Quantitative Analysis by High-Performance Liquid Chromatography Atmospheric Pressure Chemical Ionization Mass Spectrometry: The Determination of the Renin Inhibitor CP-80,794 in Human Serum

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A specific method was developed for the quantitative determination of the renin inhibitor CP-80,794. Serum extracts containing the drug and an internal standard were injected into a standard reverse-phase high-performance liquid chromatography (HPLC) column. The mobile phase, methanol/water (8/2), flowed at 1 mL/min through the column and then via a heated nebulizer interface into a corona discharge atmospheric pressure chemical ionization source. The assay minimum limit of quantification was 50 pg/mL. It exhibits satisfactory accuracy and precision over the range 50 pg/mL to 10 ng/mL. A minor modification of the HPLC mobile phase was necessary to attain extremely low detection limits. The addition of a structural analogue contributed to enhancing the precision of the assay. *(J Am Soc Mass Spectrom 1991, 2, 164–167)*

Much of the current excitement about high-performance liquid chromatography (HPLC) atmospheric pressure mass spectrometry (APMS) is due to the generation of multiply charged ions by electrospray [1, 2], which extends mass spectrometry to peptides and other large molecules [1, 3]. Other approaches to interfacing HPLC and APMS have been partially overlooked. The heated nebulizer interface developed by Thomson [4] permitted gas-phase atmospheric pressure chemical ionization (APCI) of materials eluting from standard HPLC columns. In our laboratory, the interface was applied to the analysis of several drugs and has resulted in detection limits and chromatographic fidelities approaching and sometimes exceeding those possible by gas chromatography mass spectrometry (GC/MS).

The modified peptide CP-80,794 (Figure 1, I) inhibits serum renin of various species and promises to be an efficacious orally active drug for the management of hypertension. Its in vitro inhibitory potency against human renin (IC_{50} = 0.7 nM) suggests pharmacological action at concentrations below 200 pg/mL [5]. A specific method for trace-level quantification of CP-80,794 in human serum was desired to investigate the drug's pharmacokinetics and pharmacodynamics in human subjects.

This article describes an assay employing the heated nebulizer interface for the quantitative determination of CP-80,794 by HPLC/APCI. Concentrations as low as 50 pg/mL of serum have been determined by selected ion monitoring.

Experimental

Serum Extraction

A 1-mL aliquot of human serum was placed in a silylated disposable culture tube containing 50 ng of the carrier (Figure 1, III) in 100 μL of methanol. To this was added 1 ng of internal standard (Figure 1, II), and the sample was vortexed. The serum was made basic with 100 μL of 1 N NaOH, vortexed, and extracted with 5 mL of *n*-butyl chloride for 10 min by using a reciprocating shaker. Following centrifugation for 5 min, the aqueous layer was frozen in a dry ice/isopropyl alcohol bath. The organic layer was decanted into a silylated conical centrifuge tube containing 2 mL of acetonitrile, and the extract was evaporated to dryness in a vortex evaporator (block temp = 37°C). An additional 1 mL of acetonitrile was added to the dried extract, vortexed, and the drying process

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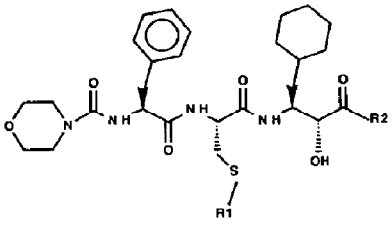

	R_1	R_2	Molecular Wt.
I. Analyte: CP-80,794	CH_3		620
II. Internal Std: CP-88,587	C_2H_5		634
III. Carrier: CP-81,489	CH_3	NH — CH_3	591

Figure 1. Structure of the subject compound and analogues used in the assay procedure.

was repeated. The dried residue was reconstituted in 60 μL of methanol, followed by the addition of 40 μL of water. Injections of 80 μL were made onto the HPLC/MS system.

Assay Calibration

Human serum was fortified with CP-80,794 at 11 concentrations (6 replicates per concentration) ranging from 0.05 to 10 ng/mL. Two standard curves were constructed to compare CP-80,794 concentrations vs. the peak height ratios of the drug over the internal standard. One curve was based on concentrations ranging from 0.1 to 10 ng/mL. To accommodate samples containing less than 0.1 ng/mL, a second curve was based on concentrations ranging from 0.05 to 1.0 ng/mL. The peak height data generated by the mass spectrometry data acquisition system were adjusted to reflect true electronic zero for a sample blank. This adjustment was arrived at by randomly measuring ion intensities of drug and internal standard in blank areas of each chromatogram and calculating mean values from those random measurements. The mean values were then subtracted from the acquired peak height data, thereby performing a background-subtracted quantitation routine.

Serum blanks and an 11-point standard curve (two samples per concentration) were run with each set of unknown samples. Data from linear regression analysis of each curve were used to calculate concentrations of the unknown samples by using the equation $y = mx + b$. The high curve was used for all samples. Samples shown to contain concentrations less than 0.1 ng/mL were recalculated by using the low curve.

Analysis

The mobile phase was prepared by mixing HPLC-grade methanol and water (8/2) and degassing through a 0.7-μm glass fiber filter. The analytical column was a WATERS (Milford, MA) C-18 Nova-Pak (3.9 × 150 mm), preceded by an SSI (State College, PA) precolumn stainless steel filter (0.5 μm). An LDC (Riviera Beach, FL) Constametric 3000 pump was used, and a flow rate of 1 mL/min was established. A Perkin-Elmer (Norwalk, CT) ISS-100 autosampler injected 80-μL sample aliquots onto the column at 4-min intervals. The column effluent was interfaced to the heated nebulizer probe of the mass spectrometer. Nonretained peaks, drug, and internal standard had elution times of 1.0, 3.1, and 3.4 min, respectively, in the system described here.

The analysis was performed on a SCIEX (Thornhill, Canada) API III HPLC/MS system. The effluent from the HPLC column flowed into the atmospheric pressure ionization source via a heated nebulizer interface (nebulizer probe temp = 500°C). The nitrogen nebulizing gas pressure of the interface was fixed at 95 psi, and auxiliary flow was set at 3.4 L/min. Prior to each period of analysis, instrumental performance was determined by injecting 10 pg (flow injection; no column) of CP-80,794. A response ≥ 3 times the mean background signal indicated adequate performance. Gas-phase chemical ionization was effected by a Corona discharge needle (−2.5 μA). Negative ions formed in the source were sampled into the quadrupole mass filter via a 0.0045″ pinhole aperture. The mass spectrometer was adjusted to selectively monitor drug anions $[M - H]^-$ at $m/z = 619$, and internal standard anions $[M - H]^-$ at $m/z = 633$, each ion at a dwell time of 500 msec. The nitrogen curtain gas was adjusted to a constant flow rate of 1.2 L/min. Voltage parameters of the quadrupoles and Brubaker lenses used during sample analysis were as follows: OR = −33 V, R0 = −30 V, R1 = −26 V, L7 = 0 V, R2 = 0 V, R3 = 0 V, L9 = −30 V. Voltages for the Faraday plate and the multiplier were −160 V and +5000 V, respectively. The channel electron multiplier was operated in a pulse-counting mode, and was capable of recording 4×10^6 counts/s. The power supplies were adjusted so that unit mass resolution was achieved (RE = 120).

Results and Discussion

The potent pharmacological activity of the renin inhibitor CP-80,794 necessitated its quantitative determination in the low pg/mL range. The low volatility and thermal instability of this modified tripeptide precluded the use of GC/MS for its analysis. In addition, the drug lacks a significant chromophore, electrophore, or fluorophore to permit its trace-level determination by standard HPLC methods. Hydrolysis of the bond between the phenylalanine and S-methyl cysteine residues of CP-80,794 followed by fluorescence derivatization of the resulting amine-containing

fragment did not provide the sensitivity required for detection of therapeutic concentrations of CP-80,794.

Although thermospray HPLC/MS has been used for low-level quantitative determination of certain compounds, the technique is known to be compound-specific [6]. For most compounds, its practical detection limits are 10 to 50 times higher than obtainable by GC/MS [7]. For CP-80,794, the minimum limit of detection by thermospray was 50 ng.

The commercial availability of a mass spectrometer designed specifically for atmospheric pressure ionization (SCIEX) provides another option for on-line HPLC/MS. The instrument supports two approaches for HPLC/MS interfacing: heated pneumatic nebulization [4] and pneumatically assisted electrospray [2]. The two approaches complement each other; together they are applicable to essentially all classes of compounds. In our laboratory they have outperformed thermospray in the analysis of many drugs and metabolites.

The heated nebulizer interface permits gas-phase APCI of analytes eluting in the HPLC mobile phase [4]. The technique generates mild ionization conditions, and the internal energy of the ions is low. The rapid desolvation of the nebulized droplets minimizes fragmentation or thermal decomposition, and provides abundant molecular ions [8, 9]. The outer jacket of the nebulizer probe is usually maintained at 350–500°C depending on the solvent composition and flow rates. However, the solvent and analyte molecules do not come in contact with this temperature. The vapor temperature was estimated to be 125–150°C [7]. Although the interface is compatible with various reverse-phase mobile-phase compositions and flow rates of up to 2 mL/min [7], there are limitations to low-level detection with buffered mobile phases. A buffered mobile phase produces an erratic and nonreproducible baseline, significantly reducing signal-to-noise ratios. The relative intensity of the baseline noise correlates with the concentration of ammonium acetate in the mobile phase (Figure 2). The increased background noise appears to be due to the formation of buffer ion clusters, which can be declustered by increasing the dry nitrogen curtain gas flow rate or by increasing the voltage of the orifice plate. This, however, decreases the signal from the analyte. The background noise can sufficiently be reduced to detect CP-80,794 concentrations of 50 pg/mL only when the buffer components are eliminated from the mobile phase.

A structural analogue of CP-80,794 served as an internal standard (Figure 1, II). A second structural analogue (III) was added to each sample to reduce adsorption losses during sample preparation and HPLC/MS analysis [10]. High concentrations of III enhanced extraction efficiency and the precision of the assay (Table 1), presumably due to carrier effects [11]. The results show that for those samples spiked with carrier, the extraction efficiency of the drug and the

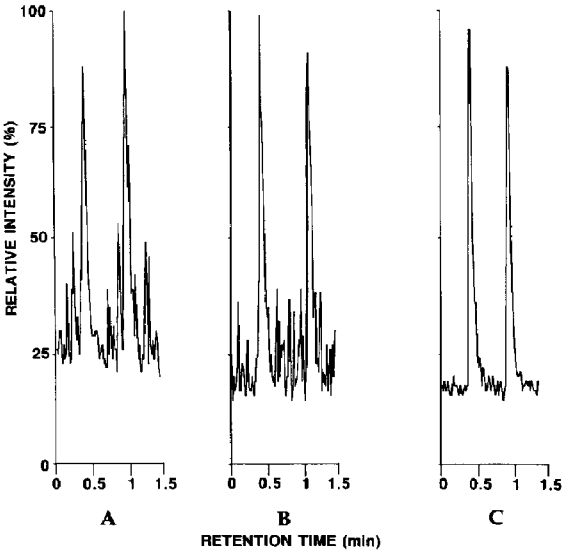

Figure 2. The effect of mobile-phase composition on signal-to-noise for duplicate 100-pg injections of CP-80,794. (a) 10 mM ammonium acetate:methanol, (b) 5 mM ammonium acetate:methanol, (c) water:methanol.

Table 1. Effect of adding 50 ng/mL of the carrier CP-81,489 (III) to serum samples fortified with CP-80,794 at 0.1 ng/mL

Sample #	Drug pk ht	IS pk ht	Adjusted D/IS ratios
Without Carrier:			
1	4420	10080	0.140
2	4400	7660	0.205
3	4140	5960	0.243
4	4560	6020	0.371
5	3900	6320	0.192
6	3880	6660	0.180
7	4000	6740	0.197
8	3860	6560	0.182
9	3840	6000	0.211
10	3640	5960	0.229
Mean	4064	6796	0.210
Std. Dev.	303	1268	0.060
C.V.[a]	7.5	18.7	29
With Carrier:			
11	5100	8300	0.127
12	5560	9740	0.186
13	4900	7160	0.162
14	4940	7660	0.178
15	5180	10440	0.167
16	5080	8500	0.099
17	4800	7920	0.175
18	4780	8340	0.181
19	4920	10200	0.159
20	4720	7820	0.179
Mean	4998	8608	0.160
Std. Dev.	247	1127	0.030
C.V.[a]	4.9	13.1	17

[a] Coefficient of variation.

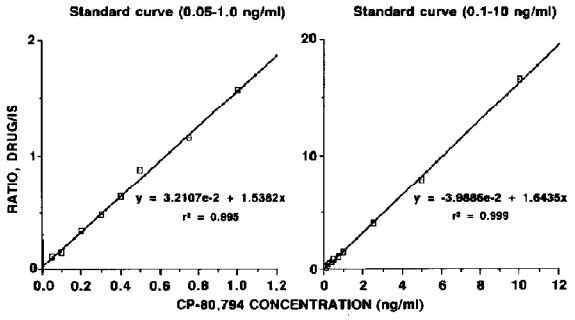

Figure 3. Standard curves of the CP-80,794 assay for concentrations ranging from 0.05 to 10 ng/mL.

internal standard was improved by approximately 24%, and the assay precision was improved by approximately 35%.

The linear dynamic range of the assay is quite narrow. For best accuracy, two standard curves (Figure 3) were required for concentrations ranging from 0.05 to 10 ng/mL. The narrow dynamic range of the assay was also evident in other APCI or electrospray methods. We cannot at this time offer a satisfactory explanation of this phenomenon. Typically, the background level of APCI is high, and background subtraction routines were required for best linear regression analyses and smallest y-intercept.

The present assay permits the quantitation of CP-80,794 in serum over the range 0.05 to 10 ng/mL with satisfactory accuracy and precision (Table 2; Figure 4). Samples containing more than 10 ng/mL can be diluted with control serum before analysis. The assay has already been used to support several clinical studies. The mass spectrometer, which has been in continuous use for over one year, proved to be reliable and rugged. No significant downtime or mass marker drift was encountered. During the continuous introduction of biological samples, the signal for the analyte steadily decreases because of the buildup of a deposit on the corona discharge needle. Cleaning or replacing the needle, an operation requiring about one minute, regenerates the initial response. During the present assay, the needle was cleaned after every 50 serum samples.

Table 2. Analysis of serum samples containing known amounts of CP-80,794; intra-assay parameters (n = 6)

Fort. conc.[a] (ng/mL)	Conc. found (ng/mL)	C.V.[b]	% accuracy
0.05	0.052	7.7	104
0.10	0.119	10.1	119
0.20	0.227	9.7	113
0.30	0.313	6.4	104
0.40	0.407	5.4	102
0.50	0.556	7.7	111
0.75	0.717	5.0	96
1.00	1.034	5.8	103
2.50	2.488	12.5	100
5.00	4.820	6.7	96
10.00	10.094	9.9	101

[a] Fortified concentration.
[b] Coefficient of variation.

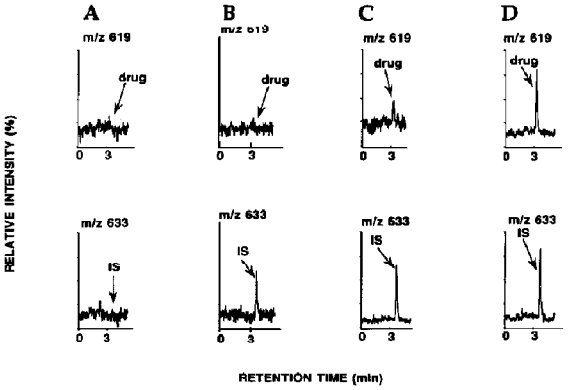

Figure 4. Representative HPLC chromatograms of human serum samples. Extracted ion currents for (a) Blank, (b) Blank + IS, (c) 0.1 ng/mL, and (d) 0.5 ng/mL.

Summary

The use of HPLC/APMS allowed the quantitative determination of the subject compound down to 50 pg/mL of serum. The assay has thus far been applied to the analysis of more than four thousand clinical samples.

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References

1. Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. *Science* **1989**, *246*, 64.
2. Bruins, A. P.; Covey, T. R.; Henion, J. D. *Anal. Chem.* **1987** *59*, 2642.
3. Covey, T. R.; Bonner, R. F.; Shushan, B. I.; Henion, J. D. *Rapid Commun. Mass Spectrom.* **1988**, *1*, 249.
4. Thomson, B. A.; Danylewych, M. L. *31st Annual Conference on Mass Spectrometry and Allied Topics*, Boston, 1983, p. 852.
5. Murphy, W. R.; Wester, R. T.; Hoover, D. J.; Holt, W. F.; Rosati, R. L.; Purcell, I. M.; MacAndrew, J. T.; Schelhorn, T. M.; Wilder, D. E.; Smith, A. H. In *Amino Acids: Chemistry, Biology, and Medicine*; Lubec, G., Rosenthal, G. A.; Eds.; ESCOM Science Publishers B. V., 1990; pp. 676–688.
6. Straub, K. M. *Progress in Drug Metabolism* **1988**, *11*, 267.
7. Covey, T. R.; Lee, E. D.; Bruins, A. P.; Henion, J. D. *Anal. Chem.* **1986**, *58*, 1451A.
8. Crowther, J. B.; Covey, T. R.; Silverstre, D.; Henion, J. D. *LC Magazine* **1985**, *3*, 240.
9. Covey, T. R.; Lee, E. D.; Henion, J. D. *Anal. Chem.* **1986**, *58*, 2453.
10. Self, R. *Biomed. Mass Spectrom.* **1978**, *6*, 315.
11. Lee, M. G.; Millard, B. J. *Biomed. Mass Spectrom.* **1975**, *2*, 78.