of the program, the total amount of aspiration volume (extract, aqueous plug(s) and air gaps) could not exceed the injection loop size (e.g. 100 µL). Depending on the batch size, the plug volume defined in the custom program may require several vials in the autosampler tray (Figure 1).

Table 1. Precision and Accuracy Parameters for QC levels of Pomalidomide

QC Level	QC LLOQ (500 pg/mL)	Low QC (1.50 ng/mL)	Medium QC (10.0 ng/mL)	High QC (112.5 ng/mL)
% CV	2.5	3.8	1.6	1.7
% Nominal	94.8	104.5	105.1	111.1

CONCLUSION

An HPLC custom injection program introducing an autosampler aqueous plug to the extracts was successfully developed for the analysis of pomalidomide in human plasma. The approach allows flexibility to inject different volumes or compositions of aqueous plugs, making the extracts compatible with HPLC conditions. This cost-effective protocol can be adopted for various compounds or solvents, negating the requirement for the time-consuming chromatographic optimization usually necessary to align extract and mobile phase compositions.

ACKNOWLEGMENTS

The authors would like to thank Hélène Montpetit for assistance in poster assembly and Jeff Plomley for technical editing.