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Improving ion-trap GC-MS quantitation limits for therapeutic agents extracted from rat plasma[1]

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Abstract

Insufficient quantitation limits using ion-trap gas-chromatography mass-spectrometry (GC-MS) prevented the assay of some samples during a preliminary screening of preclinical rat plasma samples (50 μl) containing novel, polar therapeutic agents. Few options were available for improving the lower limit of quantitation. THe limited amount of sample available precluded the extraction additional plasma. Liquid–liquid extraction recoveries were greater than 90% throughout the range of the standard curve (500–2000 ng ml^{-1}). Chromatography was optimized and multiple, equivalent sites for analyte fragmentation were precluded, using MS-MS to improve assay sensitivity. Quantitation limits were decreased 10-fold however, by using a larger syringe to increase the injection volume from 5 to 50 μl, in combination with a universal programmable injector. These large injection volumes required changes in the injector events program and in column plumbing. Additionally, evaluation of injection liner packing material demonstrated a 2-fold improvement in sensitivity, using carbofrit, relative to silanized glass wool. Converting to inert ion-trap electrodes did not appear to affect the detection limit, perhaps due to over-riding peak broadening during gas chromatography. The changes described produced a 20-fold improvement in the lower limit of quantitation.

Keywords: Ion-trap GC-MS; Therapeutic agents; Quantitation

1. Introduction

An ion-trap gas-chromatography mass-spectrometry (GC-MS) method was developed for a preliminary pharmacokinetic screening of novel, polar, therapeutic agents extracted from 50 μl of rat plasma. The region of linearity (500–2000 ng ml^{-1}) for most analytes was suitable for the assay of samples obtained shortly after dosing. However, the quantitation limit was insufficient to assay some of the samples obtained at much later time points.

Method changes were needed in order to improve the quantitation limit, but few options were available. Gas chromatography (GC) conditions

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and analyte recoveries from liquid–liquid extraction (90%) had been optimized. Lowering the detection limit by extracting more plasma was precluded by low sample volumes. A limited evaluation of GC-tandem MS indicated multiple, equivalent fragmentation sites and produced little improvement in the signal/noise. A significant improvement might be achieved by careful reconstitution in a reduced volume and using an internal standard. However, because the availability of a suitable internal standard was not assured, a reduction in the reconstitution volume, 150 μl, was not evaluated.

Therefore, the goal was to improve the quantitation limit without increasing the volume of plasma extracted, while also maintaining acceptable precision and accuracy. Any change had to be compatible with the standard automation available. Three options were evaluated for improving the quantitation limit: (1) increasing the injection volume to greater than 5 μl; (2) using a more inert packing in the GC injector liner; and (3) sharpening chromatogram peaks by using inert, Silchrom ion-trap electrodes.

Larger injection volumes have been used for GC, although these usually involved hydrocarbon or pesticide analytes, with FID, ECD or NPD detection [1,2]. Elaborate systems allowing GC injections of up to 20 ml also have been described [3]. However, using a universal temperature programmable injector [4,5] and exchanging the standard (5 μl) capacity syringe in the autosampler with a commercially available 50 μl capacity syringe, were considered to be more practical upgrades.

Another option to improve the quantitation limit was the use of a more inert injector liner packing material [6]. The packing can interact with polar analytes, especially if some of the deactivated surface loses its inert capabilities during handling, etc. These effects were evaluated at lower analyte concentrations, where adherence of analytes to packing material should be most evident.

The final option to improve the quantitation limit was the use of Silchrom ion-trap electrodes. These provide a more inert surface in the ion-trap and can result in sharper chromatographic peaks due to less interaction between the analytes and the electrode surface as analytes are ejected from the ion-trap. Sharper peaks would lead to increased sensitivity.

2. Experimental

2.1. Chemicals and reagents

Analytes were proprietary compounds, containing amine and phenol groups, with molecular weights of approximately 300. They were obtained from Parke–Davis Pharmaceutical Research (Ann Arbor, MI). Acetonitrile (HPLC grade) was from Mallinckrodt Chemical (Paris, KY). Tris (99.5 + %) was from Aldrich Chemical, (Milwaukee, WI). Methyl-*t*-butyl ether (99.9 + %) was from Baxter Healthcare (Muskegon, MI). Water was purified using a Milli-Q system (Millipore, Milford, MA).

2.2. GC-MS

The ion-trap GC-MS was a model 3400 Saturn II equipped with a model 8200 autosampler, with an injection rate of 5 μl s^{-1} (Varian, Sugarland, TX). A model 1078 temperature-programmable injector was used with a 100 μl injection (Varian). A DB-5 ms column, 15 m × 0.25 mm i.d., 0.25 μm d.f. (J & W Scientific, Folsom, CA) was used with deactivated liners and packings: carbofrit (3.4 i.d., 5.0 × 54 mm o.d., double packing; Restek, Bellefonte, PA), silanized fused silica (3.4 i.d., 5.0 × 54 mm o.d., Restek), or deactivated glass wool (2.0 i.d., 5.0 × 54 mm o.d., Varian). Helium was used as the carrier gas, at an inlet pressure of 15 psi. Silchrom electrodes were from Varian.

The GC oven temperature program was: (1) 75°C for 1.5 min; (2) 75–225°C at 30° min^{-1}; (3) 225–250°C at 5° min^{-1}; (4) 250–300°C at 20° min^{-1}. The injector temperature program was: (1) 75°C for 0.2 min; (2) 75–300°C at 150° min^{-1}; and (3) 300°C for 3.0 min. The injector purge valve events are contained in Table 1. MS conditions involved electron-impact ionization and selected ion monitoring. Other parameters were as previously reported [7,8].

Table 1
Injector events for a 50 μl injection

Time (min)	Temperature (°C)	Event
0.0	75	Injection; purge valve open
0.2	150° min^{-1}	Heating to facilitate solvent vaporization
0.6		Purge valve closed
1.0	~200	Volatilized analytes begin to transfer from the injector to the GC column
4.0	300	Analyte transfer completed; purge valve is then re-opened to allow high-boiling contaminants in the injector to be transferred to waste.
4.7		Return injector to initial conditions.

2.3. Sample preparation

Analytes from 50 μl rat plasma calibration standards were isolated using liquid–liquid extraction with 0.2 ml Tris buffer (0.1 M; pH 9.5) and 1.0 ml methyl-*t*-butyl ether. The organic layer was removed, evaporated to dryness and reconstituted in 0.15 ml acetonitrile.

3. Results and discussion

3.1. Evaluation of an increased injection volume

Four criteria were used to evaluate larger injection volumes: (1) peak shape, to indicate possible overloading; (2) linear increases in response corresponding to increased injection volume; (3) signal/noise improvements; and (4) repeatability of responses over a range of injection volumes. In principle, increasing the volume injected 10-fold should improve sensitivity 10-fold. In practice however, two significant changes were necessary to receive the full benefit of a increasing the injection volume from 5 to 50 μl.

One change was a compensation for injector cooling as the injection solvent was vaporized. This was because more injector cooling occurred when the injection volume was increased [4]. The injector was held at 75°C for 0.2 min after injection and was then heated to facilitate solvent evaporation. The timing for purge valve opening and closing for a 50 μl injection was extended by 0.4 min, relative to a 5 μl injection, to adjust for this additional cooling (Table 1).

The second change involved the installation distance of the column into the injector. This distance had a significant effect on the linearity of response for injections of the same solution over a range of injection volumes. The response appeared linear for 5–20 μl injections when using a 7.5 cm distance, but was nonlinear for injection volumes ranging from 5 to 30 μl. However, reinstalling the column to 5.9 cm produced a linear response of over 5–50 μl.

Optimized conditions for 50 μl injections resulted in a 10-fold improvement in the signal/

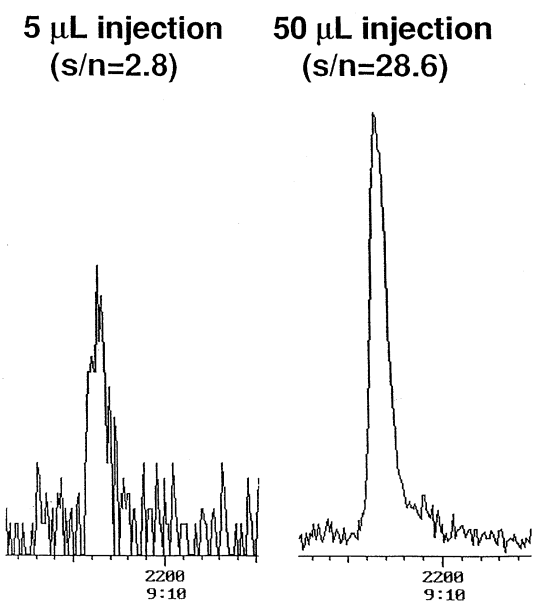

Fig. 1. Comparison showing signal/noise in selected ion responses as a function of time and scan number for different injection volumes of an extracted 100 ng ml^{-1} calibration standard.

Table 2
Repeatability of response for injections from a 50 μl syringe

Volume injected	Concentration ng ml^{-1}	Replicate injections	% RSD[a]
5 μl	4000	6	8.6
10 μl	4000	6	3.1
15 μl	4000	6	2.6
20 μl	4000	6	3.3
25 μl	4000	6	5.9
30 μl	4000	6	3.9
30 μl	800[b]	5	4.7
40 μl	800	5	4.6
50 μl	800	6	1.2

[a] Relative standard deviation of peak areas.
[b] A lower concentration was used to avoid analyte overloading with larger injection volumes.

noise for 100 ng ml^{-1} extracted calibration standard (Fig. 1). The repeatability of response areas from using 5–50 μl injection volumes was evaluated (Table 2). RSDs were usually less than 5%, but were larger for 5 μl injections.

3.2. Evaluation of injector liner packing

The effects of 50 μl injections with carbofrit, silanized fused silica or silanized glass wool liner packing material were evaluated using several criteria. First, peak tailing, signal/noise and precision obtained with each packing were compared for triplicate injections at a lower concentration on the calibration curve for extracted standards (30 ng ml^{-1}). Carbofrit produced the best results (Table 3), followed by fused silica and glass wool.

Next the ranges of standard curves from extracted calibration standards were compared (Table 3). Those standard curves with back-calculated values giving a %RE of less than 20% were considered. The quantitation limit was reduced approximately 2-fold by using Carbofrit, with fused silica producing slightly better results than glass wool.

Finally, serial dilution a fresh extract of a 2000 ng ml^{-1} plasma spike was used to provide a more accurate estimate of the best detection limit that might be achieved (signal/noise of 3). These dilutions provided information in addition to that from curves of calibration standards, in that it removed effects of extraction artifacts, such as decreased recoveries at lower analyte concentration. The detection limits obtained with glass wool were lowered 2-fold by using Carbofrit or fused silica.

3.3. Evaluation of Silchrom ion-trap electrodes

No change in peak shape was observed by using Silchrom electrodes with these analytes. This could have been because gas-phase interactions with the electrodes were not a major cause of band broadening for the analytes used in this study.

4. Conclusions

The most significant improvement in the quantitation limit for the ion-trap GC-MS of drug candidates extracted form rat plasma was 10-fold; it occurred when a large injection volume could be used in combination with concentration of analytes on the column head. Use of these larger injection volumes required adjustments in the temperature program of the injector. Repeatability studies using injections of 50 μl gave a variability of 1.2%. Variability increased upon using smaller injection volumes

Table 3
Comparison of responses and chromatographic properties upon using different injector liner packings

Criterion	Results		
	Carbofrit	Silanized fused silica	Deactivated glass wool
Peak tailing[a,b]	1.17 ± 2.5%	1.33 ± 9.4%	1.52 ± 6.9%
Signal/noise[a]	20:1 ± 11%	14:1 ± 16%	22:1 ± 34%
Peak area[a]	2778 ± 3.4%	2690 ± 2.3%	2455 ± 4.1%
Detection limit (ng ml^{-1})[c]	5	5	10
Range of extracted standards (ng ml^{-1})[d]	30–1000 (0.9964)	40–1000 (0.9905)	50–1000 (0.9897)

[a] For three replicate 50 ml injections of 30 ng ml^{-1}. The values are expressed as ± %RSD.
[b] The peak tailing factor was calculated as (peak width at 5% height)/2(distance in the baseline from the peak midpoint to a line drawn tangent to the peak front at 50% height).
[c] The minimum concentration of diluted extraction of 200 ng ml^{-1} standard giving $s/n > 3/1$.
[d] The acceptable range defined for extracted calibration standards. The figures in parentheses are values of r^2.

and the use of an internal standard is recommended to reduce variability. Carbofrit packing for liners provided the best peak shape, resulting in the best overall signal/noise, precision and detection limits. Use of ion-trap Silchrom electrodes did not improve sensitivity, perhaps because peak broadening was not limited by endcap electrode interactions. In summary, the changes incorporated produced a 20-fold improvement in the lower limit of quantitation.

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