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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

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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

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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

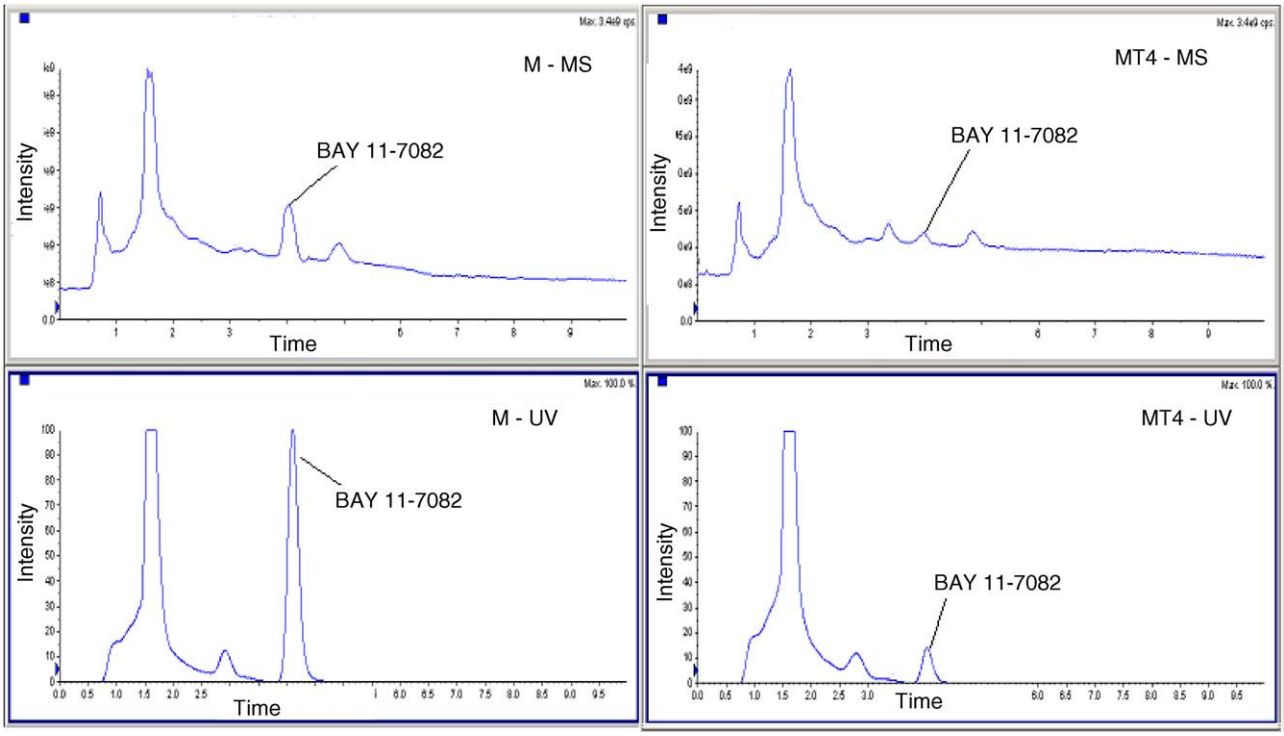

Fig. 1. Chromatogram of 100 μM BAY 11-7082 in the medium at 0 h – on the left (M) and 4 h – on the right (MT4). Both M and MT4 diagrams are on the same scale and the MS and UV detections are shown on top and bottom diagrams, respectively.

was not lost or chemically degraded with time to any significant extent, when incubated in aqueous solution, for a period of up to 4 h at 38 °C.

It is evident from the above observation that there is a clear difference in the results between those of an aqueous incubation and those following incubation in cell culture medium.

Our initial studies investigating the loss of BAY 11-7082 in aqueous conditions and in cell culture medium were undertaken using an MRM method with regard to its high sensitivity and selectivity required for quantitative analysis. However, in order to investigate further the stability of BAY 11-7082 and the possible compounds formed during the stability studies, the mass spectrometer was operated in the TIC mode, providing an opportunity to detect molecules other than BAY 11-7082. A 100 μM solution of BAY 11-7082 was prepared in cell culture medium and in water, and these were analysed by TIC MS after ultrafiltration. A blank medium was also included to examine the changes with time. All samples were analysed after 0 and 4 h of incubation. As described in Section 2 a shallow gradient was used in the separation so that all possible compounds in the samples would elute and separate. At this elevated concentration no significant differences were observed in water or the cell culture medium of the amounts of BAY 11-7082 at zero time. However, an approximately 80% reduction in BAY 11-7082 concentration was observed following 4 h incubation in the medium, as shown in Fig. 1, while the difference in concentration with time was again shown to be not significant when in water. A careful and thorough scanning and comparison of the MS spectra of each peak in the chromatograms at 0 and 4 h showed no differences except for that of the BAY 11-7082 peak. This suggests that degradation products were not present in the cell culture medium incubated for 4 h although there was a reduction of BAY 11-7082 by 80%. If the degradation products are not ionisable at the pH of the mobile phase they may not produce a significant MS signal. However, as there is an 80% reduction in the BAY 11-7082 a significant change can be expected in UV chromatogram as UV detection is unaffected by the ionisability of the molecule and the detection wavelength used (254 nm) is versatile. These studies provide evidence that the change in Bay 11-7082 concentration does not result in the formation of compounds within the molecular weight range of 70–300 (range used in MS-TIC studies).

The above results suggest that it is likely that BAY 11-7082 will have been removed when the sample of cell culture medium was ultrafiltered to eliminate proteins, i.e. some of the BAY 11-7082 must have bound to proteins possibly in a manner long recognised for drugs and other xenobiotics [8] and been filtered and removed concomitantly. If this were to be the case, the removal of protein by precipitation should release the protein bound BAY 11-7082 [3], since this method is recognised to denature proteins. To test this hypothesis we undertook a similar experiment using 2 μM BAY 11-7082 in the cell culture medium incubated for varying lengths of time, removing the proteins by the precipitation method using acetonitrile as described in Section 2, and the sample was quantitated by mass spectrometry using the MRM mode. The reduction in peak areas observed were 3, 60 and 99% at 1, 2, and 4 h incubation times, respectively, again confirming significant loss of the compound with time. A similar result was also observed at 50 μM concentration of BAY 11-7082 in the medium with

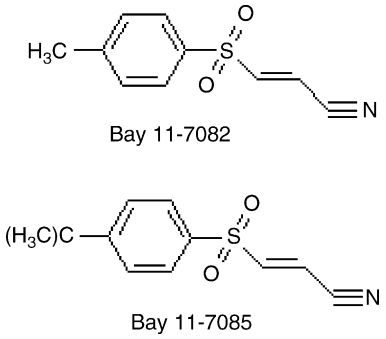

Fig. 2. Structures of BAY 11-7082 and BAY 11-7085.

time when the same precipitation method was used to remove proteins.

The structure of BAY 11-7082 (Fig. 2) suggests that a proton alpha to the sulfone would be relatively easily extracted and the subsequent carbanion stabilised by the sulfone. The stabilisation of carbanions by adjacent sulfone groups has been long recognised [9,10]. The carbanion would be an electron-rich centre and therefore susceptible to reaction with a variety of electrophiles. It is possible to speculate that electrophilic groups capable of this interaction may be present in the proteins in the cell culture medium to form covalent adducts with BAY 11-7082. Such covalent adducts are not likely to dissociate at the denaturing process during precipitation. To test this hypothesis a buffer solution containing bovine serum albumin was used in place of the cell culture medium. As described in Section 2, a 10 μM concentration of BAY 11-7082 was added to all samples, each treatment was carried out in triplicate and quantitation carried out using both MS (MRM) and UV. A blank was also included from which BAY 11-7082 had been omitted. Protein was removed by ultrafiltration or by precipitation with acetonitrile as described in Section 2. The results are shown in Table 1. The results are similar to those obtained with the tissue culture medium in that the concentration of BAY 11-7082 was reduced by over 95% following both ultrafiltration and precipitation of the bovine serum albumin while the loss of BAY 11-7082 in buffer (with no albumin) was reduced only about 5%.

We therefore conclude that it appears that BAY 11-7082 reacts with the protein components of the tissue culture medium potentially to form covalent adducts. Such adducts are then removed when the protein is separated during sample preparation either by ultrafiltration or by precipitation. This is a potential problem in quantitative analysis of small molecules in biological samples if protein removal is used for sample cleanup.

Although the recovery of the method is poor due to the reasons described so far in this paper we have nevertheless carried out the validation of the method for its potential use for quantitation of BAY 11-7082 as there were no reported methods available for the quantitation of this compound using any analytical technique. Several different volumes (1, 5, 10 and 50 μL) of a 4 μM standard were injected and peak heights were plotted against the amounts injected. The on-column limit of detection (LOD) was determined by computing the peak height corresponding to 3× noise. The LOD of BAY 11-7082 with MS (MRM method) was 0.45 pmol, and that with UV was 6.2 pmol. For a typical volume of injection in this study of 50 μL the corresponding LOD values are 9 nM with MS and 0.12 μM with UV detection. The detector responses were linear at least up to 0.5 nmol. The repeatability coefficient of variation ($n = 9$) was 7.5% when ultrafiltration method was used for sample clean up. The recovery studies were carried out at two levels (4 and 8 μM additions to the blank culture medium followed by ultrafiltration and LC–MS–MS) using five replicates each. The mean recovery values were 0.33 μM (±0.02) for 4 μM addition and 0.89 μM (±0.06) for 8 μM addition (i.e. 8.4 and 11% recovery, respectively). The reason for low recovery is the possible covalent binding of BAY 11-7082 to proteins in the medium as discussed earlier.

We have also investigated the possibility of incorporating an internal standard to this method so that if the losses of both BAY 11-7082 and the internal standard were comparable then BAY 11-7082 could be quantitated even after protein removal. Fortunately, a compound of very similar chemical structure to BAY 11-7082 was commercially available: BAY 11-7085, the only difference in structure being that a methyl group of BAY 11-7085 is replaced by a tertiary butyl group (Fig. 2). The recovery study was carried out using the putative internal standard (BAY 11-7085). The recovery of BAY 11-7082 was 8.4% (%S.D. = 5.8%, $n = 5$) and that of BAY 11-7085 was 40% (%S.D. = 5.8%, $n = 5$). The internal standard was also lost in the same manner as BAY 11-7082, following the protein precipitation sample preparation but the percentage loss (∼60%) was different to that of BAY 11-7082 (∼90%). Therefore, when internal standard method is used for calculations the recovery of BAY 11-7082 changed from 8.4 to 21% for 4 μM addition of the compound and from 11 to 27% for a 8 μM addition. This means that using BAY 11-7085 as an internal standard to compensate for the loss of BAY 11-7082 due to protein removal is not a feasible option.

An isotope labelled (deuterated or C^{13} labelled) BAY 11-7082 could have been a better alternative to BAY 11-7085 as the internal standard because there is a better chance of the recoveries being similar. However, isotope labelled BAY 11-7082 was not commercially available yet. If this become available,

Table 1
Percentage loss of BAY 11-7082 with time at various conditions

Sample	2 h (MS)	2 h (UV)	4 h (MS)	4 h (UV)
B	6.2% (9.3%)	0.10% (1.5%)	4.7% (12%)	−1.3% (1.3%)
PB-uf	–	–	95% (1.3%)	97% (5.2%)
PB-ppt	82% (20%)	77% (2.3%)	98% (20%)	97% (2.2%)

Sample key: B, compound in buffer; PB-uf, compound in buffer and protein, after ultrafiltration; PB-ppt, compound in buffer and protein after protein precipitation. All numbers are means of triplicates with the coefficients of variation in parentheses. The 2 h experiment was not carried out for PB-uf sample.

provided that the recoveries of BAY 11-7082 and the internal standard have very similar recoveries when mixed with the cell culture medium the loss of analyte due to protein binding can be corrected.

4. Conclusion

A potential problem in quantitative analysis of small molecules in biological samples has been identified and investigated using the drug BAY 11-7082. This problem was also evident with the drug BAY 11-7085 which we attempted to use as an internal standard. Protein removal by ultrafiltration and precipitation resulted in similar losses when the compound was in solution either in tissue culture medium or in PBS buffer-albumin solution implying that the losses are due to covalent binding to protein. A validated method with high sensitivity and specificity is presented for quantitative analysis of BAY 11-7082.

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