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LC/MS/MS quantitation of an anti-cancer drug in human plasma using a solid-phase extraction workstation: application to population pharmacokinetics

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Abstract

A liquid chromatographic/mass spectrometric (LC/MS/MS) method to quantitate an anti-cancer drug in human plasma was validated. The method has proven suitable for routine quantitation of the experimental anti-cancer compound at concentrations from 1 to 400 ng/ml. Retention times of the compound and internal standard (compounds I and II, respectively) were 1.8 and 2.1 min, respectively. No interfering endogenous peaks were observed throughout the validation process. Precision estimates for this approach were typically less than 5% relative standard deviation (RSD) across the calibration range. Other validation parameters studied included specificity, system reproducibility, limit of quantitation, accuracy, linear range, and stability of the compound and internal standard in plasma and injection solvent. This method was used to quantify drug for population pharmacokinetic studies.

Keywords: Electrospray; Solid-phase extraction; Atmospheric pressure ionization; Biological fluids; Pharmacokinetics

1. Introduction

CI-994 (compound I) (4-acetylamino-*N*-(2′-[aminophenyl]benzamide) has shown considerable potential as a novel anti-cancer agent. This compound is currently in clinical development, after demonstrating anti-tumor activity against many drug-refractory in vivo tumor models [1–7]. Compound I has been shown to inhibit cell growth by blocking cells in the G_1–S phase of the cell cycle [8–11], but the exact mechanism of its anti-tumor activity remains a mystery. Studies done by Rummel et al. [12] have demonstrated that the drug causes loss of a 16 kDa nuclear phosphoprotein, which precedes a block in cell proliferation. The loss of this phosphoprotein occurs primarily through inhibition of phosphorylation. Because this protein was believed to be a member of the histone family, additional in vitro studies have been conducted [13] showing that the drug causes

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an increase in histone acetylation rather than inhibiting histone deacetylase. A more complete assessment of the in vivo efficacy of the anti-cancer compound occurs when it is first dosed in man during Phase I clinical trials and then expanded to Phase II trials. Evaluation of the pharmacokinetic profiles of a select population of patients enrolled in these trials should provide a greater understanding of the anti-cancer mechanism of action for this drug.

Tandem liquid chromatography/mass spectrometry (LC/MS/MS) has become a well-established technique for rapidly obtaining pharmacokinetic profiles [14,15]. Using this technique, a variety of compounds in a wide array of matrices such as blood, plasma, bile, or urine can be assayed by methods that have been shown to be selective, linear, sensitive, and rugged. Due to the complexity of these matrices, sample preparation via extraction or protein precipitation remains necessary prior to LC/MS/MS.

Solid-phase extraction (SPE) continues to evolve in both utility and range of bioanalytical applications. Recent advances in automated workstations and 96 well technology have broadened the scope of this technique [16–19]. Both method development and sample preparation times can be abbreviated by automated SPE. In addition to analyst timesavings, automated SPE offers the advantages of selectivity, improved performance for polar or ionic compounds compared to liquid/liquid extractions, and minimal solvent waste generation [19]. Automated SPE combined with LC/MS/MS has been utilized in the determination of several compounds including indomethacin [20], cortisol and prednisolone [21], iloperidone [22], and glucocorticoid fluticasone proprionate [23].

In this study, we combined automated SPE with LC/MS/MS for the quantitation of compound I in human plasma, with application to population pharmacokinetics. The pharmacokinetic disposition of compound I in patients enrolled in Phase I clinical trials with advanced solid tumors whose disease is refractory to conventional treatment was determined [24]. Plasma samples were collected at pre-determined times after dosing on Days 1, 8 and 15 of a 21-day regimen, with compound I given once daily. Compound I was administered without regard to meals. The initial dose level of the compound was 4 mg/m^2. If a given dose level was well-tolerated, subsequent patients dose levels were increased by two until the maximum tolerated dose was reached. A total of 20 patients were studied with doses ranging from 4 to 8 mg/m^2. The results for 10 patients receiving the 6 mg/m^2 dose are summarized here.

2. Experimental

2.1. Materials

Test compounds were synthesized by Parke-Davis Pharmaceutical Research (Ann Arbor, Michigan). The structures for these are given in Fig. 1. They are designated as compound I (4-acetylamino-*N*-(2′-[aminophenyl]benzamide) and compound II (internal standard) (4-(acetylamino)-*N*-(2-acetylamino-phenyl)benzamide). Liquid nitrogen, used in the mass spectrometer ion source as curtain, drying, and nebulizing gas was purchased from AGA, (Maumee, OH). Ammonium acetate (analytical-reagent grade), glacial acetic acid (HPLC grade), acetonitrile (HPLC grade), and methanol (HPLC grade) were obtained from Mallinckrodt (Paris, KT). Reagent grade water

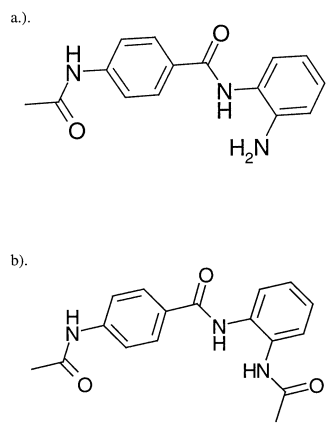

Fig. 1. Chemical Structures for (a) 4-acetylamino-*N*-(2′-[aminophenyl]benzamide), compound I; and (b) 4-acetylamino-*N*-(2-[acetylamino-phenyl]benzamide), compound II.

was prepared from in-house reversed osmosis, using a Milli-Q system (Millipore, Milford, MA). Pooled heparinized human plasma was obtained from Interstate Blood Bank, Inc. (Memphis, TN) and Valley Biomedical (Winchester, VA).

2.2. Apparatus

Automated solid-phase extractions were performed on a Zymark RapidTrace workstation (Zymark, Hopkington, MA) equipped with 10 extraction modules, operating in parallel, and utilizing 100 mg Isolute C-18, 1 cc cartridge (Part # 221-0010A, Jones Chromatography, Lakewood, CO). The workstation was controlled by RapidTrace software operating under Windows for Workgroups (Microsoft, Bellevue, WA) on a laptop computer (Xpi, Dell Computer, Round Rock, TX).

LC/MS/MS experiments were performed on a model API-3000 LC/MS/MS system (Sciex, Concord, Ontario, Canada), outfitted with a quaternary solvent delivery system and autosampler (Perkin Elmer series 200, Norwalk, CT). The ion source was a Turbo-Ionspray, capable of operating at pneumatically assisted electrospray flow rates (100–500 μl/min). Ion molecule precusor: product transitions of 269.9 → 161.3 and 312.4 → 162.4 were used for compounds I and II, respectively, in multiple-reaction monitoring (MRM) mode.

A J' Sphere H80 chromatography column (Part # JH08S04-0502WTA, 4 μm particles, 50 mm × 2.0 mm I.D., YMC, Inc., Milford, MA) was employed and run under ambient isocratic reversed phase conditions. The mobile phase was composed of 64:20:16 ammonium acetate (0.005 M, pH 5.8): acetonitrile: methanol at a flow rate of 200 μl/min. Injections of 5.00 μl were utilized.

2.3. Preparation of standard

Stock solution I (200 μg/ml) was prepared fresh with the weighing of compound I and diluted with methanol. Stock solution I was diluted 10- and 100-fold to yield stock solutions II and III, respectively, with 50:50 methanol:water. Stock solution II (20 μg/ml) was diluted 25-, 50-, 100-, 200-, and 1000-fold with 50:50 methanol:water to produce 8000, 4000, 2000, 1000, and 200 ng/ml working stocks of compound I, respectively. Stock solution III (2.00 μg/ml) was diluted 20- and 100-fold with 50:50 methanol:water to produce 100 and 20.0 ng/ml working stocks of compound I, respectively. A 100 μl aliquot of each working stock was added to 1.90 ml of blank human plasma to prepare calibration standards for validation runs. These standards were subdivided into 0.600 ml aliquots and stored in polypropylene tubes at approximately −20°C until the time of assay.

2.4. Preparation of internal standard

A 0.100 mg/ml (free base) stock solution was prepared in methanol. This solution was diluted with 50:50 methanol:water to prepare a 500 ng/ml working internal standard solution. Addition of 25 μl aliquots of the working solution to 0.500 ml human heparin plasma gave a concentration of 25.0 ng/ml.

2.5. Preparation of quality controls

Stock solution II (20 μg/ml) was diluted 8-, 13-, 40-, and 1000-fold with 50:50 methanol:water to produce 2500, 1500, 500, and 20.0 ng/ml working stocks of compound I, respectively. Quality controls were prepared by diluting 5.00 ml aliquots of these working stock solutions to 100 ml with blank heparinized human plasma. These controls were subdivided into 0.600-ml aliquots and stored in polypropylene tubes at approximately −20°C until the time of assay.

2.6. Preparation of samples

The following components were added to borosilicate glass test tubes (13 × 100 mm): 500 μl heparinized human plasma (standard, sample, QC or blank plasma), 25.0 μl internal standard, and 25.0 μl water. Samples were vortexed approximately 5 s. Sample tubes were placed in the Zymark RapidTrace workstation that was programmed to process the samples by solid-phase extraction on a C18 sorbent, according to the procedure in Table 1. Analytes were eluted into

Table 1
Workstation program sequence for solid-phase extraction of compounds I and II from plasma

Step	Process	Reagent	Volume (ml)	Flow rate (ml/min)
1	Purge cannula	H_2O	3.00	10
2	Cartridge precondition	CH_3OH	0.75	10
3	Cartridge precondition	CH_3CN	0.75	10
4	Cartridge precondition	H_2O	0.75	10
5	Load sample	Sample	1.00	1
6	Wash cartridges	H_2O	0.75	2
7	Wash cartridges	(90:5:5) H_2O:CH_3OH:CH_3CN	0.75	2
8	Elute	(50:50) CH_3OH:CH_3CN	2	1
9	Purge cannula	H_2O	3.00	10

clean 12×75 mm borosilicate glass test tubes. Eluents were evaporated to dryness at 50°C under N_2 and residues were manually reconstituted with 200 μl of mobile phase.

3. Results and discussion

3.1. Sample throughput

A single RapidTrace workstation module can process up to 10 samples sequentially [17]. The workstation used for these experiments was configured with ten modules allowing for a total sample capacity of 100 (10×10). An extraction of compounds I and II from one sample would take 10 min; thus, 100 samples could be processed, unattended, in less than 2 h. This time is equal to or less than that required for manual solid-phase extractions. Carryover was nonexistent when using this configuration.

3.2. Specificity

Chromatograms representing the separation of the analytes from matrix are shown in Fig. 2. No plasma components were detected at the retention times for compound I (1.8 min) or compound II (2.1 min) in blank plasma samples from six independent sources. Compounds I and II were well retained from the void (Table 2), with k' values of 1.90 and 2.50, respectively. System specificity was gauged by α, the selectivity factor, and by R_s, the chromatographic resolution, in standard and sample chromatograms. Collision cell cross-talk originating from the internal standard was noted at a retention time of 2.1 min in the compound I channel, but because of adequate chromatographic separation, it did not interfere with the quantitation of the peak at 1.8 min. Compounds I and II were sufficiently well resolved ($R_s > 1.5$) from each other. No significant matrix components were detected under these MRM conditions. For a variety of samples from patients orally dosed with compound I, no metabolite peaks were apparent in the MRM channels used for this method.

3.3. Recovery

Mean recoveries (%RSD) of compounds I and II from human plasma were 106% recovery (25% RSD), 110% recovery (26% RSD), 105%recovery (19% RSD) and 77% recovery, (6% RSD), respectively, using solid-phase extraction at concentrations of 25, 75, and 125 ng/ml of compound I and 25 ng/ml of compound II (internal standard), respectively. The variability of the recovery within different batch runs was large, but was manageable in that it did not affect the overall precision of the assay, as discussed below.

3.4. Precision, accuracy, and linearity

This technique exhibited detection linearity from at least 1 to 400 ng/ml, based on seven point calibration curves. Calibrating curve regression was weighted as $1/x^2$ and performed using a

linear fit. A typical curve equation for compound I was $y = 0.072*x + 0.030$ with correlation coefficients (r^2) greater than 0.99. Mean relative errors in back calculated values for standards ($n = 3$) ranged from −8.2 to 5.2% for calibration curves generated on three separate days, suggesting linearity consistent with typical instrument performance.

Intra-day precision for 3 levels of quality controls (Table 3) ranged from 0.72 to 13.9% RSD intra-day, and was typically less than 5%. Inter-day precision ranged from 3.55 to 8.72%. Intra-day relative error estimates ranged from −6.35 to 3.24%. Inter-day relative error ranged from −1.73 to 0.24. These values suggest acceptable performance for a quantitative bioanalytical method.

3.5. Lower limit of quantitation (LLOQ)

The LLOQ was defined as the lowest concentration that could be determined with acceptable precision and accuracy, as demonstrated by repli-

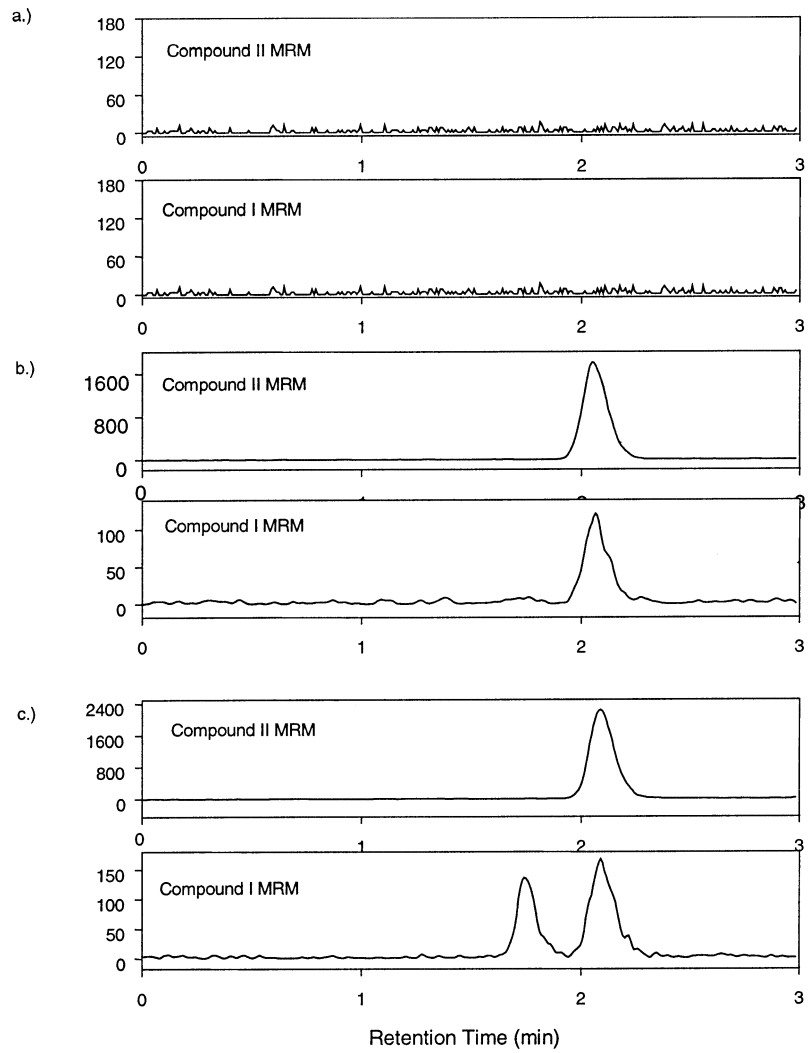

Fig. 2. Representative chromatograms of (a) blank human plasma without compounds I or II; (b) blank human plasma with compound II; and (c) 1.00 ng/ml compound I calibration standard in human plasma.

Table 2
System suitability parameters for compounds I and II

	Compound I	Compound II
Retention time (min)	1.77	2.11
Capacity factor (k')	1.90	2.50
Resolution (R_s)		1.5
Selectivity (α)		1.3

cate determinations. The intra- and inter-assay precision of LLOQ samples were 12.2 and 15.0% RSD, respectively, for compound I at 1 ng/ml. The intra- and inter-assay accuracy were -14.6 and -14.6 %RE, respectively, for compound I at 1 ng/ml. These results suggest that 1 ng/ml was an acceptable LLOQ for this assay method.

3.6. Stability

Compound I stability in plasma stored at −20°C was investigated by comparing the accuracy of quality controls at three levels over a given time interval. It was found that the quality control samples were stable for up to nearly 16 months. The quality control stability was not evaluated beyond this time period. Compound II was found to be stable in plasma for at least 2 h after spiking into plasma at ambient temperature. It was also observed that samples could be stored for nearly 3 weeks in chromatographic mobile phase at 4°C after preparation (extraction, dry down, and reconstitution). Thus, the overall stabilities of compounds I and II were generally well behaved.

Table 4
Compound I pharmacokinetic parameter summary of 10 patients receiving 6 mg/m^2 once daily[a]

	Vd/F	Cl/F	Ka	lag
Mean	59.8	2.7	2.5	1.36
%RSD	26%	23%	34%	20%

[a] Vd/F, Apparent volume of distribution (l); Cl/F, Apparent clearance (l/h); Ka, Absorption rate constant (per hour); lag, Absorption lag time (h).

3.7. Pharmacokinetics

Resulting concentration-time profiles were analyzed using conventional population pharmacokinetic methods. Table 4 summarizes the pharmacokinetic disposition of the anti-cancer

Table 3
Human heparin plasma compound I concentrations in quality controls for three separate batches

Concentration added (ng/ml) (Free-base equivalents)	25.0	75.0	125
Batch Run 1			
Intrarun mean	25.8	75.7	122
Intrarun %RSD	2.26	0.72	0.89
Intrarun %RE	3.13	0.94	−2.09
Batch Run 2			
Intrarun Mean	24.4	74.3	117
Intrarun %RSD	1.17	4.38	4.68
Intrarun %RE	−2.31	−0.97	−6.35
Batch Run 3			
Intrarun mean	25.0	72.0	129
Intrarun %RSD	7.84	3.48	13.9
Intrarun %RE	−0.10	−4.00	3.24
N	9	9	9
Mean concentration found (ng/ml)	25.1	74.0	123
Interrun SD	1.19	2.63	10.7
Interrun %RSD	4.75	3.55	8.72
Interrun %RE	0.24	−1.34	−1.73

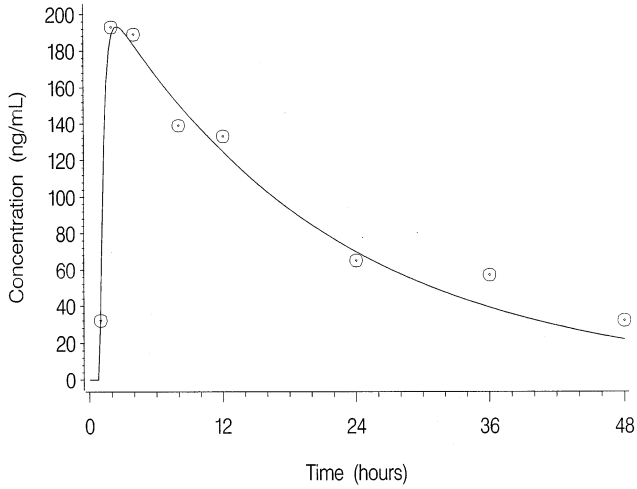

Fig. 3. Plasma profile of a typical patient receiving the 6 mg/m^2 dose.

agent. The plasma profile for a typical patient is presented in Fig. 3. Apparent volume of distribution and clearance were 59.8 l and 2.7 l/h, respectively with an estimation error of less than 10% for each parameter. The resulting elimination half-life averages 8.5 h. Interindividual variability in distribution volume and clearance was modeled as log–normal and averaged 26 and 23%, respectively. Estimation error for these same respective parameters was 24 and 38%. These results are consistent with those from earlier Phase I studies wherein an older, less sensitive analytical method was employed. An absorption half-life of approximately 15 min was estimated, however, due to the sparse sampling scheme used in this study there is a considerable degree of estimation error associated with this parameter. Similarly, the absorption lag time of over an hour is suspect due to infrequent sampling over the initial absorption window.

4. Conclusions

An automated SPE LC/MS/MS method to quantify an anti-cancer drug in heparinized human plasma was validated and applied. No human plasma components, drug metabolites, or internal standard cross talk interfered with quantitation of compound I. Compound I and II were sufficiently well resolved from each other and from all human plasma components. The method has proven suitable for routine quantitation of compound I in human heparinized plasma at concentrations from 1 to 400 ng/ml. The recovery, accuracy, and precision were found to be acceptable for a typical automated solid-phase extraction assay. This approach allowed for unattended, high throughput solid-phase extraction requiring less sample processing when compared to similar manual solid-phase extractions. Concentration-time profiles were generated from the resulting data and were analyzed using conventional population pharmacokinetic methods. These results are consistent with those from earlier Phase I studies wherein an older, less sensitive analytical method was employed.

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