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Shortcomings of protein removal prior to high performance liquid chromatographic analysis—A case study using method development for BAY 11-7082

A.K. Hewavitharana [a,*], C. Hyde [b], R. Thomas [b], P.N. Shaw [a]

[a] *School of Pharmacy, University of Queensland, Brisbane, Qld 4072, Australia*
[b] *Centre for Immunology and Cancer Research, Princess Alexandra Hospital, Brisbane, Qld, Australia*

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Abstract

During the analytical method development for BAY 11-7082 ((E)-3-[4-methylphenylsulfonyl]-2-propenenitrile), using HPLC-MS–MS and HPLC-UV, we observed that the protein removal process (both ultrafiltration and precipitation method using organic solvents) prior to HPLC brought about a significant reduction in the concentration of this compound. The use of a structurally similar internal standard, BAY 11-7085 ((E)-3-[4-*t*-butylphenylsulfonyl]-2-propenenitrile), was not effective in compensating for the loss of analyte as the extent of reduction was different to that of the analyte. We present here a systematic investigation of this problem and a new validated method for the determination of BAY 11-7082.

Keywords: BAY 11-7082; BAY 11-7085; HPLC-MS–MS; Protein precipitation; Ultrafiltration

1. Introduction

BAY 11-7082 ((E)-3-[4-methylphenylsulfonyl]-2-propenenitrile) is widely used as an inhibitor of cytokine-induced IκBα phosphorylation and consequently as an agent which results in decreased expression of NF-κB [1]. This compound is also reported to have some potential as an anti-inflammatory agent, or for use in antigen-specific tolerance [1,2] thus gaining popularity in biological and medical research. However, to date, there are no reported methods available for the quantitative analysis of BAY 11-7082. There are also no reports in the literature relating to the stability of this compound. We therefore decided to develop an analytical method for the quantitation of BAY 11-7082 using HPLC-UV and HPLC-MS–MS techniques.

It is a common practice to remove proteins from biological samples such as plasma and serum prior to their introduction into a reversed-phase HPLC system [3,4]. Proteins in the samples cause back pressure problems and a deterioration of the column performance due to the precipitation of proteins by organic solvents and buffers in the mobile phase [3,5].

During the analytical method development for BAY 11-7082 using an established protein precipitation method for sample clean-up, we noted that the compound was no longer detectable after incubation in tissue culture medium at 37 °C for 48 h. These observations, coupled with the knowledge of its use in cellular applications, and the lack of previous studies in this area, prompted us to undertake a systematic investigation on the stability of this compound. The aims of this study were to attempt to understand the observed reduction in BAY 11-7082 concentration, and to determine under what conditions the reduction occurred, including the role of tissue culture medium and the time course of any reduction in BAY 11-7082 concentration. We have also developed and validated a sensitive and highly specific method for the quantitative analysis of BAY 11-7082.

2. Experimental

BAY 11-7082 ((E)-3-[4-methylphenylsulfonyl]-2-propenenitrile) and BAY 11-7085 ((E)-3-[4-*t*-butylphenylsulfonyl]-2-propenenitrile) were purchased from United Bioresearch Products (Sydney, NSW, Australia). Stock solutions (1000 μM) of

* Corresponding author. Tel.: +61 7 3365 8853; fax: +61 7 3365 1688.
E-mail address: a.hewavitharana@pharmacy.uq.edu.au (A.K. Hewavitharana).

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both compounds were prepared in methanol, and were diluted to prepare working standards. All solutions were stored at −20 °C. Bovine serum albumin and phosphate buffer saline (PBS) were obtained from Sigma (St. Louis, MO, USA). The tissue culture medium used was X-VIVO 20 (Bio-Whittaker, Walkersville, MD, USA). Incubations were carried out in a humidified environment at 37 °C and 5% CO_2. All solvents used were of HPLC grade.

Chromatography was performed using an Agilent binary HPLC system consisting of an Agilent 1100 LC pump, an Agilent 1100 well plate autosampler, and a Cogent Aclarity-C18 (2.0 mm × 50 mm, 5 μM) HPLC column (Microsolv Technology Corporation, Long Branch, NJ, USA). An API 3000 tandem mass spectrometer equipped with a turbo ion spray interface and supported by Analyst 1.4 software (Applied Biosystems, Foster City, CA, USA) was used to detect the separated compounds and process all data. A Shimadzu model SPD-10A ultraviolet/visible spectrophotometric HPLC detector (Shimadzu Corporation, Kyoto, Japan) was also connected in parallel (to the waste line) and operated at 254 nm.

All solvents used (water and acetonitrile) contained formic acid (0.1%, v/v). An isocratic program consisting of 40% (v/v) acetonitrile–60% (v/v) aqueous was used for all separations when the multiple reaction monitoring (MRM) mode of detection was employed. When the total ion chromatography (TIC) mode of detection (below) was used the separation was undertaken using gradient elution in order to separate the early eluting peaks. The gradient was preceded by a 5 min isocratic segment consisting of 10% (v/v) acetonitrile–60% (v/v) water. The percentage of acetonitrile was changed from 10 to 40% within a 10 min period and then remained at this composition for 5 min before re-equilibrating the column with the original conditions (10%, v/v, acetonitrile–60%, v/v, water) for 6 min. The flow rate was 200 μL/min through the column. The post-column flow was split 60 μL/min to the electrospray of the mass spectrometer and 140 μL/min to the ultra-violet/visible detector (using a splitter and appropriate lengths of tubing). The sample injection volume was 100 μL unless otherwise stated.

Multiple reaction monitoring (MRM) mass spectrometry was performed and monitored the transition (*m*/*z*) of 208.3 → 91.6 (protonated molecular ion → major fragment of BAY 11-7082) at a dwell time of 1 s. An ion spray voltage (IS) of 5000, orifice/declustering potential (DP) of 26, ring/focusing potential (FP) of 110, entrance potential (EP) of 10, collision energy (CE) of 25 and collision exit potential (CXP) of 10 V were used for all experiments. The curtain gas (CUR), nebulizer gas (NEB) and collision gas (CAD) flows were maintained at 8 (in arbitrary units used in the instrument). The temperature of the ion spray was maintained at ambient and the resolution of both quadrupoles (Q1 and Q3) was 1 amu.

Total ion chromatography was undertaken in the range 70–300 (*m*/*z*) with 1 s scans. Other operating conditions were same as those for MRM above except CE, CXP and CAD which are not relevant for TIC.

Proteins were removed using two methods: precipitation of protein with acetonitrile (by adding 3 volumes of acetonitrile to 1 volume of sample) [3] followed by evaporation of the solvent (Savant Speedvac plus concentrator operated at 43 °C for approximately 2 h) and subsequent reconstitution in a volume of water similar to that of the original sample and ultrafiltration using Microcon YM-10 (10,000 Da cut off) centrifugal filter devices (Millipore Corporation, MA, USA). Each 0.5 mL sample aliquot was centrifuged at 12,000 × *g* for 1 h at 4 °C to remove molecules larger than 10,000 Da prior to injection on the HPLC.

In experiments using PBS buffer and albumin the buffer was prepared as per manufacturer specifications (0.01 M phosphate buffer saline) and the albumin concentration used in all samples was 0.5% (w/v), similar to the protein content of the cell culture medium. All samples contained 10 μM BAY 11-7082 unless otherwise stated, and the volume of injection was 50 μL.

3. Results and discussion

MRM mass spectrometry was performed to monitor the transition (*m*/*z*) of 208.3 → 91.6 (protonated molecular ion → major fragment of BAY 11-7082) in order to obtain a high degree of specificity in this study. In addition to the traditional LC–UV where the specificity is solely dependant upon the similarities in retention times between the sample and the standard LC–MRM mass spectrometry offers two additional degrees of specificity (mass of the analyte molecule and that of a collision induced fragment). This high degree of specificity is an asset in this type of studies where the matrixes such as cell culture medium are likely to contain compounds that are similar to the analyte and co-elute with it.

As part of an investigation into the use of BAY 11-7082 in tissue culture [6,7] we were interested to learn how much of this compound is retained by cells when incubated in an in vitro tissue culture medium. BAY 11-7082 was added to five samples of medium to a final concentration of 4 μM. The samples were ultrafiltered to remove proteins and quantitated as described in Section 2. The mean concentration obtained (±S.D.) was 0.33 μM (±0.02). These experiments indicated that there was very little BAY 11-7082 remaining in solution in the tissue culture medium after a few hours of incubation. This, coupled with the observation that the concentration of the compound was also markedly reduced in medium where cells were present (data not shown), suggested that the concentration of BAY 11-7082 was affected in some manner by the tissue culture medium.

To investigate whether the loss of BAY 11-7082 was due to degradation with time in aqueous conditions the following study was undertaken. A 10 μM aqueous solution of BAY 11-7082 was incubated at 38 °C in the dark for periods 0, 1, 2, 3 or 4 h and was then analysed by isocratic LC–MS (operating in MRM mode) and LC–UV (λ = 254 nm). The peak areas of the MRM mode (×10^6) were 1.67, 1.51, 1.38, 1.52 and 1.50, respectively and those of UV (×10^2) were 1.72, 1.61, 1.52, 1.54 and 1.59, respectively.

These results for both MS and UV indicated that there was no significant change in concentration after incubation in water even when the temperature was elevated beyond ambient. It is likely that the minor variations observed arise from variability in peak integration. These results suggest that the compound