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Direct injection for high sample throughput capillary gas chromatographic–mass spectrometric bioanalysis

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Abstract

Because of the drawback of the relatively long analysis times inherent to temperature-programmed splitless injection capillary GC–MS, isothermal direct injection capillary GC–MS was investigated for quantitative bioanalysis. Using extracts from spiked plasma samples, we showed that high quality chromatography with a run time much shorter than that achievable with splitless injection can be achieved with direct injection. Sensitivity and other performance parameters were as good as or better than those of the splitless method. Since sample throughput is of great importance in laboratories that analyze thousands of biological samples, it is recommended that, when possible, splitless injection, which has traditionally been used in trace level GC–MS bioanalytical methods, be replaced by direct injection.

1. Introduction

Capillary GC–MS is widely used for the quantitative determination of drugs and metabolites in biological matrices such as blood, plasma and urine for studies of bioavailability and pharmacokinetics. The main advantages of GC–MS are excellent sensitivity, specificity, and the ability to utilize a stable isotope analogue of the drug for co-administration with the drug or as the analytical internal standard [1–3]. For trace level (sensitive) bioanalytical methods based on capillary GC or GC–MS, the most commonly used mode of introducing the sample onto the chromatographic column has been the splitless injection, which permits transfer of the injected sample onto the column without splitting the sample in the inlet. The splitless and other modes of capillary GC injection are thoroughly treated in a recent book by Grob [4]. There are basically two ways to carry out splitless injection successfully. Both approaches rely on reconcentrating bands broadened in time as the result of slow introduction of sample vapor onto the column, which is inherent to splitless injection. One method utilizes cold trapping in which band reconcentration is achieved by the use of a relatively low initial column temperature so that the solute (analyte) does not migrate in the column during the period of the slow transfer of the vaporized sample into the column. In practice, cold trapping is achieved by keeping the column temperature about 90°C below the elution temperature of the solute (analyte) for about one minute and then starting the temperature programming. The other method utilizes the

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solvent effect (solvent focusing) in which band reconcentration is achieved by recondensation of the solvent in the column; thereby the solvent acts as part of the stationary phase which results in a temporarily increased retention of the solute. This effect is achieved by employing an initial column temperature which is lower than the boiling point of the solvent by at least 20–30°C. In the authors' laboratory, both modes of splitless injection have successfully been used for bioanalysis. In two examples where cold trapping was utilized [5,6], the solvent used for injection was toluene (b.p. 110.6°C) and the initial column temperature was 220°C in one and 180°C in the other example. Although the initial column temperature in each case was above the boiling point of toluene, the column temperature was low enough to cold trap the analyte. In the example where the solvent effect was used [7], the solvent used for injection was *n*-tetradecane (b.p. 253°C) and the initial column temperature was 210°C, well below the boiling point of the solvent.

Although splitless injection via cold trapping or solvent effect works fine, it usually requires column temperature programming. The undesirable consequence of temperature programming is a relatively long analysis time. For instance, if the method employs an initial column temperature of 210°C and an elution temperature of 300°C, it will take 3.0 min just to get to the elution temperature when the column temperature is programmed at a rate of 30°C per min. Adding a pre-programming splitless period of 1.0 min and a cooling period of 1.0 min, a recycle time of at least 5.0 min is thus required. For GC–MS methods developed for one or two analytes, the need for a long chromatographic run time to achieve chromatographic separation usually does not exist. Thus, the relatively long time required for the temperature program cycle is wasted time. This overhead time of the splitless injection is costly for a high sample-throughput laboratory charged with analyzing thousands of biological samples. The overhead could be eliminated by using an injection mode, which, like splitless injection, allows the transfer of all injected sample into the chromatographic column but which, unlike splitless injection, does not rely on cold trapping or solvent effect. Thus, temperature programming would not be required. The so-called direct injection technique [8–10], in which the sample is flash vaporized in a liner which makes a leak-tight seal to the capillary column via a press-tight connection (Fig. 1) meets this objective. The direct injection configuration has no split outlet and thus rules out split injection or purging the vaporizing chamber after splitless sample transfer. While the direct injection technique has been promoted [8] as more desirable than splitless injection due to its higher sensitivity and reduced adsorption of active compounds, its potentially great advantage of routinely allowing isothermal chromatography was not highlighted.

The results of the work undertaken to investigate direct injection and isothermal chromatography when applied to a capillary GC–MS bioanalytical method originally developed using splitless injection and temperature-programmed chromatography are presented in this paper. The focus here is on the injection and chromatography aspect, and not on the extraction, de-

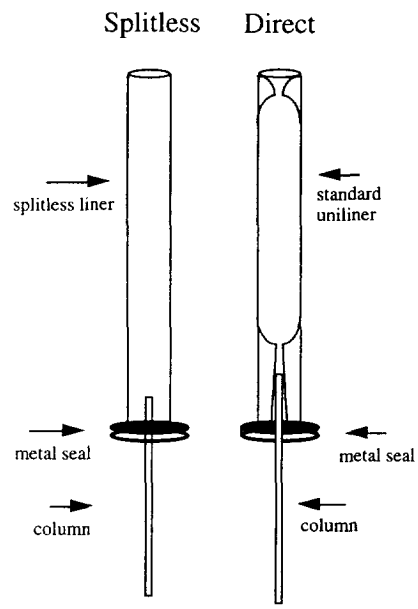

Fig. 1. Schematic drawing of column installation in relation to the inlet liner for direct injection and splitless injection.

rivatization, or mass spectrometric aspect of the method.

2. Experimental

2.1. Samples

BMS180291 (Fig. 2), the analyte, is a drug under development by Bristol-Myers Squibb Pharmaceutical Research Institute. The analytical internal standard, [$^{13}C_3$]BMS180291 (Fig. 2), is also a product of the Institute. All other chemicals were obtained from commercial sources and were used as received.

Standards and quality control samples (QCs) were prepared by spiking human plasma with BMS180291. The standard curve range was 0.4857–485.7 ng/ml of plasma. The standard curve consisted of nine concentrations, with two preparations at each concentration. The QCs, which were prepared from a stock solution different from that used for the standard curve set, were of three concentrations–low, medium and high. There were six preparations for each level of QC.

Each tube containing 0.5 ml of standard or QC plasma sample was spiked with the internal standard and then passed through a cyclohexyl solid-phase extraction (SPE) column to isolate BMS180291 and the internal standard, which were retained on the SPE column. The compounds were eluted from the SPE column with methanol. After removing the methanol by evaporation, the pentafluorobenzyl esters of the analyte and internal standard were formed (Fig. 2) by reacting the residue with pentafluorobenzyl bromide in acetone using N,N-diisopropylethylamine as a catalyst. After removing the reagents by evaporation, the residue was reconstituted with 0.4 ml of *n*-tetradecane for injection into the GC–MS system.

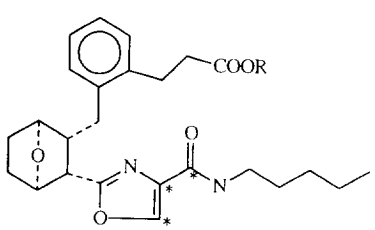

Fig. 2. Structures of BMS180291, the analyte, and [$^{13}C_3$]BMS180291, the stable isotope analogue used as analytical internal standard. For BMS180291, R = H. For BMS180291 pentafluorobenzyl ester, R = $C_6F_5CH_2$. The position of the ^{13}C in the stable isotope analogue is denoted by *.

2.2. Instrumentation

The GC–MS system consisted of an HP5890 Series II GC equipped with electronic pressure control (EPC), HP7673 automated liquid autosampler, HP5989 mass spectrometer and HP MS chemstation (DOS series). A 15 m × 0.32 mm I.D. DB-1 capillary column with 0.25 μm film thickness (J and W Scientific) was used. The mass spectrometer was operated in the negative-ion chemical ionization (NICI) mode using ammonia as the NICI buffer gas.

The split/splitless inlet of the HP5890 GC was operated either in the splitless or direct injection mode. For direct injection, the inlet purge was operated in the off mode continuously. The direct injection Uniliner (Restek No. 20335), which incorporates a press-fit taper at the bottom (Fig. 1), was used. The capillary column was connected to the Uniliner via press-fit sealing, as described in the manual [8]. The leak-tight seal between the column inlet end and the Uniliner press-fit taper isolates the injected sample from the split outlet. The column helium flow was set at 5.0 ml/min in the constant-flow mode. The linear velocity was 124 cm/s at a column head pressure of 20.1 psig. The total carrier gas flow to the inlet zone was set so that the flow measured at the split vent was about 5.0 ml/min. Thus, the total flow was estimated to be 12 ml/min, with a 5.0 ml/min portion going to the column, 2.0 ml/min portion to septum purge, and 5.0 ml/min portion to the split vent. It is important to note that there is no splitting of the sample in the inlet as the Uniliner is directly sealed to the column inlet end, and hence the whole sample injected into the Uniliner goes to the column. The oven temperature was operated isothermally at 290°C. The injection and GC–

MS interface temperatures were set at 300°C. The NICI ammonia gas flow-rate was set to obtain a source pressure of 1.8 Torr, unless indicated otherwise. After setting all the other parameters, the mass spectrometer was calibrated with the NICI autotune program using perfluorotributylamine (PFTBA) as the calibration compound.

For splitless injection, the column was installed as described in the HP5890 GC manual, using a 4 mm I.D. splitless liner (Restek No. 20772). As shown in Fig. 1, the inlet end of the capillary column was inside the open tube liner, with the column inlet end not physically connected to the liner. The helium carrier gas flow was set at 2.0 ml/min, operated in the constant flow mode. The linear velocity was 78.3 cm/s with the column head pressure of 7.5 psig (at 295°C). The oven temperature was 220°C for 1.0 min after injection and then heated at a rate of 30°C/min to 295°C, where it was held for 0.60 min. The septum purge was 2.0 ml/min and the split flow was 50 ml/min. The inlet purge was set to turn on at 0.90 min after injection. The ammonia gas flow-rate was left unchanged from that of the direct injection mode but the source pressure gauge reading decreased to 1.4 Torr as the result of the lower helium flow-rate in the GC column. After setting all other parameters, the mass spectrometer was calibrated with the NICI autotune program using perfluorotributylamine (PFTBA) as the calibration compound.

2.3. Sample analysis

A set consisting of standards and QCs was first injected using the direct injection method. All injections (1.0 μl) were performed with the autosampler. The mass spectrometer was used in the selected-ion monitoring (SIM) mode. The ions monitored were m/z 439 for BMS180291 and m/z 442 for the internal standard. The precise mass of each ion to 0.1 mass unit was determined by creating a window of monitored ions around the nominal values of m/z 439 and m/z 442. The other parameters were set as follows: solvent delay 0.4 min, dwell time for each ion monitored 0.20 s, source temperature 260°C, and quadrupole temperature 120°C. Following the completion of the set of the standards and QCs, a low-level and a medium-level standard sample were each injected 10 times to gauge the reproducibility of absolute area abundances and area ratios. The GC–MS was then configured for the splitless method, viz.: changing the liner and installing the same column in the splitless mode, programming the inlet purge off/on, increasing the total flow to the inlet, reducing the column flow, programming the column temperature, changing injection volume to 2.0 μl, changing solvent delay to 3.0 min, and autotuning under the new set of conditions. The same set of standards and QCs were injected using the splitless method. Ten replicate injections were then obtained from each of the same single vials used for replicate injections for the direct injection mode.

3. Results and discussion

As shown in Figs. 3 and 4, the retention time of BMS180291 using the direct injection method

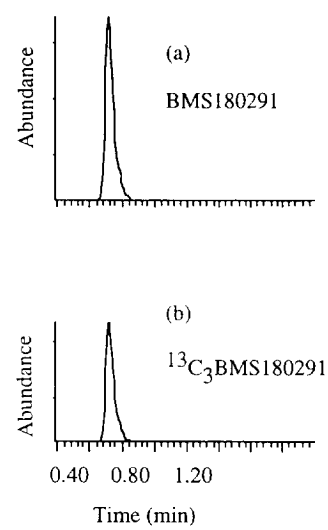

Fig. 3. Ion chromatograms obtained from a direct injection of 194.24 ng/ml spiked plasma standard: (a) ion chromatogram for BMS180291, m/z 439.3, retention time 0.79 min; (b) ion chromatogram for [$^{13}C_3$]BMS180291, m/z 442.3, retention time 0.79 min.

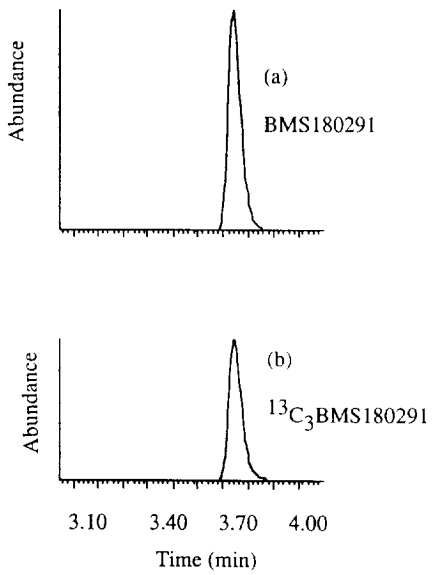

Fig. 4. Ion chromatograms obtained from a splitless injection of 194.24 ng/ml spiked plasma standard: (a) ion chromatogram for BMS180291, m/z 439.3, retention time 3.75 min; (b) ion chromatogram for [$^{13}C_3$]BMS180291, m/z 442.3, retention time 3.75 min.

was 0.79 min, which is significantly shorter than the 3.75 min retention time obtained using the splitless method. The peak shapes for the two injections were equally good. The peak widths at half height were not significantly different: 3.60 s for the splitless mode and 3.54 s for the direct mode. Thus, a high quality chromatogram was obtained with the direct injection method in a fraction of the time required for the splitless method.

Tables 1 and 2 show the results obtained for the set of standards and QCs by direct and splitless injection methods, respectively. The data for the standards consisting of the nanogram values (x) and area-ratio values (y) were analyzed by weighted $1/x$ linear regression for each injection method. The predicted concentrations of the QCs were then determined using the area ratios and the respective regression lines. There was no significant difference between the two injections in the performance of the QCs as judged by percentage deviation (accuracy) or coefficient of variation (precision).

Table 1
Accuracy and precision for direct injection method

QC nominal concentration (ng/ml)	QC mean predicted concentration (ng/ml)	Deviation (%)	C.V. (%)
24.28	23.18	−4.5	2.1
242.85	255.07	5.0	2.6
437.04	442.50	1.3	1.5

There were six preparations for each level of QC. Nominal concentrations were obtained from the weight of the reference standard. Predicted concentrations were obtained from the regression line. Deviation (%) is predicted value minus nominal value, normalized to nominal value. The standard curve consisted of 9 concentrations, with duplicate preparations for each level: 0.4857, 0.9714, 1.943, 4.857, 9.714, 97.12, 194.2, 388.6, 485.7 ng/ml. Thus, there were 18 data points. Linear regression results: slope 0.00782061, intercept 0.00706827, r^2 0.999.

There was no matrix interference in either injection method.

As shown in Table 3, the reproducibility of the area-ratio values obtained from replicate injections from a single standard vial was very good (C.V. of less than 1%) for both splitless and direct methods and there was no significant difference between the two. However, the reproducibility of the absolute area values was not as good, which points out the importance of the use of the internal standard for both injection methods.

Table 2
Accuracy and precision for splitless injection method

QC nominal concentration (ng/ml)	QC mean predicted concentration (ng/ml)	Deviation (%)	C.V. (%)
24.28	23.06	−5.0	2.1
242.85	255.37	5.2	1.4
437.04	449.38	2.8	1.1

The standards and QCs were the same ones used in Table 1. Linear regression results: slope 0.008241668, intercept 0.009125725, r^2 0.998.

Table 3
Reproducibility of replicate injections for direct and splitless injection methods

	Replicate direct injections	Replicate splitless injections
C.V. (%) of absolute abundance (area) of analyte	11.9[a], 11.9[b], 7.1[bc]	8.7[a], 8.4[b], 7.9[bc]
C.V. (%) of absolute abundance (area) of internal standard	11.9[a], 11.7[b], 7.1[bc]	8.3[a], 8.8[b], 7.7[bc]
C.V. (%) of area ratio	0.89[a], 0.39[b], 0.22[bc]	0.86[a], 0.88[b], 0.36[bc]

Each value shown in the table was obtained from 10 replicate injections. Area ratio is analyte area/internal standard area. The values designated with letter "a" were obtained from the injection of the low 4.857 ng/ml spiked plasma standard. The values designated with letter "b" were obtained from the injection of the medium 97.12 ng/ml spiked plasma standard. In both cases, the normal source pressure was used: 1.4 Torr for splitless and 1.8 Torr for direct. The values designated with letter "c" were obtained from the injection of the medium 97.12 ng/ml spiked plasma standard using higher source pressures: 1.8 Torr for splitless and 2.1 Torr for direct.

Table 4 shows the summary of the comparison of the absolute abundance values (area counts) obtained for the 97.12 ng/ml standard using direct and splitless injections. The response from the direct injection was 3 to 7 times higher than that from the splitless injection depending on the ion-source pressure. Although the reason for the enhanced response with the direct injection was not systematically investigated as this was not the focus of this study, this finding was not surprising. The increased response could be due to chromatographic or mass spectrometric origin. Since direct injection provides a more inert inlet environment and a shorter sample residence time in the inlet, loss of sample in the inlet is expected to be minimized [8]. In addition, the retention time is much shorter with the direct injection and hence residence time for the compounds in the GC column is much shorter, which should theoretically minimize any loss of the compounds to the column [11,12]. On the other hand, the

Table 4
Comparison of absolute abundances obtained from direct and splitless injection methods

Condition	Abundance ratio
Source pressure 1.8 Torr for direct, 1.4 Torr for splitless: condition 1	6.8
Source pressure 1.8 Torr for direct, 1.8 Torr for splitless: condition 2	2.8
Source pressure 2.1 Torr for direct, 1.8 Torr for splitless: condition 3	4.4

Abundance ratio was obtained by dividing the area response value of the analyte obtained from direct injection by that obtained from splitless injection and then multiplying by 2 to correct for the volume of injection, 1.0 μl for direct and 2.0 μl for splitless. Conditions 1 and 3 are obtained by keeping the same ammonia flow settings for both direct and splitless, with condition 3 being of higher ammonia flow setting for both injections. Condition 2 was obtained by using a higher ammonia flow setting for splitless than for direct so that the source pressure readings for the two injection modes were the same.

increased abundance value that was obtained when employing a higher ion-source pressure for either injection mode was also in accordance with a published report on the effect of source pressure on abundance [11].

Thus, a capillary GC–MS bioanalytical method employing isothermal direct injection with a short run time was shown to perform as well as or better than a method employing a temperature programmed splitless injection with a much longer run time. The relatively high carrier gas flow required for efficiently transporting the vaporized sample to the column [8,9] was easily tolerated by the mass spectrometer used. The volume of vapor generated by the injection of 1.0 μl of the relatively high-molecular-mass tetradecane solvent was calculated [8,13] to be less than 100 μl, which was easily handled by the combination of the high carrier gas flow and the size of the vaporizing chamber provided by the volume of the Uniliner. This was in spite of the fast injection rate of the autosampler. Thus, the conditions used resulted in the delivery of the injected sample vapor cloud into the column in an adequately narrow band and there was no

need to use either cold trapping or solvent recondensation to reconcentrate (focus) the initial band.

In conclusion, wherever practical, it is recommended that the splitless injection, which has been traditionally used in trace level capillary GC–MS bioanalytical methods, be replaced by direct injection without column temperature programming. This will increase the sample throughput of bioanalytical laboratories that analyze thousands of samples.

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