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Development and validation of a high-sensitivity assay for an antipsychotic agent, CP-88,059, with solid-phase extraction and narrow-bore high-performance liquid chromatography

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Abstract

An analytical method has been developed and validated for the quantitation of CP-88,059 in human serum. The compound and internal standard were extracted from serum by solid-phase extraction with a weak cation-exchange phase. The analytes were resolved from endogenous interferences using narrow-bore (2.1 mm I.D.) C_{18} reversed-phase HPLC. Column effluent was monitored by UV absorbance detection at 215 nm. The standard curve range was 1 to 250 ng/ml. The accuracy and precision values for the method were within ±10% and ±15%, respectively. A four-fold detectability enhancement was achieved using a 2.1 mm I.D. HPLC column relative to the more common 4.6 mm I.D. column. A performance comparison was made between the 2.1 mm I.D. column used for validation and a 4.6 mm I.D. column with the same stationary phase.

1. Introduction

CP-88,059, 5-[2-(4-benzo[*d*]isothiazol-3-yl-piperazin-1-yl)ethyl]-6-chloro-1,3-dihydroindol-2-one (I, see Fig. 1), is an antipsychotic agent structurally related to tiosperone. To full characterize the pharmacokinetics of this drug, an analytical method with a lower limit (LLOQ) of at least 1 ng/ml in serum was required. We first developed a quantitative method utilizing HPLC–mass spectrometry (MS)[1]. This method, with a LLOQ of 0.5 ng/ml, was used to support phase I clinical studies. Due to the high cost and unavailability of the HPLC–MS for routine sample analysis, an alternative method was sought. It was necessary for the new method to be rugged and easily transferred to other users. I is non-fluorescent, non-volatile, and is not easily derivatized. In order to meet these requirements and conditions, HPLC with absorbance detection was our first choice. The absorbance maximum of I is below 220 nm, requiring very efficient removal of endogenous serum interferences. To achieve these goals, a very selective solid-phase serum extraction was coupled with absorbance detection at 215 nm. In addition, narrow-bore HPLC was used to increase detectability.

Described herein is the development and validation of a highly sensitive, accurate and precise

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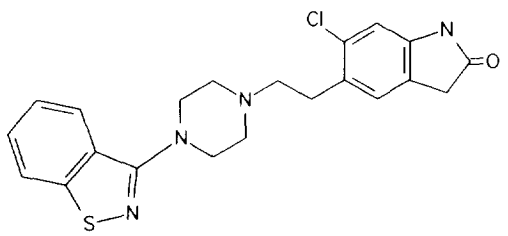
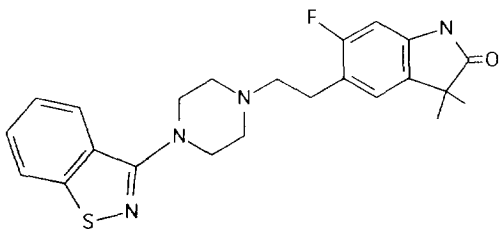


CP-88,059



Internal Standard

Fig. 1. Structures of I and I.S.

analytical method for the determination of I in serum.

2. Experimental

2.1. Chemicals and reagents

All solvents used were of HPLC grade or better. Water was purified with a Milli-Q system (Millipore, Marlborough, MA, USA) and had a resistivity of 18.2 Ω/cm at the outlet. The triethylamine (TEA) used in all reagents was of 99.9 + % grade (Aldrich, Milwaukee, WI, USA) and was stored at 4°C. Freshly opened containers were assigned an expiration date of 1 month. Solid-phase extraction (SPE) reagents containing TEA were prepared fresh daily. Serum quality control (QC) and standard samples were prepared from serum obtained in-house and from commercial sources (Cocalico Biologicals, Reamstown, PA, USA). All serum samples and controls were stored at −20°C. I and the internal standard (I.S.) were obtained from the pharmacy and sample bank at Pfizer Central Research (Groton, CT, USA).

2.2. Separation conditions

The HPLC system consisted of an Laboratory Data Control CM 3500 pump and SM 3200 variable wavelength detector (LDC Analytical, Riviera Beach, FL, USA), HP 1050 autosampler (Hewlett-Packard, Avondale, PA, USA) PE 1020 personal integrator (Perkin-Elmer, Norwalk, CT, USA) and LC-22C column heater (Biolanalytical Systems, West Lafayette, IN, USA). All separations were carried out on a 25 cm × 2.1 mm I.D. LC-18 DB HPLC column (Supelco, Bellefonte, PA, USA). A 25 cm × 4.6 mm LC-18 DB Supelco column was used during comparison experiments. Mobile phase was prepared by combining 600 ml water, 300 ml acetonitrile, 0.45 ml trifluoroacetic acid, and 0.72 ml triethylamine. This mixture was mixed well and passed through a 0.45-μm filter under vacuum. All separations were conducted at a flow-rate of 0.27 ml/min. The column temperature was maintained at 35°C.

2.3. Serum extraction

The serum extraction procedure utilizes weak cation-exchange (WCX) solid-phase extraction (SPE) columns (Supelclean LC-WCX, 3 ml, Cat. no. 57061, Lot no. SPO585, Supelco). A Vac Elut SPS 24 vacuum manifold (Jones Chromatography, Littleton, CO, USA) was used to process the SPE columns. Prior to extraction the columns were conditioned with one column volume (3 ml) of methanol followed by two column volumes of 1% acetic acid. The column bed was not allowed to dry during conditioning steps. The vacuum was then turned off and a 0.5-ml serum sample was applied to the individual columns followed by 50 μl of I.S. (equivalent concentration = 100 ng/ml). The samples were aspirated through the columns at an applied vacuum of approximately 5 in. Hg (ca. $16.9 \cdot 10^3$ Pa). The columns were then washed in the following order with a single column volume of each reagent; 1% acetic acid, methanol and 0.25% (v/v) triethylamine in acetonitrile. Compound I and I.S. were eluted into 16 × 100 mm silated glass tubes with a single column volume

of 1.0% triethylamine in acetonitrile. The samples were evaporated to dryness using a TurboVap analytical evaporator (Zymark, Hopkinton, MA, USA) under a gentle stream of nitrogen at 42°C. The dried residue was reconstituted in 40 μl of HPLC mobile phase and 30 μl injected on to the column.

2.4. Calculations

A seven-point standard curve from 1 to 250 ng/ml was prepared in control human serum by serial dilution of a stock prepared at 250 ng/ml. The curve was constructed by analyzing freshly prepared serum standards and plotting peak area ratio versus concentration. The line of regression was determined by the method of least squares with a weighting factor of 1/concentration. Quality control samples (QCs) prepared at five concentrations in serum were used to assess assay accuracy and precision. The QC concentrations corresponded to LLOQ, upper limit of quantitation (ULOQ), lower, mid-point, and upper portions of the standard curve range. The concentration of I in QC samples was calculated relative to the regression line.

3. Results and discussion

Representative chromatograms of human serum extracts at 0.0 and 1.0 ng/ml are shown in Fig. 2. Assay specificity was evaluated by analysis of control serum drawn from 5 individuals, several lots of serum from a commercial source, and predose clinical samples. In all cases, the region in which I elutes was free of endogenous interference. All known metabolites of I are very polar relative to the parent and are expected to elute in the void [2]. In addition, the circulating metabolites are present at very low levels.

Compound I was found to be stable at −20°C for at least six months. QC samples were subjected to three freeze–thaw cycles and analyzed to assess freeze–thaw stability. No significant deviation from nominal values was found.

Intra-assay performance was assessed by assaying QCs at five different concentrations in replicates of five on the same day. The results of this experiment are presented in Table 1. Intra-assay accuracy and precision values were within ±10% and ±15%, respectively. Inter-assay performance was assessed by analyzing QC samples prepared at 6.0, 100.0 and 200.0 ng/ml in duplicate on four different days. The results of the inter-assay experiment is presented in Table 2. Inter-assay accuracy and precision values were within ±4.0% and ±7.0%, respectively.

3.1. Extraction performance

Initial assay development approaches involved liquid–liquid extraction into normal butyl chloride. This method provided adequate recovery of drug, but resulted in unacceptable interference and late eluting peaks, presumably due to the low detection wavelength used (215 nm). A more successful approach, based on ion-exchange SPE, involves exploitation of the adsorptive characteristics of I afforded by the basic nitrogen atoms present in the structure. In the SPE scheme used, the drug is adsorbed to the weak cation phase at acidic pH. The binding of the drug to the phase is tight enough to allow washes with pure methanol prior to elution of the analyte. Elution was accomplished with 3 ml of 1% (v/v) triethylamine in acetonitrile. Interestingly, it was found that all of the analyte was present in the third ml of elution solvent. TEA apparently competes with the adsorbed I, saturating the adsorption sites and eluting it from the column. The TEA–acetonitrile eluent is removed by evaporation leaving a barely discernible residue in the collection tube.

Extraction recovery of I was evaluated by comparison to standards prepared in mobile phase at concentrations equivalent to serum QCs at 6.0, 100.0 and 200.0 ng/ml. The average recovery of drug over this concentration range was 66%.

3.2. Narrow-bore column performance

Narrow-bore columns (2–3 mm I.D.) were used in this work with the primary intent of achieving greater detection sensitivity. Reduced

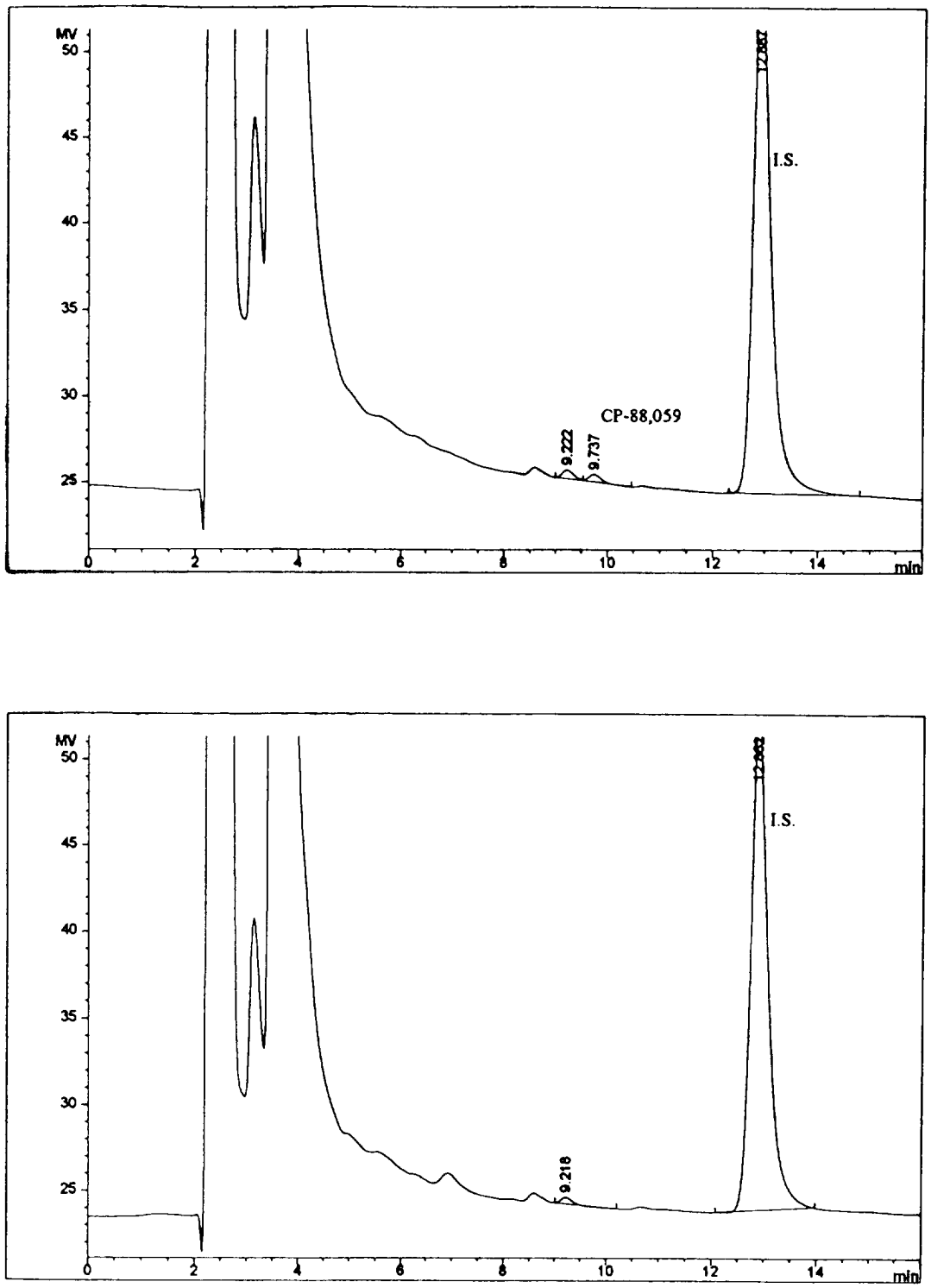


Fig. 2. Representative chromatograms of human serum extracts. Upper: I at 1.0 ng/ml. Lower: Blank serum with I.S.

Table 1
Intra-assay precision and accuracy

Concentration, (n = 5) (ng/ml)			R.S.D. (%)	Deviation (%)
Nominal	Calculated	S.D.		
1	1.04	0.15	14.58	4.00
2	2.18	0.18	8.21	9.00
6	6.42	0.73	11.41	7.00
100	101.53	9.25	9.11	1.53
200	204.57	16.24	7.94	2.28
250	226.78	14.37	6.33	−9.29

diameter columns dilute the chromatographic band to a lesser extent, and concentration-sensitive detectors exhibit enhanced detectability. In theory, peak height is 4.8 times larger on a 2.1 mm I.D. column than on a 4.6 mm I.D. column of the same length and particle size [3]. Fig. 3 shows an overlay of neat standards on columns differing only in internal diameter. During assay development, it was found that use of narrow-bore columns resulted in four-fold enhancement for I relative to 4.6 mm I.D. columns.

While the advantages of these columns are well known [3–5], their use in bioanalysis has not been extensive. The primary reason for this has been the perception that band-broadening and loss of resolution would occur with conventional instrumentation [4,5]. Chromatographic resolution is of particular importance in the trace analysis of complex biological matrices. Since most drugs have UV absorbance maxima in the low (<220 nm) UV, resolution from endogenous interference is essential in achieving robust LLOQs.

To characterize the impact of narrow-bore columns on assay performance, several variables were studied. Comparisons of chromatographic efficiency and peak shape were made between columns identical in all respects except for internal diameter. Of specific interest were the effects of injection volume and conventional equipment on efficiency.

Peak efficiency and asymmetry were compared for the 2.1 mm I.D. versus 4.6 mm I.D. columns by injecting equivalent amounts of a standard prepared in mobile phase at increasing injection volumes. The results of this experiment are presented in Table 3. Theoretical plate values were approximately 3.5 times less with the 2.1

Table 2
Inter-assay precision and accuracy

Analysis sequence	Mean calculated concentration (ng/ml)		
	6.0 ng/ml[a]	100.0 ng/ml[a]	200.0 ng/ml[a]
1	6.4	101.5	204.6
2	5.7	95.1	194.3
3	5.7	98.8	192.2
4	5.6	91.8	184.8
Inter-assay			
R.S.D. (%)	6.3	4.4	4.2
Deviation (%)	−2.5	−3.2	−3.0

[a] Nominal concentrations.

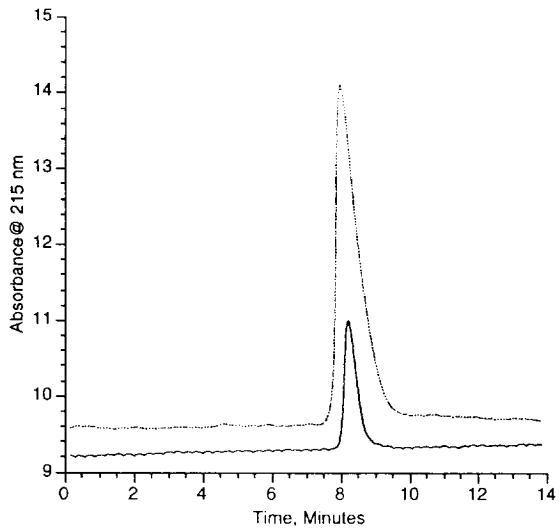


Fig. 3. Overlay chromatograms which depict the sensitivity enhancement using the narrow-bore HPLC column. Dotted line: 2.1 mm I.D. column. Solid line: 4.6 mm I.D. column. Equivalent amounts were injected.

mm I.D. column, presumably due to the pronounced extra column band broadening encountered when using conventional instrumentation coupled with narrow-bore columns [4]. HPLC tubing of 0.010 in. (1 in. = 2.54 cm) I.D. and a relatively large detector flow cell (10 mm pathlength, 14 μl volume) were used for this experiment. While peak efficiency should improve by reduction of extra column volumes at this end of the system, method performance was adequate under these conditions.

In addition to reduced efficiency, narrow-bore columns exhibited increased peak asymmetry relative to conventional diameters. The increased peak asymmetry was likely due to a combination of the amplified response and efficiency loss observed under these conditions.

Concurrent with reduced flow-rates, narrow-bore columns require reduced injection volumes to maintain chromatographic performance. As detection sensitivity was a primary consideration in this case, it was desirable to maximize sample loading. Plate numbers for I were approximately equivalent for volumes of 1 to 30 μl. However, injection volumes of 40 and 50 μl resulted in further loss of efficiency relative to the smaller injection volumes, presumably due to precolumn band broadening effects.

The decline in chromatographic performance with the 2.1 mm I.D. column was not unexpected. The tradeoff in this case was the significant increase in detectability achieved with the smaller diameter column. A further advantage of these columns is that increased absorbance response and reduced solvent consumptions can be attained with unaltered conventional instrumentation. Changes in flow cell design and attention to extra column volume effects should result in improved chromatographic performance.

4. Conclusions

The validated method for I is sensitive, accurate, and precise. Use of narrow-bore columns results in a four-fold increase in detector re-

Table 3
Peak efficiency and asymmetry comparison ($n = 5$)

2.1 mm I.D.			4.6 mm I.D.		
Volume injected (μl)	Efficiency	A_S[a]	Volume injected (μl)	Efficiency	A_S[a]
1	3174	1.5	1	10946	1.1
5	2893	1.5	5	10483	1.2
10	3116	1.5	10	10551	1.1
20	3106	1.5	20	12007	1.1
30	2810	1.5	30	10265	1.1
40	2168	1.4	40	10938	1.1
50	2109	1.4	50	10862	1.2

[a] A_S = asymmetry.

sponse for I, and is key to achieving the required detection limit. While narrow-bore columns can be used with conventional HPLC instrumentation, some losses in chromatographic performance due to extra column effects are observed.

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