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Trace quantitation of the novel cholinesterase inhibitor in human plasma by capillary gas chromatography/ion trap mass spectrometry

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Abstract

This paper demonstrates the utility of an ion trap mass spectrometer as a detector for trace quantitative determinations of pharmaceuticals in human plasma by capillary gas chromatography/mass spectrometry. A novel acetylcholinesterase inhibitor (CI-1002) was selected as an illustrative example for the technique. When coupled with a selective solid-phase extraction, this approach was capable of quantifying as little as 34 pg (0.50 ng ml^{-1}, RSD = 12.7%) of compound on the column, and the inter-run precision was typically 3–4% RSD over a 0.5–25 ng ml^{-1} linear range. The advantages and requirements of the technqiue, in addition to the propects for improvements in the detection limit, are discussed.

Keywords: Cholinesterase inihibitor; Cognition activator; Gas chromatography/mass spectrometry; Human plasma; Ion trap; Selected ion storage; Solid-phase extraction

1. Introduction

The quadrupole ion trap (IT) has received much attention in the recent literature as a mass spectrometry (MS) detector providing a significant efficiency advantage over beam-type scanning instruments. This advantage stems from a high duty cycle (fraction of time for which a given ion can be transmitted to the detector) and a high transmission rate (fraction of ions from the source which reach the detector for a selected m/z). In practical terms, this efficiency advantage can result in lower detection limits for an IT instrument relative to a beam-type scanning instrument. Two recent papers review the fundamental aspects of IT instruments [1,2].

Bioanalytical applications of the IT as a selective detector for gas chromatography (GC) are somewhat limited. They include trace quantitation of compounds of pharmaceutical interest in tissues [3], urine [4], milk [5] and serum [6], the latter reporting detection limits of 500 pg ml^{-1}. Applications of the IT to forensics and drug-abuse testing have been reported [7–9], as have practical comparisons of IT performance relative to a quadrupole based mass-selective detector [10,11].

This work describes the application of IT GC/MS to the trace quantitation of drug substances in human plasma. CI-1002, a novel cholinesterase inhibitor and potential cognition activator, was chosen to illustrate the approach. The chemical structure of CI-1002 and an internal standard, PD 146023, are given in Fig. 1. The objective of the method was to provide as low a detection limit as practical, to

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support a thorough study of the pharmacokinetics of the drug. The desired performance characteristics of the method were achieved by minimizing undesired matrix ions through the use of a highly selective extraction. Ion production, storage and detection parameters and other factors crucial for sensitive detection will also be discussed.

2. Experimental

2.1. Materials

CI-1002 HCl (> 99% purity, molecular mass of free base = 269.4), 1,3-dichloro-6,7,8,9,10,12-hexahydro-azepino[2,1-b]quinazoline monohydro-chloride, and PD 146023-2 (> 99% purity, molecular mass of free base = 283.2), 1,3-dichloro- 6,7,8,9,10,12-hexahydro-2-mehyl-azepino[2,1-b]-quinazoline monohydrochloride, were prepared by Parke-Davis Pharmaceutical Research (Ann Arbor, MI, USA). Ultrapure helium (99.999%) was purchased from AGA (Maumee, OH, USA). Sodium phosphate, acetonitrile, methanol, water and trifluoroacetic acid were either reagent or HPLC grade and were purchased from EM Science (Gibbstown, NJ, USA) or Mallinkrodt (Paris, KT, USA) and used as received. Solid-phase extraction (SPE) columns (Bond Elut™ C-18 200 mg, 3 ml cartridges, Manufacturing Number 1210-2025) were purchased from Varian Analytichem (Harbor City, CA, USA). Blank human plasma was obtained from Interstate Blood Bank Incorporated (Memphis, TN, USA).

2.2. Preparation of standards and controls

A fresh aqueous stock solution, containing 200 μg ml^{-1} of CI-1002 (free-base equivalent) was prepared for each batch run, from which a 2.0 μg ml^{-1} solution was prepared by volumetric dilution with water. This dilute stock solution was further diluted volumetrically with water to prepare working standards at 2, 4, 10, 20, 40 and 100 ng ml^{-1}. A 250 μl aliquot of each working standard was added to 1.0 ml of blank human plasma to prepare standards for calibration. A 200 μg ml^{-1} (free-base equivalent) stock solution IS was prepared for each batch run. This solution was diluted (1:100) with water to prepare a stock IS solution of

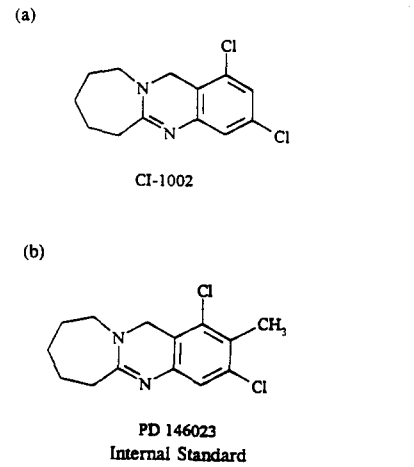

Fig. 1. Chemical structures of (a) CI-1002 and (b) internal standard.

2.0 μg ml^{-1}. This 2.0 μg ml^{-1} solution was diluted with water to prepare IS solution of 100 ng ml^{-1}. Addition of 250 μl aliquots of the working IS solution to human plasma samples gave a concentration of 25 ng ml^{-1}.

Human plasma quality controls containing 1.5, 7.0 and 20.0 ng ml^{-1} CI-1002 (free-base equivalents) were prepared by diluting aliquots of 2500 and 250 ng ml^{-1} stock solutions with human plasma. The biological component constituted more than 95% of these controls. Controls were subdivided into 3.5 ml aliquots and stored in glass 2 dram vials with foil lined screw caps at −20 °C until use.

2.3. Instrumentation

A Varian Saturn II GC MS equipped with an 8200 autosamppler and a Compaq Prolinea 4/33 data system was utilized. Sample injection was performed with a 1093/1094 septum-equipped programmable injector (SPI) and silanized glass on-column inserts. The sample injection volume was 2.0 μl (in acetonitrile), sandwiched by air gaps of 0.5 μl. After septum penetration, the pre- and post-injection delays were 0.05 and 0.10 min, respectively. Sample injection was at 70 °C, at a rate of 5 μl s^{-1}. At injection, the SPI injector manifold was heated to 150 °C at 300 °C min^{-1} and held for 7.25 min, then heated at 300 °C min^{-1} to 300 °C and held for 3.5 min. The temperature of the transfer line was held constant (250 °C). Electron ionization (EI) was performed at 70 eV at a manifold temperature of 220 °C. Positive ions were monitored.

A DB-5ms fused silica capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness; J&W, Folsom, CA, USA) was utilized. A guard column of fused silica (0.5 × 0.25 mm, J&W) was connected between the injector and the analytical column. The column was initially operated at 100 °C for 0.25 min, after which the temperature was increased at 20 °C min^{-1}, to 300 °C, with no hold time. The carrier gas was helium with a column inlet pressure of 8 psi, resulting in a linear velocity (based on diethyl ether) of 1.6 m s^{-1} at an oven temperature of 100 °C.

The Saturn autotune program was used to tune the mass spectrometer. To improve signal-to-noise ratio (S/N), the mass range was limited from m/z 265 to 285. Ions of m/z 267 and 281 were selected to monitor CI-1002 and IS, respectively. This limited scan range allowed eight microscans to be averaged for each mass scan (430 ms per scan). Data collection began 5.5 min after injection, with a duration of 4.5 min. Chromatographic retention times for CI-1002 and the internal standard were 6.27 and 7.04 min, respectively.

2.4. Assay procedures

To 1.0 ml of human plasma, 0.250 ml of CI-1002 working standard or water (for samples, quality controls, or blanks), 0.250 ml of IS working standard, and 1.0 ml of sodium phosphate buffer (pH 6.0; 0.05 M) were added. Bond-Elut™ solid-phase cartridges (200 mg, C18) were conditioned by rinsing with 3 ml each of methanol, acetonitrile, water, and sodium phosphate (5 in. Hg for about 5 s). Samples were loaded on cartridges at full vacuum (20 in. Hg) to ensure initial even flow of plasma. The vacuum was then reduced (5 in. Hg) and the remaining sample volumes were drawn through the cartridges. The cartridges were then washed with 1 ml of water and 1 ml of acetonitrile (5 in. Hg) and dried under full vacuum for 10 min. Analytes were eluted into 10 × 75 mm glass tubes with 1 ml of 2% trifluoro-acetic acid in HPLC grade methanol (5 in. Hg).

The samples were dried under nitrogen (10 psi, 50 °C), reconstituted with 30 μl of acetonitrile, and transferred into low volume glass injection vials. Volumes of 2.0 μl of each sample or standard were injected into the GC column. Between each sample, a 2 μl injection of acetonitrile was made to wash the injector liner. This wash step had a 1 min run time, with the column temperture staying at 150 °C, and the injection temperature increasing from 70 to 150 °C in 0.25 min.

2.5. Data reduction

The assay was validated over a CI-1002 plasma concentration range of 0.50–25.0 ng ml^{-1}, by assaying six calibration standards and three quality control samples in triplicate, in three separate batch runs. The best-fit line was determined by least-squares linear regression of the peak-area ratio (peak area of CI-1002 at $m/z = 267$ to peak area of IS at $m/z = 281$) vs. concentration from each batch run, using a weighting factor of 1/concentration. Concentrations of CI-1002 in quality controls and samples were calculated using peak-area ratios and regression parameters.

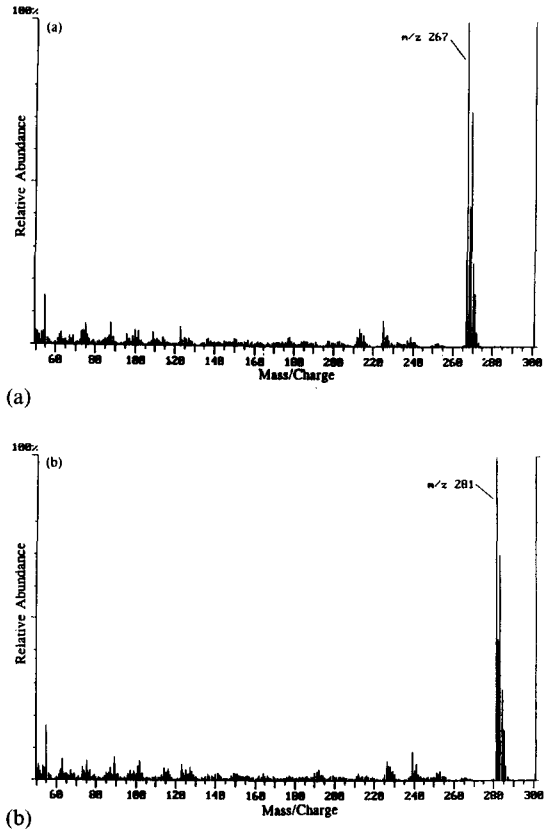

Fig. 2. Electron ionization ion trap mass spectra (m/z 50–300) for (a) CI-1002 and (b) internal standard, from acetonitrile solution following capillary gas chromatographic separation.

3. Results

This method was validated for CI-1002 in human plasma over the concentration range of 0.50–25.0 ng ml^{-1}. Selectivity, chromatographic performance, system reproducibility, recovery, limit of quantification, linearity, precision, accuracy, and stability were evaluated for the method.

3.1. Stability

The response of CI-1002 in injection solution after 24 h was 100% of the initial reading, indicating stability over this time period at least. No drug degradation was observed during sample assay.

3.2. Mass spectra of analytes

Mass spectra for 25 ng ml^{-1} pure standards of CI-1002 and IS were evaluated from m/z 50 to 300 and are presented in Fig. 2. Predominant ions for CI-1002 and IS (m/z 267 and 281) were chosen for monitoring the compounds in the GC separations because they provided the best sensitivity as well as selectivity. A unique feature of the IT, relative to the quadrupole, is its ability to collect a range of masses without any appreciable loss in mass throughput. As noted earlier, the mass range for data collection was limited from m/z 265 to 285, to maximize S/N while retaining most of the qualitative information. Minimal fragmentation was noted with EI, so chemical ionization was not considered.

3.3. Chromatographic selectivity

Chromatograms representing the separation of analytes from human plasma are shown in Figs. 3–5. As can be seen in Fig. 3, no components coeluted with CI-1002 (m/z 267, 6 min 15 s) or IS (m/z 281, 6 min 52 s) in 20 independent sources of blank human plasma. Selectivity was gauged by R_s, the chromatographic resolution, in standard and sample chromatograms. CI-1002 and IS were sufficiently well resolved ($R_s > 2.8$) from human plasma components at their respective m/z.

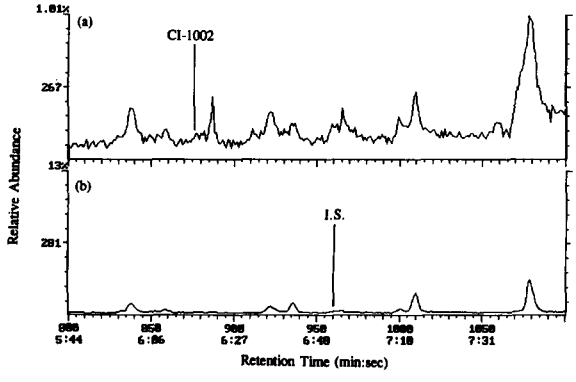

Fig. 3. Chromatograms for representative blank human plasma at (a) m/z 267 and (b) m/z 281.

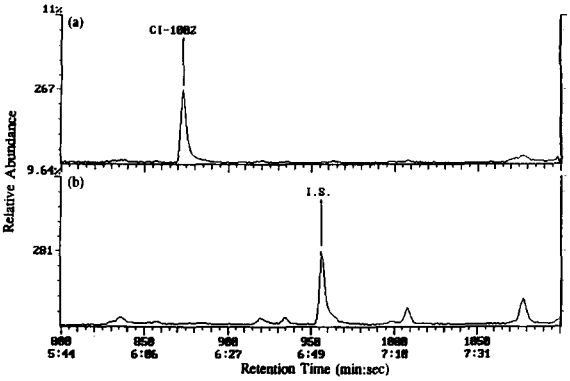

Fig. 4. Chromatograms for plasma spikes (25 ng ml^{-1}) of (a) CI-1002 (m/z 267) and (b) internal standard (m/z 281).

3.4. System reproducibility

The reproductibility for nine replicate injections of extracted biological samples at low and high CI-1002 concentrations was 4.4% and 3.7% RSD for CI-1002 peak-area ratios, at concentrations of 1.0 and 25.0 ng ml^{-1}, respectively.

3.5. Recovery from human plasma

Using a C18 solid-phase sorbent, extraction efficiencies of CI-1002 from human plasma

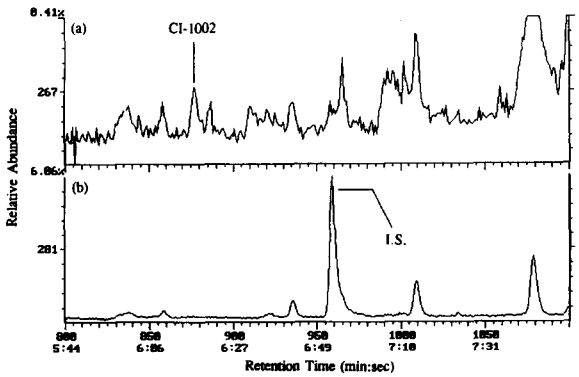

Fig. 5. Chromatograms of plasma spikes for (a) 0.5 ng ml^{-1} of CI-1002 (m/z 267) and (b) 25 ng ml^{-1} internal standard (m/z 281).

Table 1
Percentage recovery of CI-1002 and IS from human plasma

Sample	CI-1002			IS		
	1.50 ng ml^{-1}	7.00 ng ml^{-1}	25.0 ng ml^{-1}	25.0 ng ml^{-1}		
1	–	85.4	84.7		117.0	117.1
2	108.6	90.9	93.1	91.7	109.9	106.5
3	99.0	88.3	93.6	100.6	113.2	106.0
4	98.5	88.1	96.2	101.1	113.4	103.1
5	103.0	93.7	113.4	96.7	106.6	87.5
6	93.1	91.2	101.3	107.0	109.6	97.9
7	85.6	104.3	102.9	116.3	95.8	96.4
8	96.5	96.4	93.1	103.2	103.7	106.5
9	95.7	103.8	105.0	104.1	96.3	94.4
N	8	9	9		26	
Mean	97.5	94.6	99.8		104	
SD	6.8	6.8	8.4		7.9	
RSD(%)	7.0	7.1	8.4		7.6	

were determined by assaying nine samples at each of three concentrations and comparing the results with unextracted solution standards run in triplicate. The results are summarized in Table 1. The mean (RSD) CI-1002 recoveries were 97.5 (7.0)%, 94.6 (7.1)%, and 99.8 (8.4)% at 1.5, 7.0 and 20 ng ml^{-1}, respectively. The pooled mean (RSD) recovery at all levels was 96.4 (7.5%). The mean (RSD) internal standard recovery was 104 (7.6)% at 25 ng ml^{-1}.

Several other solid-phase sorbents, including C8, C2, cyclohexyl, phenyl, cyano and diol, were evaluated. The C18 sorbent was chosen because of its relatively high recovery and comparable selectivity.

3.6. Limit of quantitation (LOQ)

The LOQ, defined as the lowest concentration on the standard curve, was evaluated by obtaining acceptable intra-run precision (RSD) of replicate spiked plasma samples, as well as intra-run precision of replicate standards. The values obtained for five replicates at 0.25, 0.50, 0.75, and 1.0 ng ml^{-1} were 16.2, 12.7, 5.5, and 5.1% RSD, respectively. These data suggested that 0.50 ng ml^{-1} would be an acceptable LOQ. At 0.50 ng ml^{-1}, the inter- and intra-run RSD and RE accuracies of replicate standards were always less than 9.3%, also indicating that acceptable results could be achieved at 0.50 ng ml^{-1} for 1.0 ml aliquots of human plasma. A representative chromatogram of a 0.5 ng ml^{-1} plasma sample is shown in Fig. 5(a).

3.7. Linearity, precision, and accuracy of calibration curves

Assay calibration was validated with human standards containing 0.50–25.0 ng ml^{-1} CI-1002 in triplicate, in three separate batch runs. Peak-area ratios were proportional to the amount of CI-1002 added to human plasma over these ranges. The best-fit line was determined for each batch run as described earlier.

Calibration curve reproducibility, evaluated by variations in individual back-calculated standards from the regression line, ranged from 2.0 to 8.8% RSD over three batch runs, with relative errors between −3.6% and 5.0%. Based on the accuracy (%RE) and precision (%RSD) of calibration standards, the method demonstrates sufficient adherence to a linear model over the concentration range of 0.50–25.0 ng ml^{-1} CI-1002. If no precautions were taken, some carryover was evident at the highest concentrations. To minimize carryover, the injection syringe was thoroughly washed with methanol and acetonitrile between sample injections. Blank solvent injections were made between samples to wash the injector liner. Silanized glass injector liners were replaced daily, also to minimize carryover.

3.8. Intra-run precision and accuracy

The intra-run (within run) precision and accuracy were determined by assaying quality controls in triplicate, at each of three levels, in three separate batch runs. The intra-run

Table 2
CI-1002 concentrations in human plasma quality controls over three separate batch runs

	Concentration added (ng ml^{-1}) (free-base equivalents)		
	1.50	7.00	20.0
Batch Run 1	1.54	7.04	19.3
	1.40	7.00	19.1
	1.59	7.25	19.3
Intra-run mean	1.51	7.10	19.2
Intra-run SD	0.098	0.134	0.115
Intra-run RSD(%)	6.5	1.9	6.0
Intra-run RE(%)	0.7	1.4	−4.0
Batch Run 2	1.42	7.20	20.6
	1.48	7.42	19.5
	1.46	6.99	19.8
Intra-run mean	1.45	7.20	20.0
Intra-run SD	0.030	0.215	0.569
Intra-run RSD(%)	2.1	3.0	2.8
Intra-run RE(%)	−3.3	2.8	0.0
Batch Run 3	1.54	6.94	18.9
	1.48	6.64	18.9
	1.48	6.99	18.9
Intra-run mean	1.50	6.86	18.9
Intra-run SD	0.035	0.189	0.0
Intra-run RSD(%)	2.3	2.8	0.0
Intra-run RE(%)	0.0	−2.0	−5.5
N	9	9	9
Mean concentration found (ng ml^{-1})	1.49	7.05	19.4
Inter-run SD	0.060	0.218	0.562
Inter-run RSD(%)	4.1	3.1	2.9
Inter-run RE(%)	−0.67	0.71	−3.0

precision (RSD) estimates for CI-1002 were less than 6.5%, 3.0%, and 6.0% for controls containing 1.50, 7.00, and 25.0 ng ml^{-1} CI-1002, respectively. The intra-run RE for CI-1002 was ±5.5% (Table 2).

3.9. Inter-run precision and accuracy

The inter-run (between-run) precision and accuracy were determined by pooling individual assay results of triplicate quality controls over three separate batch runs. The inter-run precision estimates for CI-1002 were 4.1%, 3.1%, and 2.9% (RSD) for controls containing 1.50, 7.00, and 20.0 ng ml^{-1} CI-1002, respectively. The inter-run RE was ±3.0% (Table 2).

4. Discussion

Matrix problems can have a pronounced effect on the dynamic range and detection limits of an IT detector owing to its finite capacity to store ions. If the trap is filled predominantly with ions which arise from the sample matrix, then only a small fraction of trap capacity is available for holding ions which are of analytical interest. Consumption of trap capacity with ions that are not of interest results in both a decreased dynamic range and detection limits. Although advances in selected ion storage techniques allow accumulation of ions of analytical interest at the expense of undesired matrix ions [2], these approaches have not yet been widely applied. A more elementary, but still effective, approach is to develop a selective, efficient sample cleanup procedure prior to sample introduction. The use of a highly selective extraction should complement future gains made by selected ion storage.

The SPE techniques used here relies on a mixed retention mechanism which has been shown to be highly selective for amines [12]. This extraction combines a reverse-phase parti-

tioning mechanism on C18 material with cation exchange on the residual unfunctionalized silica support. At pH 6.0, the tertiary amine ($pK_a = 8.6$) in the analyte molecule is almost completely protonated, while unfunctionalized silanol moieties on the silica support are partially deprotonated and capable of weak cation exchange. It is this weak cation exchange mechanism which allows retention of analyte molecules on the solid phase during the rigorous acetonitrile wash step. Any matrix molecules which do not have a net positive charge at pH 6.0 are washed to waste. After washing, pH adjustment to below 1 with 2% trifluoroacetic acid results in protonation of virtually all the silanol sites on the silica, effectively eliminating cation exchange. Analyte molecules are then eluted by simply overcoming the partitioning mechanism associated with C18. The resulting sample has been stripped of many undesired matrix molecules, allowing for sample concentration prior to injection. This effective sample cleanup allows better utilization of the finite capacity of the IT, and will complement any selectivity gains made by selective ion ejection techniques. The low quantitation limits for this method (34 pg on column, RSD = 12.7%) can be attributed to the high intrinsic sensitivity of the IT, but were realized only because of an effective sample cleanup.

5. Conclusions

Analytical methodology for the trace quantitation of a novel acetylcholinesterase inhibitor in human plasma has been developed and validated using capillary gas chromatography with IT detection. The approach is capable of quantitation limits as low as 500 pg ml^{-1} (estimated imprecision of 12.7% RSD). A selective mixed mechanism solid-phase extraction contributes to the selectivity and quantitation limits of the method.

A future extension of this application will be to generate and apply one of the several types of waveforms to the end electrodes of the IT during ion accumulation. This so-called selected ion storage will result in the removal of many of the remaining matrix ions, allowing for the accumulation of more analyte ions. Additional improvements in detection limits and dynamic range of IT-MS are anticipated as the selected ion storage technique is brought into wider use.

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