ELSEVIER

Journal of Chromatography B, 718 (1998) 87–94

JOURNAL OF CHROMATOGRAPHY B

# Direct plasma injection for high-performance liquid chromatographic–mass spectrometric quantitation of the anxiolytic agent CP-93 393

Shane R. Needham*, Mark J. Cole, Hassan G. Fouda

*Department of Drug Metabolism, Central Research Division, Pfizer Inc, Groton, CT 06340, USA*

Received 13 March 1998; received in revised form 30 June 1998; accepted 17 July 1998

**Abstract**

A direct plasma injection method has been developed for the rapid analysis of drugs in biological fluids. A new generation restricted access media column specifically designed to accommodate direct injection of plasma and other fluids is utilized for on-line HPLC–ESI-MS analysis. For rapid analysis the on-line extraction column is linked to a HPLC–ESI-MS system. Good results are obtained for the quantitation of CP-93 393 and deuterated internal standard over the range of 10–1000 ng/ml. The lower limit of detection for the assay was 58 pg injected on column. Accuracy and precision values are 9.0% or better over the entire range of the assay. In addition, more than 200 injections (100 μl) were performed per column with unattended, automated analysis. 

*Keywords:* CP-93 393

## 1. Introduction

Sample preparation is now the rate-limiting step to higher throughput HPLC–MS analysis of drugs in biological fluids. To facilitate faster sample preparation times several groups [1,2] have developed off-line SPE assays in the 96-well plate format. Automated on-line SPE has been reported [3,4] for quantitation of several pharmaceutical compounds. Another, possibly more efficient, approach for sample preparation is to directly inject the biological fluid onto a restricted access media HPLC column. Direct injection of the fluid eliminates the time consuming sample extraction step and facilitates complete on-line automation. A recently available restricted access media (RAM), HPLC column, BioTrap 500, is specifically designed to accommodate the direct injection of plasma and other fluids. The column permits the rapid elution of water-soluble salts and large molecules while retaining smaller hydrophobic molecules. The BioTrap 500 column is a porous silica based extraction column with a biocompatible external surface and a hydrophobic, C-18 internal surface. The pores of the column are small enough to exclude proteins and other large endogenous compounds while smaller molecules penetrate the pores and adsorb onto the C-18 groups. The biocompatibility has been obtained by coating the outer silica surface with $\alpha_1$-acid glycoprotein (AGP). Unlike more traditional re-

*Corresponding author.

0378-4347/98/$19.00 
PII: S0378-4347(98)00353-3

stricted access media columns, AGP tolerates high concentrations of organic solvents and large volume (up to 1.0 ml) injections of plasma and other biological fluids. Similar columns have been used for quantitation of pharmaceutical compounds; [5–11] however, the advantages of coupling the technique with the selectivity and sensitivity of API-MS have not been fully exploited. The majority of previous direct plasma injection assays [5–7,9–11] possessed long analysis times (>15 min), which are not acceptable in a high-throughput, drug discovery environment. Total analysis times of less than 5 min per sample are achievable using dual BioTrap 500 columns set up on-line with HPLC–MS detection.

CP-93 393 currently undergoing clinical evaluation as an anxiolytic agent was chosen as a model analyte for this on-line extraction and analysis method. A previous HPLC–API-MS method was validated to determine concentrations of CP-93 393 in human, rat and mouse plasma and determine drug stability in plasma at room temperature [12]. Since samples may await analysis in an autosampler for an extended period of time (>8 h), stability of the drug in the biological fluid is an important consideration when using an on-line extraction method. Therefore, prior to analysis, analyte stability in the biological matrix must be confirmed under appropriate conditions.

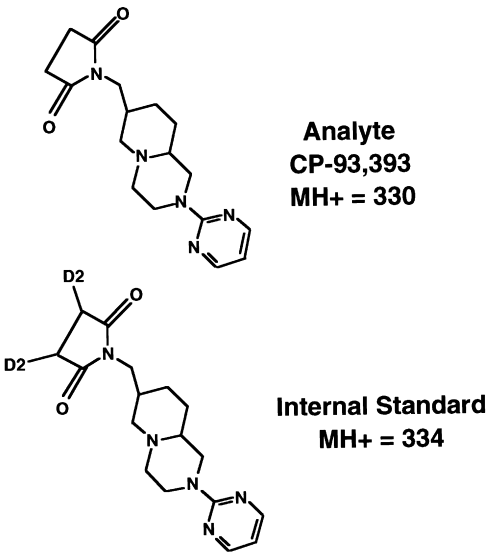


Fig. 1. Structures of CP-93 393 and CP-93 393-*d*4.

## 2. Experimental

### *2.1. Materials*

CP-93 393 [1-{2-pyrimidin-2-yl-octahydro-pyrido-[1,2-a]pyrazin-7-ylmethyl}-pyrrolidine-2,5-dione] was obtained from Pfizer Central Research, Groton, CT, USA.

CP-93 393-*d*4 (Fig. 1) was synthesized in-house. Isotopic purity was 99%.

Other reagents were of HPLC grade or better and were used without further purification. Control human plasma was obtained from in-house volunteers.

### *2.2. Column switching setup*

For accelerated sample analysis, a dual extraction column set up was used with two Gilson (Middleton, WI, USA) valve actuators fitted with Rheodyne

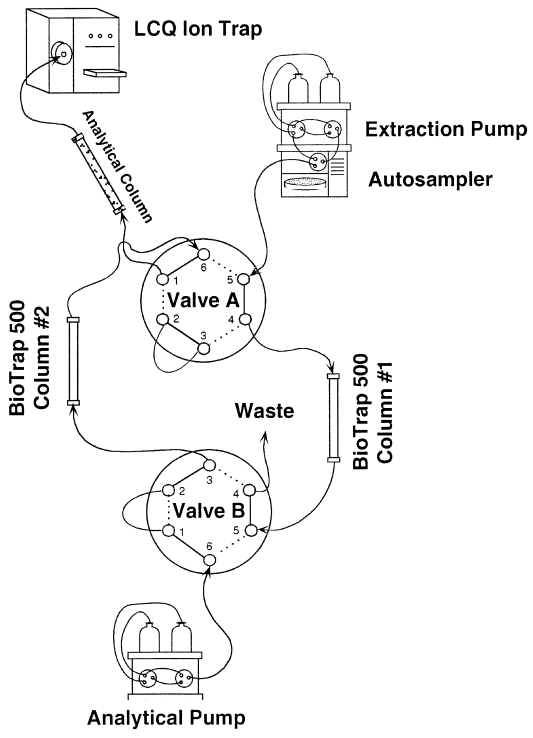


Fig. 2. Flow diagram for dual BioTrap column plasma extraction and analysis. Valve position for equilibrating and loading BioTrap 500 column 1 while transferring analytes from BioTrap 500 column 2 onto the analytical column.

(Cotati, CA, USA) 7000 six-port injector valves. The switching valve configuration is diagrammed in Fig. 2 and is similar to that described by Ramsteiner [13]. The dual column arrangement allows the extraction of one column while the other column is equilibrating and rinsing from the previous injection. After 2.5 min of extracting on one of the BioTrap columns, the valves are switched, allowing the extraction column to be backflushed with analytical mobile phase thereby eluting the analytes from the BioTrap column onto the analytical column.

### 2.3. HPLC

A LDC Analytical constaMetric 3200 series pump (Riviera Beach, FL, USA) delivered the mobile phase for extraction on the 20 mm×4.0 mm I.D. BioTrap 500 column (ChromTech, Hagersten, Sweden). The flow-rate for extraction was maintained at 2 ml/min using 99:1 50 m*M* ammonium acetate–isopropanol with 2 m*M* triethylamine as the mobile phase. A Hewlett-Packard 1050 series autosampler (Waldbronn, Germany) injected 100 μl aliquots of human plasma fortified with drug and internal standard onto the BioTrap 500 extraction column. A Hewlett-Packard 1050 series HPLC pump (Waldbronn, Germany) delivered the analytical mobile phase at a flow-rate of 0.5 ml/min causing the elution of the analytes from the BioTrap 500 column onto the analytical HPLC column. A Hewlett-Packard 1050 series multiple wavelength detector (Waldbronn, Germany) set at 238 nm was employed to perform the extraction recovery experiments. The analytical mobile phase was a 25:75 methanol–10 m*M* ammonium acetate mixture adjusted to pH 3 with formic acid. A Keystone Scientific (Bellefonte, PA, USA) Hypersil phenyl column (30 mm×2 mm I.D., dp=5 μm) was used as an analytical column to provide further separation of analytes from other endogenous plasma components before detection. A 2-μm and a 0.5-μm Upchurch filter (Oak Harbor, WA, USA) preceeded the analytical HPLC column.

### 2.4. Mass spectrometry

A Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) operating in the positive ion electrospray ionization mode was employed for the detection of analytes. Detection was performed using selected ion monitoring (SIM). The heated capillary

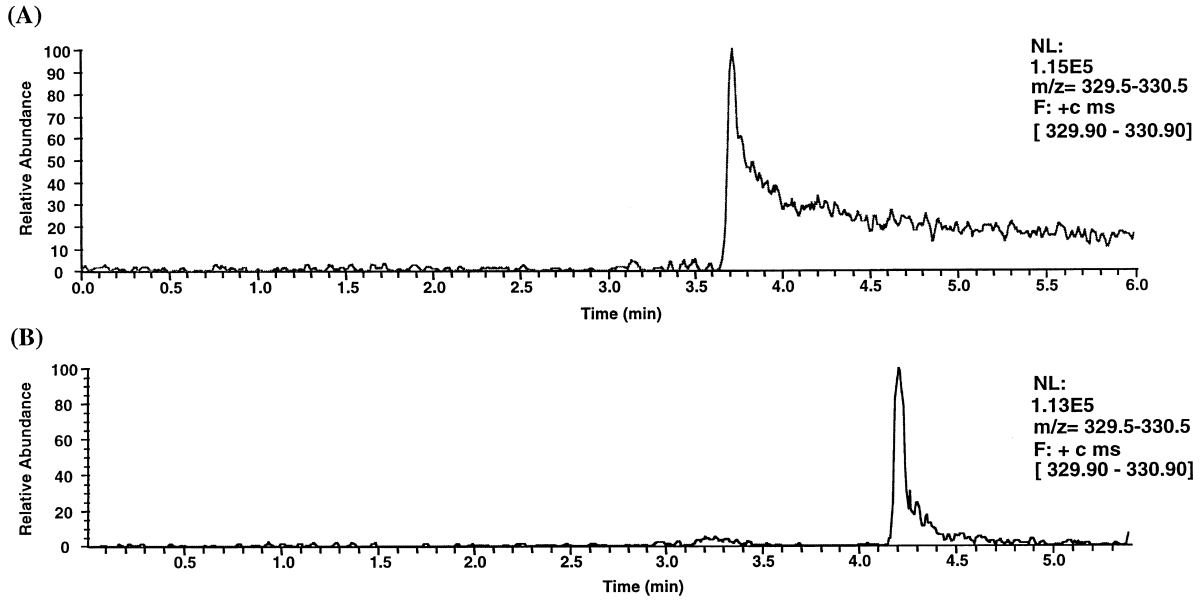


Fig. 3. HPLC–MS chromatograms comparing peak shape of CP-93 393 taken through the entire extraction and analysis procedure (A) without triethylamine in the extraction mobile phase and (B) with triethylamine in the extraction mobile phase.

temperature was set to 275°C, while the sheath and auxiliary gases were operated at 85 psi and 15 l/min, respectively. Primary ions of $m/z$ 330 and 334 were monitored for the drug and internal standard, respectively. Peak height ratios for the selected ions were determined using Finnigan LCQ software version 1.0.

### 2.5. Calibration and quantitation

Plasma samples were centrifuged at 2400×$g$ for 10 min before being fortified with CP-93 393. Calibration and calibration verification samples were prepared in plasma to cover the range 10–1000 ng/ml. Plasma was fortified to give calibration points of 10, 20, 50, 100, 500 and 1000 ng/ml and calibration curve verification points of 10, 20, 100 and 1000 ng/ml, respectively. Aliquots (125 μl) of the samples were then placed in autosampler vials where 100 ng of the internal standard was added and the mixture was vortexed for 5 s. Calibration was performed by plotting peak height ratios of drug to internal standard against drug concentration. A polynomial, cubic spline fit was employed for all cali-

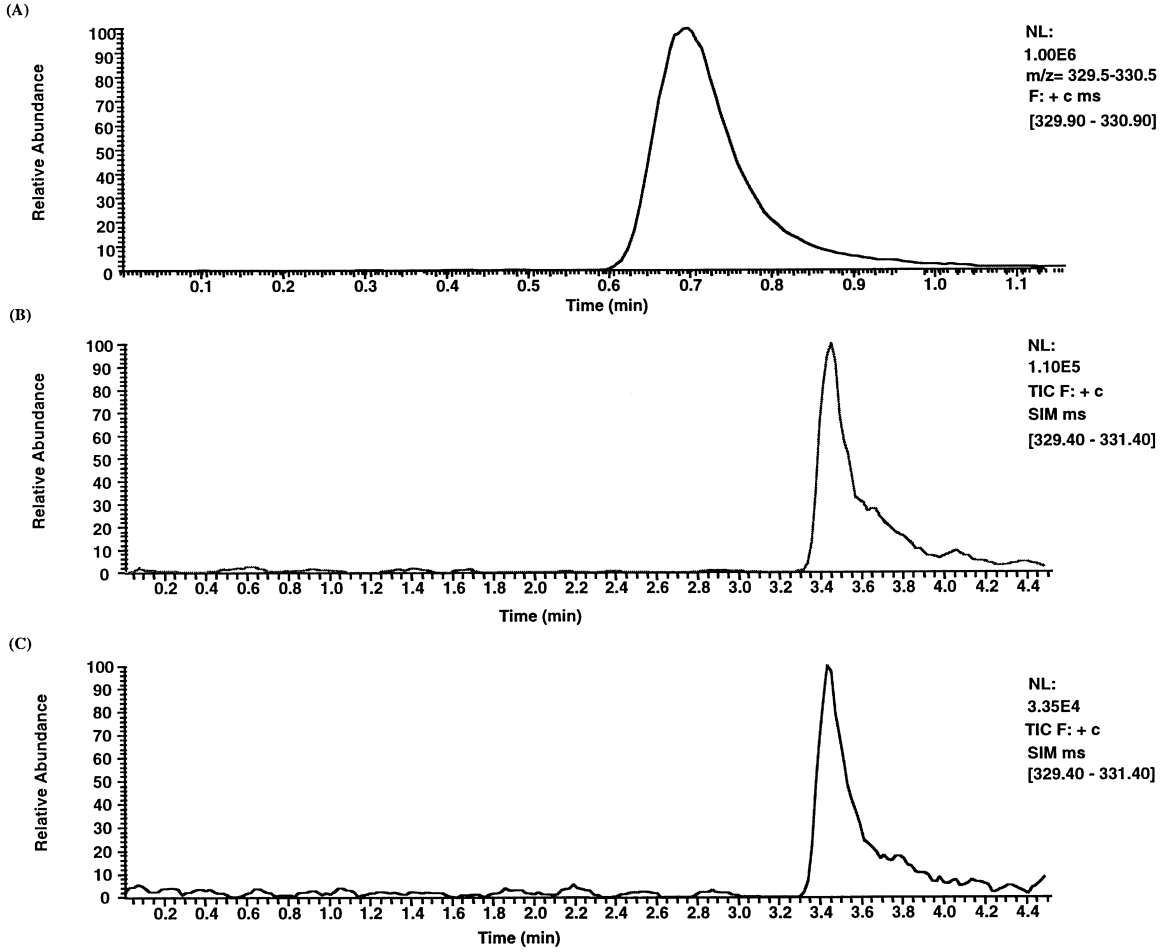


Fig. 4. HPLC–MS chromatogram from the analysis of CP-93 393. (A) 1.0 ng of CP-93 393 injected onto the analytical column only. (B) Direct injection of 100 μl plasma fortified with CP-93 393 at 10 ng/ml taken through the entire extraction procedure. (C) Injection of 1.0 μl of buffer fortified with CP-93 393 at 1.0 μg/ml taken through the entire extraction procedure.

bration curves. Concentrations for replicates of fortified drug samples were calculated from the calibration fit. Recovery experiments were performed using UV by comparing the peak height for a 100 ng injection of CP-93 393 from buffer to that of an injection of a 100 μl aliquot of plasma fortified with CP-93 393 at 1000 ng/ml.

## 3. Results and discussion

The selectivity of HPLC–API-MS detection allows the use of short analytical HPLC columns yielding typical analysis times of less than 3 min per sample. As a result, sample preparation is the most time consuming and thus rate-limiting step of quan-

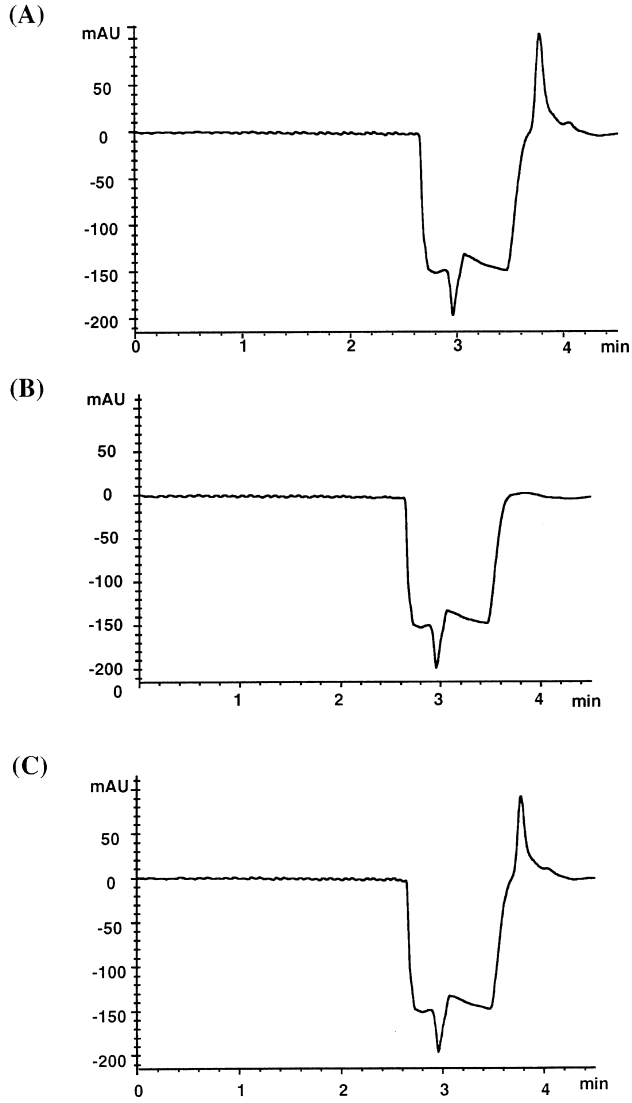


Fig. 5. UV ($\lambda$=238 nm) chromatograms used to calculate extraction recovery of CP-93 393 from human plasma. (A) Injection of 100 ng of CP-93 393 standard from buffer. (B) Extraction and analysis of 100 μl of blank human plasma. (C) Extraction and analysis of 100 μl of human plasma fortified with CP-93 393 at 1000 ng/ml.

Table 1
Inter-assay accuracy and precision for the determination of CP-93 393 by the direct injection of human plasma[a]

| Fortified concentration (ng/ml) | Calculated concentration (ng/ml) | *n* | % Accuracy | % R.S.D. |
|---|---|---|---|---|
| 10 | 10.0 | 17 | 100 | 2.7 |
| 20 | 18.3 | 18 | 91.5 | 2.6 |
| 100 | 102 | 17 | 102 | 1.2 |
| 1000 | 1070 | 18 | 107 | 7.9 |

[a] Combined data from three separate runs. Accuracy [(mean of calculated concentration−fortified concentration)/(fortified concentration)]× 100.

titative analyses of drugs in biological fluids. A method that combines a faster rate of sample extraction with on-line automation provides a significant advantage over conventional off-line, liquid–liquid and solid-phase extraction techniques. The above on-line extraction and analysis method performs the preparation and analysis step with no manual intervention necessary, thus allowing the analyst to concentrate on less tedious tasks.

Several challenges were encountered during the method development stages of this on-line extraction technique coupled with API-MS. For example, reported previously [14] addition of triethylamine to the extraction mobile phase was necessary to reduce the secondary interactions associated with the tertiary amine functionality of the analyte and the free silanol groups on the silica based column as shown in Fig. 3A and 3B. However, by adding triethylamine to the mobile phase in API-MS, suppression of basic analyte ionization often occurs which effects the lower limits of detection [15] as shown in Fig. 4. Despite the expected ionization suppression by triethylamine the lower limit of quantitation and the limit of detection for the assay were quite commendable at 190 pg and 58 pg, respectively, calculated according to Ref. [16].

Since detectability in API-MS is affected by various mobile phase compositions including pH [17] and buffer concentration [18], calculating extraction recovery is another challenge associated with interfacing on-line extraction techniques with HPLC–API-MS. The challenge is especially true when the extraction mobile phase varies from the analytical mobile phase as with the assay described in this manuscript. When direct injection of 1.0 ng CP-93 393 was performed on the analytical column only using the analytical mobile phase (Fig. 4A) and compared with the signal of 1.0 ng CP-93 393 injected from 100 μl plasma taken through the extraction process (Fig. 4B) the apparent recovery was ~10%. The apparent low recovery is most likely due to that the analytical mobile phase and the extraction mobile phase are different in pH and solvent composition which as stated above can significantly effect ESI-MS response. In addition,

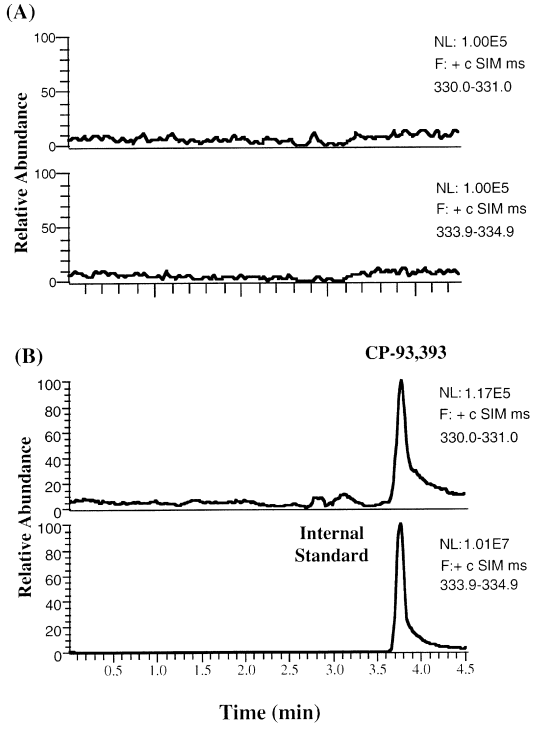


Fig. 6. HPLC–MS chromatograms from the direct injection of 100 μl of human plasma fortified with CP-93 393 at (A) 0 ng/ml and (B) 10 ng/ml.

when injecting 1.0 ng of CP-93 393 from buffer and comparing that with a 100 μl injection of 1.0 ng CP-93 393 from fortified plasma the apparent extraction recovery was >300% when using ESI-MS, as shown in Fig. 4B and 4C. The apparent high recovery may be due to endogenous plasma components extracted with CP-93 393 improving the ESI-MS response for CP-93 393 when injected from plasma. Obviously these plasma components have different masses than CP-93 393 since a blank 100 μl plasma sample produces no response using this method. However, further investigation is necessary to fully explain this phenomena. Nonetheless, since the UV trace of blank, on-line extracted plasma was free from endogenous peaks at the retention time of CP-93 393, a multiple wavelength detector was employed at the maximum absorbance of the drug (238 nm) to compare peak heights and calculate recovery. Fig. 5 shows the UV traces for a 100 μl blank human plasma sample, a 100 ng injection of CP-93 393 standard from buffer and a 100 μl injection of a human plasma sample fortified with CP-93 393 at 1000 ng/ml. Extraction recovery determined by UV was 81±6%. Recovery of the deuterated internal standard was assumed to be equivalent to the analyte and the recovery was assumed to be constant over the entire dynamic range of the assay.

Precision and accuracy of the procedure were determined by analyzing batches of replicate fortified samples on three separate occasions. Mean data is shown in Table 1. CP-93 393 and internal standard were co-eluted at retention times of about 3.8 min. Total analysis times between injections was less than 5 min per sample. A typical 100 μl injection of human plasma fortified with CP-93 393 and internal

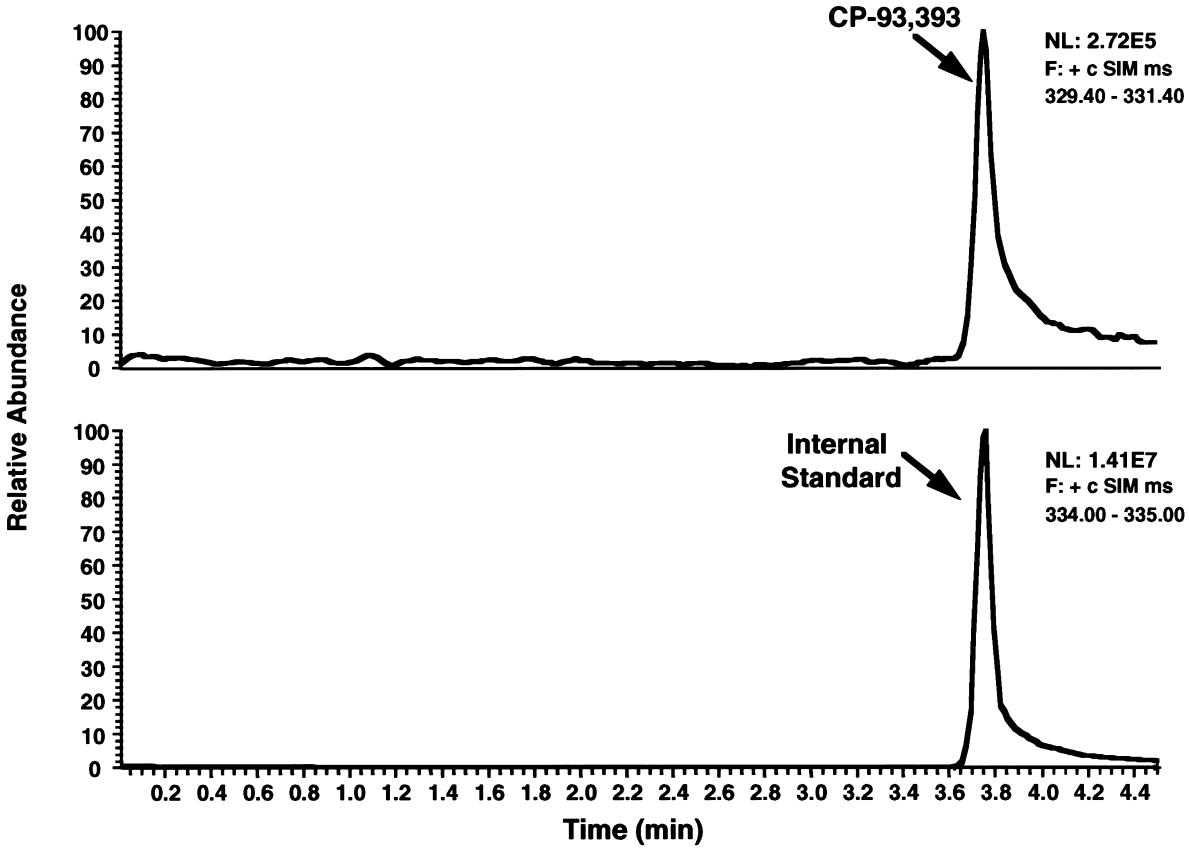


Fig. 7. HPLC–MS chromatogram from the direct injection of 100 μl of plasma of rat dosed with CP-93 393. The upper trace is the drug total ion current at 17 ng/ml and the lower trace is the internal standard total ion current.

standard is shown in Fig. 6. Fig. 6 also shows a 100 μl injection of blank human plasma. In Fig. 7 is presented a typical drug discovery example of this method for the analysis of 100 μl of plasma of rat dosed with CP-93 393. The clean chromatogram from the patient sample demonstrates the selectivity of this on-line extraction method when coupled with ESI-MS. Using the on-line dual column plasma extraction and analysis method described above, more than 250 samples have been extracted and quantitated in a 24 h period with total on-line automation. In addition, the ruggedness of the method was demonstrated by extracting more than 200 samples with a single BioTrap 500 column. Seemingly more samples could be analyzed per extraction and analytical column with no manual intervention if the increasing back pressure on both columns after each plasma injection was not a factor. However, this phenomena is unavoidable due to the adsorption of plasma components to sealings and other column components [19].

SIM mode was employed to demonstrate applicability of this technique to relatively specific detectors. Operation in MS–MS mode would, obviously, increase specificity. Performing direct plasma injections using a general on-line HPLC extraction method with API-MS detection for diverse analytical structure classes is currently under investigation. This method has the potential of being broadly applicable.

## Acknowledgements

The authors are thankful to Dr. Chandra Prakash for the synthesizing and supplying the deuterated internal standard. The authors would also like to thank Dr. Bob Ronfeld for his review of this manuscript.

## References

[1] J. Janiszewski, R. Schneider, K. Hoffmaster, M. Swyden, D. Wells, H. Fouda, Rapid Commun. Mass Spectrom. 11 (1997) 1033.
[2] B. Kaye, W. Herron, P. Macrae, S. Robinson, D. Stopher, R. Venn, W. Wild, Anal. Chem. 68 (1996) 1658.
[3] J. McElvain, J. Mooney, Proceedings of the 45th ASMS Conference on Mass Spectrometry and Allied Topics. Palm Springs, CA. June 1997.
[4] M. Zell, C. Husser, G. Hopfgartner, J. Mass Spectrom. 32 (1997) 23.
[5] J. Hermansson, A. Grahn, J. Chromatogr. A 660 (1994) 119.
[6] H. Lee, H. Shim, S. Yu, Chromatographia 42 (1996) 405.
[7] H. Lee, J. Lee, H.S. Lee, Chromatographia 37 (1993) 618.
[8] H. Lee, O. Zee, K. Kwon, Chromatographia 42 (1996) 39.
[9] H. Svennberg, P. Lagerstrom, J. Chromatogr. B. 689 (1997) 371.
[10] Z. Yu, D. Westerlund, K. Boos, J. Chromatogr. B. 689 (1997) 379.
[11] S. Vielhauer, A. Rudolphi, K. Boos, D. Seidel, J. Chromatogr. B. 666 (1995) 315.
[12] M. Avery, H. Fouda, Proceedings of the 44th ASMS Conference on Mass Spectrometry and Allied Topics, Portland, OR. May 1996.
[13] K. Ramsteiner, J. Chromatogr. 456 (1988) 3.
[14] L.R. Snyder, J.J. Kirkland, in: Introduction to Modern Liquid Chromatography. John Wiley and Sons. New York. 1979. Ch. 19. p. 801.
[15] M. Greig, R.H. Griffey, Rapid Commun. Mass Spectrom. 9 (1995) 97.
[16] ACS Committee on Environmental Improvement, ACS Subcommittee on Environmental Analytical Chemistry Anal. Chem. 52 (1980) 2242.
[17] W.H. Schaefer, F. Dixon Jr., J. Am. Soc. Mass Spectrom. 7 (1996) 1059.
[18] A.P. Bruins, Adv. Mass Spectrom. 10 (1986) 119.
[19] Z. Yu, D. Westerlund, K. Boos, J. Chromatogr. B. 704 (1997) 53.