ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2000) 325–333

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Development and validation of a semi-automated method for L-dopa and dopamine in rat plasma using electrospray LC/MS/MS

Wenlin Li, David T. Rossi, Scott T. Fountain *

Bioanalytical Core Group, Department of Pharmacokinetics, Dynamics, Metabolism, Pfiezer Global Research and Development, Ann Arbor, 2800 Plymouth Road, Ann Arbor, MI 48105, USA

Received 17 April 2000; received in revised form 7 July 2000; accepted 10 July 2000

Abstract

A semi-automated alumina-based extraction method for the determination of L-dopa and dopamine in plasma using liquid chromatography/mass spectrometry was validated. The method exploited the use of a Tomtec Quadra 96 liquid handing robot to expedite aluminum oxide extraction for sample clean up. Two 96-well sample plates can be processed in less than 2 h and extracts, collected in a 96-well plate format, can be directly injected onto the ESI/LC/MS/MS instrumentation. Chromatographic separation of the analytes was performed on a reverse-phase ODS column (TosoHaas ODS-80) with a mobile phase of acetonitrile/0.1% formic acid (5/95 v/v) at a flow rate of 0.22 ml/min. Analytes were detected by a triple–quadruple mass spectrometer equipped with an electrospray ionization source (ESI). Recoveries were evaluated for a number of pH modifiers and elution solvents. Under optimized conditions, the mean recoveries of L-dopa and dopamine were 56 and 67%, respectively. Intra-run and inter-run precision, calculated as percent relative standard deviation of replicate quality controls, was in the range of 1.45–10.8% for both L-dopa and dopamine. Intra-run and inter-run accuracy, calculated as percent error, was in the range −2.5 to 6.69% for both analytes. The limit of quantitaiton was 2.5 ng/ml for both L-dopa and dopamine when 100 μl of plasma was extracted. The method is simple, rapid, accurate and suitable for the quantification of L-dopa and dopamine in plasma or other biological fluid samples from clinical, preclinical, or pharmacological studies.

Keywords: L-Dopa; Dopamine; Parallel sample processing; ESI/LC/MS/MS

1. Introduction

The catachol amines L-dopa and dopamine are important endogenous neurotransmitters known to play a significant role in neurodegenerative disorders, including Parkinson's disease [1,2]. As such, there has been a continuing interest in ana-

* Corresponding author. Tel.: +1-734-6223087; fax: +1-734-6225115.
E-mail address: scott.fountain@pfizer.com (S.T. Fountain) (S.T. Fountain).

0731-7085/00/$ - see front matter
PII: S0731-7085(00)00422-2

lytical methodology for quantitative determinations of these molecules in a variety of biological materials, including plasma, blood, urine and cerebrospinal fluid. A number of well established procedures for their routine determination have reported the use of liquid chromatographic or capillary electrophoretic separations with various types of detection [3–8]. One of the most widely used approaches has been liquid chromatography with amperometric or coulometric electrochemical detection (LC/EC) [9]. Although this approach has received a great deal of attention because of the sensitivity that it offers for catachol amines, it is not without cost in terms of reliability, selectivity and ease of use.

Atmospheric pressure ionization mass spectrometry (API/MS) techniques such as electrospray (ESI) have recently made a large impact in bioanalytical chemistry by providing a highly selective, sensitive, and robust technique for detection and quantitation of a wide variety of compounds after an appropriate analytical separation [10,11]. The most dramatic impact of this approach has been the drastic reduction in method-development time for readily ionizable analytes. This is possible because of the dramatic improvement in selectivity offered by tandem mass spectrometry [12]. It is now possible to develop and apply a bioanalytical method after a development time of only a few days.

This paper reports an improved bioanalytical methodology for quantifying L-dopa and dopamine in rat plasma using a combination of solid-phase extraction (SPE) and liquid chromatography-electrospray tandem mass spectrometry (ESI/LC/MS/MS). This method offers a number of analytical advantages, including excellent selectivity, quantitation limits and improved sample throughput in the form of decreased run time. Although the sample preparation approach used here has been reported previously for the isolation and trace enrichment of these neuorotransmitters from biological samples [3], we have redesigned and optimized the choice of extraction conditions so that the procedure is readily compatible with ESI/LC/MS/MS. The procedure has been characterized by assessing the precision, accuracy, recovery and selectivity of the method in accordance with standards generally accepted [13] and has found utility in studies of the central nervous system where elevated levels of L-dopa and dopamine are anticipated after appropriate doses.

2. Experimental

2.1. Materials

L-dopa (> 99%), dopamine (> 98%), and stable isotope labeled [$^2H_3$] Dopa (> 98%) were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Chemical structures of test compounds are given in Fig. 1. Acid washed aluminum oxide was supplied by Bioanalytical Systems Inc. (Lafayette, IN, USA) and was used as received. Hydroxymethyl aminomethane (Tris) was obtained from Bio-Rad laboratories (Hercules, CA, USA). Sodium metabisulfite, acetonitrile, and EDTA were obtained from Mallinckrodt (Paris, KY, USA). All other reagents and solvents were analytical or HPLC grade, respectively, and were used as received. Rat plasma (heparinized) was provided by Pel-Freeze Biological (Rogers, AK, USA).

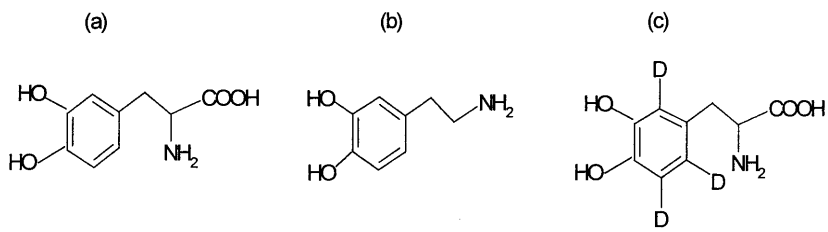

Fig. 1. Chemical structures of test analytes, (a), L-dopa; (b), dopamine; (c), deuterium labeled dopa used as internal standard.

Table 1
MRM scan parameters

	Ion mode	Parent (m/z)	Daughter (m/z)	Dwell (s)	Collision energy (eV)	Cone energy (V)
Dopamine	Positive	154.1	91.2	0.2	20	25
L-Dopa	Positive	198.1	152.1	0.2	15	20
[$^2H_3$] Dopa (IS)	Positive	201.1	154.9	0.1	20	25

2.2. Liquid chromatography and mass spectrometry

Assays were performed on a PE Series 200 LC pump and autosampler (Norwalk, CT, USA) with a Micromass Quattro II triple–quadrupole mass spectrometer (Beverly, MA, USA) detection system. A TosoHaas (Montgomeryville, PA, USA) ODS-80TS 2 mm × 15 cm × 5 μm reverse-phase column was used. Acetonitrile/0.1% formic acid (5/95 v/v) was used as mobile phase at a flow rate of 0.22 ml/min. The sample injection volume was 5 μl. The mass spectrometer was equipped with a Z-spray electrospray ionization source. Typical source conditions were as follows: capillary 3.5 kV, skimmer 1.5 V, RF lens 0.2 V, source temperature 130°C, desolvation temperature 280°C. MS1 parameters were LM resolution, 14; HM resolution, 14; ion energy, 1.0; and lens 6,5 V. MS2 parameters were LM resolution, 14; HM resolution, 14; ion energy, 1.0; lens 8,40 V; lens 9,0 V; and multiplier 650 V. Spectra were acquired in positive ionization multiple reaction monitoring (MRM) mode with interchannel delay of 0.03 s, mass span of 0 Da, and an acquisition time of 4.0 min (Table 1).

2.3. Standard preparation and sample quantification

Stock solutions of L-dopa, dopamine and internal standard were prepared by accurately weighing approximately 5 mg of compound, dissolving with 0.1% aqueous formic acid and diluting to volume in a 10-ml volumetric flask. Concentration was adjusted for percent purity and percent free base. A working standard containing 20 μg/ml of L-dopa and dopamine was prepared fresh for each batch run by adding appropriate aliquots of corresponding stock solutions in rat plasma. A combined standard curve of L-dopa and dopamine was prepared in rat plasma by serial dilution of the 20 μg/ml working stock to give standards at 2.5, 5, 10, 25, 50, 100, 250, 500, and 1000 ng/ml. Quality controls of 25, 100, and 500 ng/ml, used to evaluate assay accuracy and precision, were prepared from a separate weighing of compounds using a similar dilution scheme. The standard curve was constructed based on the ratio of peak area ($P_{\text{analyte}}/P_{\text{IS}}$) versus nominal standard concentrations using Micromass MassLynx quantitation software version 3.2. The linear regression model was used with a weighing factor of 1/concentration.

2.4. Extraction procedure

An alumina-based solid-phase sample preparation approach [14] was modified and characterized on a Tomtec Quadra 96 Model 320 workstation (Hamden, CT, USA). The Tomtec can handle 96 pipetting operations in parallel by downloading a control program from a PC to the solvent handling workstation. Rat plasma standards or samples (100 μl aliquot) were transferred manually into a 'sample cluster' containing 96 1.2-ml polypropylene tubes (Costar Corp., Cambridge, MA, USA). To each tube were added 25 μl (400 ng/ml) internal standard, 25 μl of aqueous 10% sodium metabisulfite, and 300 μl of 2-M tris with 5% EDTA w/v (pH 9). This could be accomplished manually using a repeater pipette or via automation using the Tomtec. Following the qualitative addition of approximately 30 mg aluminum oxide, sample tubes were capped with 8-cap strips (Costar Corp., Cambridge, MA, USA) and reciprocally shaken for 10 min. After the alumina settled to the bottom of tubes, as

much supernatant as possible was transferred by programming the stage height of the Tomtec. New pipette tips were changed and 300 μl of wash solvent (water) was added to each tube in the 'sample cluster' and the tubes vortexed for 20 s. This wash cycle was applied a total of four times. Desorption from the aluminum oxide was initiated with 100 μl of 2.5% formic acid added into the 'sample cluster' and vortexed for 10 min. After centrifugation, 50 μl of supernatant was transferred into a 1 ml 96 deep well plate via the Tomtec. The plate was sealed with a Scotch pad (3 M, St. Paul, MN, USA) from which injections were directly made for ESI/LC/MS/MS analysis.

3. Results and discussion

3.1. Method characterization

The effect of acetonitrile on the retention time of the analytes was investigated by varying its content from 0 to 20% in the mobile phase (Fig. 2). Five percent was preferred to achieve a balance between run time and sufficient separation from the chromatographic solvent front. Ion suppression was noticed when the capacity factor dropped below 1, but slight variations of acetonitrile from 5% in the mobile phase did not affect the sensitivity of the assay.

3.1.1. Elution solvent

Extraction conditions were optimized with respect to, (a), elution solvent; (b), pH modifiers; and (c), alumina washing cycle. Seven elution solvents were examined for desorption efficiency and compatibility with ESI/LC/MS/MS. An aliquot of 100 μl rat plasma containing 500 ng/ml of L-dopa and dopamine was adsorbed to aluminum oxide using a generic pH 8.7 tris buffer and eluted with 100 μl of each solvent. A 5 μl aliquot of each elution solvent was injected into the ESI/LC/MS/MS system. The results of this screening demonstrated that the highest recovered peak area was achieved by using 2.5% formic acid (Fig. 3). As a comparison, mixtures of L-dopa and

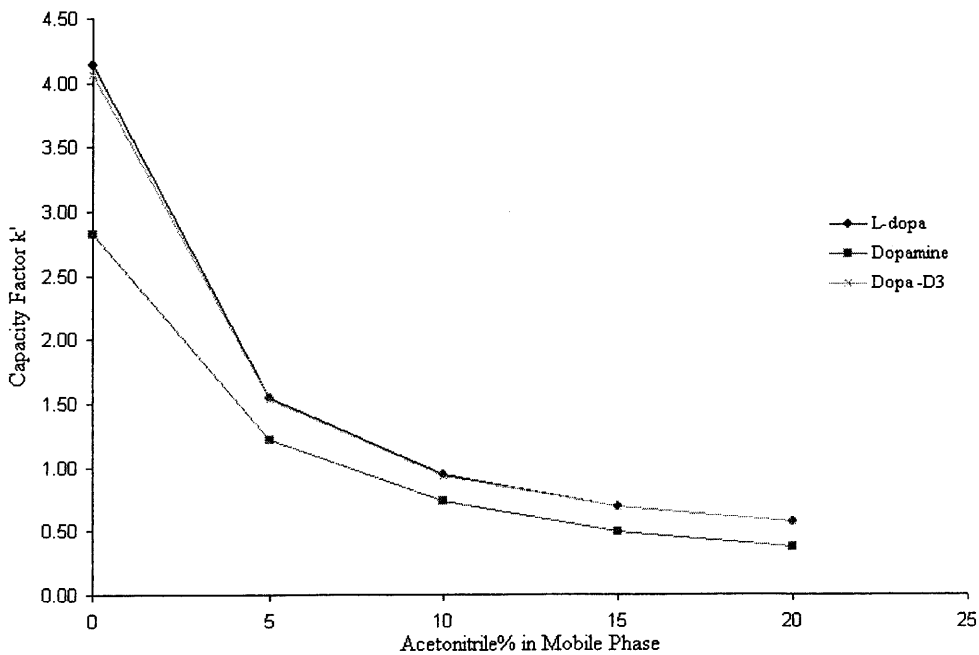

Fig. 2. Capacity factor 'k' of test analytes as a function of acetonitrile content in the mobile phase. Dots (●), squares (□) and crosses (×) indicate the capacity factors for L-dopa, dopamine and [$^2H_3$] dopa, respectively.$k' = t_r/t_v - 1$; where t_r = retention time of analyte, and t_v = time required for an unretained component (Na^+) to move through column.

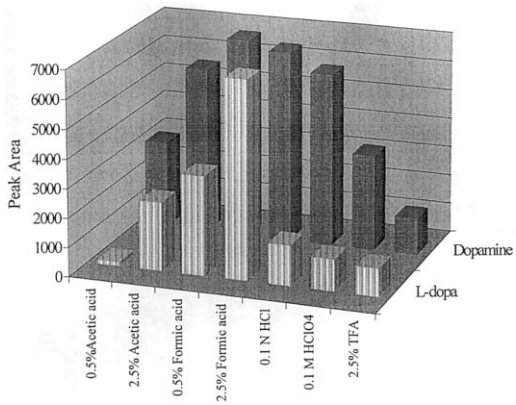

Fig. 3. Recovered peak area of rat plasma containing 500 ng/ml L-dopa and dopamine from seven different elution solvents, using aluminum oxide as solid-phase sorbent.

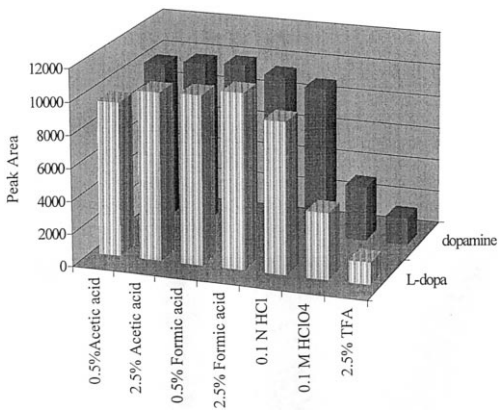

Fig. 4. Peak area of 500 ng/ml L-dopa and dopamine spiked in seven different elution solvents and directly injected into ESI/LC/MS/MS.

dopamine (500 ng/ml) were prepared in the same elution solvents, and injected into the ESI/LC/MS/MS directly. Detector response results are indicated in Fig. 4. Apparently, strong acids are unsuitable for ESI/LC/MS/MS due to the deterioration of sensitivity, although 0.1 M perchloric acid or 0.1 N hydrochloric acid was commonly used in aluminum oxide methods [4]. It has been reported that strong acids like trifluoroacetic acid induce signal suppression through an ion pairing mechanism between the acid anion and protonated analytes, as well as through the lower volatility, higher conductivity and surface tension of the solutions [15]. An aqueous solution containing 2.5% formic acid was chosen as the eluting solvent, based on its optimal combination of elution efficiency and compatibility with ESI/LC/MS/MS.

3.1.2. pH Modifiers

An additional experiment to determine the effect of pH modifiers on extraction efficiency indicated that the low pH of tris buffer (pH ~ 6) gave poor recovery (Fig. 5). The extraction efficiency increased significantly as the pH of tris buffer increased from ~ 6 to 8 and remained essentially constant as the pH of tris buffer increased from 8 to 11. This observation was in agreement with previous reports that the adsorption of catecholes to aluminum oxide is pH dependent, being optimal at pH 8.7 [14]. Higher tris buffer pH was not recommended because catecholamines are readily oxidized under basic conditions.

3.1.3. Washing cycle

Washing also had a pronounced effect on the sensitivity of the assay. Most of the protein, salts and unretained water-soluble substances were removed through washing, which was reflected by the decrease of solvent front peaks and noise. In traditional aluminum oxide assays, washing was the most time-consuming and labor-intensive step. Wash cycle time was reduced dramatically

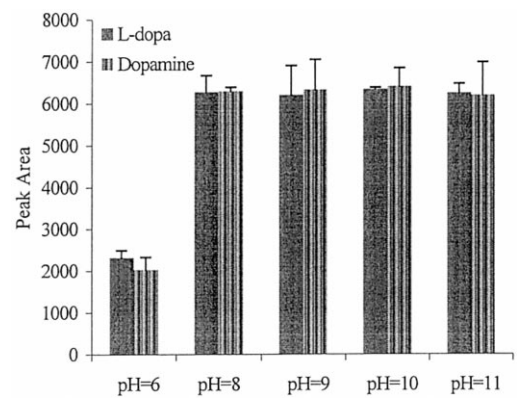

Fig. 5. Recovered peak area of rat plasma containing 500 ng/ml L-dopa and dopamine from five different pH modifiers, using aluminum oxide as solid-phase sorbent and 2.5% formic acid as elution solvent.

Table 2
Recovery

Concentration (ng/ml)	L-Dopa		Dopamine	
	Recovery (%)	S.D. (%)	Recovery (%)	S.D. (%)
10	57.3	7.98	67.0	4.10
25	54.4	7.04	67.8	4.38
100	58.5	3.39	67.6	2.63
500	55.0	3.27	66.7	2.74

through automation of liquid handling processes. With the system described here, it took less than 10 min to accomplish a washing process of 96 samples compared with approximately 2 h required in a manual process. A four-wash cycle was incorporated in the extraction procedure, which provided cleaner samples and enhanced sensitivity.

3.2. Recovery

The recovery of L-dopa and dopamine from rat plasma was assessed by comparing the concentrations of extracted standards in rat plasma with that of unextracted samples assayed in quadruplicate at each of four different concentration levels. The results are summarized in Table 2. The data indicated that the recoveries of L-dopa and dopamine from rat plasma were concentration independent in the evaluated concentration range.

Poor recovery was indicted as a significant problem encountered with the aluminum oxide extraction [14]. Overall recovery was reported previously as $42 \pm 2\%$ for dopamine and $25 \pm 2\%$ for dopa [3]. Using a larger volume of elution solvent (0.5 ml) increased the overall recovery to 80–90%, but the trade off was undesirable dilution. Higher recoveries were achieved using other forms of SPE for sample clean up. The recoveries were 64% for L-dopa by a Toyopak IC-SP S cartridge [16] and $>90\%$ for other catecholes by Bond-Elut C18 [17]. All the approaches involved predenaturation of plasma by perchloric acid or hydrochloric acid and pH adjustment of supernatant before loading on SPE cartridges, which limited the application of high sample throughput. The present sample clean up method gave mean recoveries of 56% for L-dopa and 67% for dopamine, which were comparable with the other approaches.

3.3. Selectivity

As described previously [5], alumina extraction was not selective enough to remove all the interfering substance in the plasma. Comprehensive chromatographic conditions had to be optimized to resolve the interference peaks, resulting in long chromatographic run times. This drawback has been overcome by using tandem mass spectrometry and multiple reaction monitoring (MRM). Chemical interference can be filtered out in most cases and enhanced assay selectivity achieved. Precursor $[M + H]^{+1}$ and product ions of L-dopa (198 $m/z \rightarrow 152$ m/z) and dopamine (154 $m/z \rightarrow 91$ m/z) were determined by full scan and product ion scanning using authentic standards in positive ion mode. It was noticed that the intensity and pattern of the product ions were very sensitive to the collision energy due to the existence of amine, carboxyl and hydroxyl groups. The collision energy was optimized to give the most predominant fragment ion with the precursor reduced by more than 95% of its original abundance. The mass spectrometric specificity of the analytes was confirmed by the absence of collision-cell 'crosstalk' peaks observed in the mass chromatograph when a standard containing individual compound was assayed. The overall selectivity of this assay was demonstrated by the absence of significant endogenous interference peaks in blank rat plasma (Fig. 5). Although enhanced chromatographic resolution could potentially improve selectivity by reducing competitive ionization from co-eluting endogenous material, the LC/MS/MS parameters described appear sufficient for this assay.

3.4. Linearity

Calibration curves ranging from 2.5–1000 ng/ml appeared to be linear. Deviations between back-calculated standard concentrations and nominal values were typically within $+/-8\%$ ($+17\%$ for limit of quantitation). The Pearson correlation (r^2) was generally >0.997 and y-intercept was statistically indistinguishable from zero.

3.5. Precision and accuracy

Precision and accuracy of the method, which are expressed as percent standard deviation (S.D.%) and percent relative error (R.E.%), respectively, were determined by assaying 25, 100, and 500 ng/ml quality control samples prepared in rat plasma. Triplicate samples at each of the concentration levels were determined over three independent batch runs. Acceptable precision and accuracy was achieved and the results are demonstrated in Table 3. The intra-run precision was within $\pm 11\%$ for both L-dopa and dopamine, and typically ranged between 2.0–7.5%, while the intra-day accuracy was from -2.4 to 6.7%. The inter-run precision was in the range 3.0–7.2% and inter-run accuracy in the range 0.9–7.8% for both of the analytes. These results are comparable or superior to other published procedures used to determine L-dopa and dopamine [3–9].

3.6. Limit of quantitation

The limit of quantitation (LOQ), defined as the lowest concentration of the standard curve with an acceptable precision and accuracy ($<20\%$ S.D. and R.E.), was found to be 2.5 ng/ml for

Table 3
Accuracy and precision data for L-dopa and dopamine in heparinized rat plasma

Nominal		L-Dopa			Dopamine		
Concentration (ng/ml)	Run number	Concentration found (ng/ml)	R.S.D. (%)[c]	R.E. (%)[d]	Concentration found (ng/ml)	R.S.D. (%)[c]	R.E. (%)[d]
Intra-run							
2.5[a]	1	2.69	4.04	7.47	2.80	9.43	11.9
	2	2.61	6.78	4.37	2.42	10.9	−3.19
	3	2.74	9.61	9.55	2.87	4.48	14.8
25[b]	1	24.8	10.8	−0.98	26.0	6.53	3.99
	2	25.3	8.37	1.37	25.6	3.70	2.53
	3	25.5	5.35	2.09	24.9	3.08	−0.50
100[b]	1	104	2.90	3.56	107	6.20	6.69
	2	104	4.68	3.76	105	4.48	4.69
	3	101	1.82	1.30	101	2.31	0.92
500[b]	1	525	2.42	5.01	524	1.45	4.87
	2	519	4.92	3.78	501	4.63	0.190
	3	509	4.92	1.89	488	4.63	−2.36
Inter-run							
2.5[a]		2.68	6.86	7.13	2.70	10.8	7.83
25[b]		25.2	7.40	0.83	25.5	4.54	2.00
100[b]		103	3.13	2.87	104	4.73	4.10
500[b]		518	3.13	3.56	504	5.79	0.898

[a] Limit of quantitation.
[b] Quality control.
[c] Relative standard deviation based on three replicates in each of three runs for the intra-run and nine replicates over three runs for the inter-run.
[d] Relative error based on three replicates in each of three runs for the intra-run and nine replicates over three runs for the inter-run.

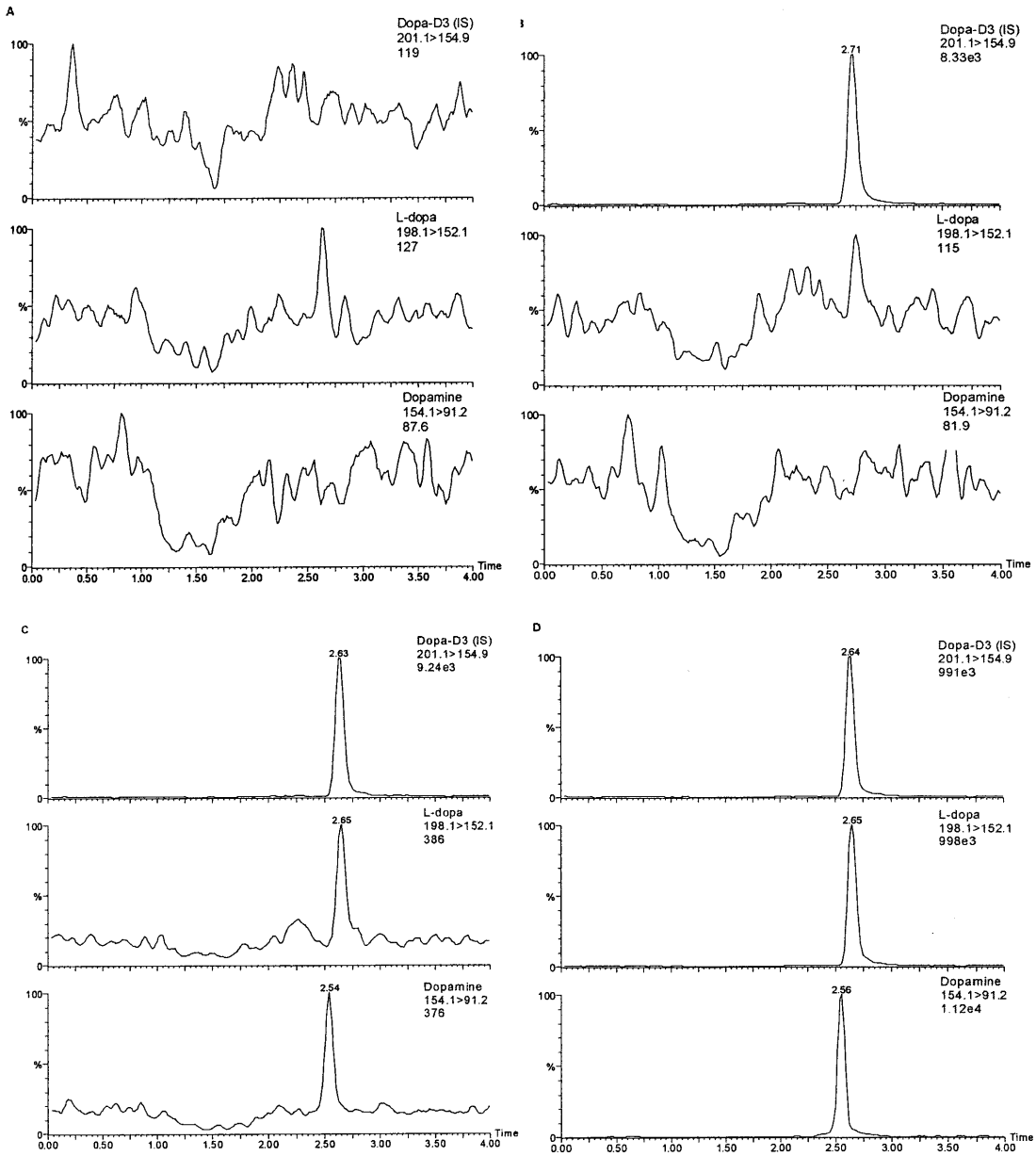

Fig. 6. Representative ESI/LC/MS/MS MRM chromatograms of internal standard [$^2H_3$] dopa (201.1 $m/z \rightarrow 154.9$ m/z), L-dopa (198.1 $m/z \rightarrow 152.1$ m/z), and dopamine (154.1 $m/z \rightarrow 91.2$ m/z), (A), MRM chromatogram of an extracted rat plasma blank; (B), MRM chromatogram of an extracted rat plasma blank with internal standard; (C), MRM chromatogram of an extracted 2.5 ng/ml rat plasma calibration standard of L-dopa and dopamine; (D), MRM chromatogram of an extracted 100 ng/ml rat plasma calibration standard of L-dopa and dopamine.

both L-dopa and dopamine. Peaks in the blank chromatogram were small and negligible, corresponding to less than 20% of the LOQ peak for L-dopa (Fig. 6). This is consistent with anticipated endogenous levels of L-dopa and dopamine in rat plasma (< 1 ng/ml) which are below the level of

detection of this assay [6]. The intra-run precision and accuracy of LOQ in rat plasma samples did not exceed ±15% for both L-dopa and dopamine. The inter-run precision and accuracy was within ±11% for both of the analytes (Table 3). These results are competitive with other assay methods for these compounds.

4. Conclusion

A liquid chromatographic mass spectrometric method has been developed and validated for the assay of L-dopa and dopamine in rat plasma samples using an automated aluminum oxide solid-phase extraction procedure for sample clean up prior to ESI/LC/MS/MS analysis. The method is simple, rapid, accurate and suitable for the quantification of these analytes in plasma or other biological fluid samples from clinical, preclinical, or pharmacological studies. The limit of quantitaiton was 2.5 ng/ml for both L-dopa and dopamine when 100-μl plasma sample was extracted. Additional sensitivity was achievable by increasing the sample aliquot. Although improvements in the limit of quantitation would be required to measure endogenous levels of L-dopa or dopamine in rat or human plasma [6,18], this method provides a fast, sensitive, and reliable assay for therapeutic studies.

One major advantage of automated aluminum oxide extraction using robotic liquid handling and 96-well sample clusters in conjunction with ESI/LC/MS/MS was improved sample preparation speed. Sample preparation time was reduced significantly compared with manual aluminum oxide extraction with roughly a four-fold improvement in sample throughput realized. Small volumes of elution solution (100 μl) allowed for direct injection without evaporation and reconstitution. The retention time was shortened significantly due to the minimized interference from sample matrix when utilizing tandem mass spectrometry. In addition to the considerable saving in labor and time, the cost associated with the clean up procedure in this assay was much less expensive than other SPE methods.

Acknowledgements

The authors would like to thank John Althaus of Pfiezer Global Research and Development, Ann Arbor and Jon Ebright for helpful discussion regarding the extraction procedure and chromatographic conditions.

References

[1] J.J. Hagan, D.N. Middlemiss, P.C. Sharpe, G.H. Poste, Trends Pharmacol. Sci. 18 (1997) 156–163.
[2] J.M. Tedroff, Rev. Neurosci. 8 (1998) 195–204.
[3] P.T. Kissinger, R.M. Riggin, R.L. Alcorn, L.D. Rau, Biochem. Med. 13 (1975) 299–306.
[4] K. Nyyssonen, M.T. Parviainen, Clin. Chem. 33 (1987) 1938–1939.
[5] C.R. Benedict, J. Chromatogr. 385 (1987) 369–375.
[6] M. Nakashima, M.F. Zhao, M.N. Nakashima, M. Sakurai, H. Sasaki, K. Matsuyama, M. Ichikawa, Bio. Pharm. Bull. 19 (1996) 988–994.
[7] P. Tuomainen, P.T. Mannisto, Eur. J. Clin. Chem. Clin. Biochem. 35 (1997) 229–235.
[8] S.M. Lunte, T.J. O'Shea, Electrophoresis 15 (1994) 79–86.
[9] A. Tolokan, I. Klebovich, K. Balogh-Nemes, G. Horvai, J. Chromatogr. B Biomed. Sci. Appl. 698 (1997) 201–207.
[10] G. Wu, M. Furlanut, Pharmacol. Res. 35 (1997) 553–556.
[11] W.M.A. Niessen, Liquid Chromatography/Mass Spectrometry, Marcel Dekker, New York, 1999.
[12] M.S. Lee, E.H. Kerns, Mass Spectrom. Rev. 18 (1999) 187–279.
[13] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Pharm. Res. 9 (1992) 588.
[14] Capsules: notes and applications from Bioanalytical Systems, Inc., Number 227, Determination of Plasma Catecholamines by LCEC, 1988, Bioanalytical Systems, Inc.
[15] A. Apffel, S. Fischer, G. Goldberg, P.C. Goodley, F.E. Kuhlmann, J. Chromatogr. A 712 (1995) 177–190.
[16] H. Nohta, E. Yamaguchi, Y. Ohkura, J. Chromatogr. 493 (1989) 15–26.
[17] K. Róna, K. Ary, B. Gachlyi, I. Klebovich, J. Chromatogr. A 730 (1996) 125–131.
[18] E. Eldrup, P. Mogensen, J. Jacobsen, H. Pakkenberg, N.J. Christensen, Acta Neurol. Scand. 92 (1995) 116–121.