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Journal of Chromatography B, 736 (1999) 167–173

JOURNAL OF CHROMATOGRAPHY B

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Determination of flunarizine in rat brain by liquid chromatography–electrospray mass spectrometry

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Received 5 May 1999; received in revised form 8 September 1999; accepted 5 October 1999

Abstract

A rapid liquid chromatography–electrospray mass spectrometry (LC–ES-MS) assay for the determination of flunarizine (FZ) in rat brain has been developed. A C_{18} column and an isocratic elution were employed for the separation. Using post-column split, 64% of the eluent was introduced into the ES-MS system for detection. The $[M+H]^+$ (m/z 406) and a fragmented ion (m/z 203) were detected using selected ion monitoring. The linear range of this assay was good, ranging from 0.05 to 5 μM ($r^2=0.99$). The intra- and inter-day precisions showed relative standard deviations ranging from 1.4% to 2.0% and 1.3% to 2.9%, respectively. The application of this newly developed method was demonstrated by examining the pharmacokinetics of FZ in rat brain.

Keywords: Flunarizine

1. Introduction

Flunarizine (FZ), [*trans*-1-cinnamyl-4-(4,4-difluorobenzenhydryl) piperazine dihydrochloride], is one of the piperazine derivatives with antihistamine properties and calcium channel blocking activity. FZ has been widely used in the treatment of cerebral and peripheral vascular insufficiency [1,2]. FZ's high lipophilic property enables it to easily penetrate the blood–brain barrier and reach the brain target. The half-life of FZ in male rats is 42 h and in female rats is 77 h in plasma [3]. The concentration of FZ in the brain is about two- to three-fold higher than its plasma concentration [4,5].

Previous studies have used gas chromatography (GC) and high-performance liquid chromatography (HPLC) methods for the determination of FZ [6–11]. Some of these reported HPLC methods required complex sample preparation to extract FZ from plasma; furthermore, some of them utilized complex mobile phase at elevated temperature. These methods are quantitative owing to the lack of information on molecular structure. Liquid chromatography–electrospray mass spectrometry (LC–ES-MS) has emerged as a sensitive and accurate analytical technique. ES generates ions under atmospheric pressure and at relatively low temperature which minimizes thermal decomposition of labile compounds. In addition, an aqueous sample can be analyzed with little or no

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0378-4347/99/$ – see front matter
PII: S0378-4347(99)00454-5

sample preparation. LC–ES-MS has proved to be very effective for the analysis of bio-polymers, environmental pollutants, drugs and so on [12–16].

This study evaluates the effectiveness of LC–ES-MS for the analysis of FZ in rat brain extract. The effects of modifier and flow-rate of mobile phase on the sensitivity of ES-MS will be presented. The linearity, detection limit and precision associated with this newly developed method will be discussed together with its application.

2. Experimental

2.1. Chemicals

HPLC grade acetonitrile (Malinckrodt Baker, KY, USA) and HPLC grade water (Labscan, Dublin, Ireland) were used throughout the experiment. Ammonia solution (28%) was purchased from Nacalai Tesque (Kyoto, Japan). Flunarizine dihydrochloride was from Sigma (MO, USA).

2.2. HPLC system

A HP1100 LC system which consisted of a quaternary pump, an on-line degaser, an autosampler and a UV–visible detector (Hewlett-Packard, CA, USA) was used. A LiChroCART RP-18e column (Purospher, 125×3 mm, 5 μm, Merck, Darmstadt, Germany) with an LiChroCART 4-4 on-line guard column was used for separation. A mixture of 0.14% of ammonia water (pH 10.4)–acetonitrile (80:20, v/v) was used a mobile phase for chromatography analysis. HPLC flow-rate was 0.5 ml/min and the injection volume was 20 μl.

2.3. Mass spectrometry

An HP-5988B mass spectrometer with a HP-59987A electrospray interface (Hewlett-Packard) was used. An HP Chemstation (G1034C, version C.03.00) was utilized for system control, data acquisition and data analysis. Heated N_2 gas (350°C, 12.5 l/min) was used to evaporate solvent from the electrospray chamber and compressed N_2 gas (80 p.s.i.) was used for neublization (1 p.s.i.=6894.76 Pa). The cylinder electrode in the electrospray chamber was set at −6000 V. The end plate and capillary entrance voltage were set at −3500 V and −4000 V, respectively. The voltages of skimmer 1, lens 1, skimmer 2, lens 2 and lens 3 were set at 33.5 V, −0.6 V, 10.6 V, 14.0 V and −68 V, respectively. The mass spectrometer was operated at positive ion mode and mass spectra collected in scan mode were obtained by scanning from 10 to 800 in 0.5 s. Nine scans were averaged with a step size of 0.1 over the range.

2.4. Standard solution

For the examination of chromatography elution conditions, linearity and detection limit associated with this method, 0.01 m*M* of flunarizine in acetonitrile–water (80:20, v/v) solution was prepared and stored at 4°C in the dark. This stock solution and the working solutions were prepared weekly. For inter- and intra-day studies, the stock and working solution were prepared daily. For the mice extracted sample analysis, standard solutions were prepared from the same solution that was used to inject the rats.

2.5. Pharmacokinetic study

Male Sprague–Dawley rats (250–350 g weight; Animal Center, National Science Council, Taiwan) were used. On the experimental day, the rats received 20 mg/kg i.p. of FZ. The injection solution was prepared by first weighing 30 mg FZ and then dissolving in three drops Tween 80 solution. Finally, we gradually added saline to the solution to 1 ml. At 20, 40, 60, 80, 100, 120, 140, 160 and 180 min post FZ administration, the rats were decapitated. The brain was quickly removed and weighed. The brain tissues were then homogenized with 5 ml HPLC mobile phase for 1 min (Ultra-turrax T25, Janke and Kunkel, Staufeni, Germany). Thereafter, samples were centrifuged at 4°C for 10 min (3500 *g*) and the supernatants were collected. The samples were stored in a 4°C refrigerator prior to analysis. The sample was filtered through a microporous syringe filter (0.2 μm, Lida, WI, USA) before HPLC analysis.

3. Results and discussion

Collision-induced dissociation (CID) has been used to promote molecular fragmentation. Two major ions (m/z M+1 and 203) were determined in positive mode detection. The structures of FZ and the proposed fragmented ion are shown in Fig. 1. Furthermore, the abundance of these two ions were largely dependent upon CID voltage. Fig. 2 is an illustration of the fragmentation of FZ at different CID voltages. The ion intensities of both ions are approximately equal at 100 V. When the voltage was increased from 100 to 125 V, the abundance of fragmented ion (m/z 203) also increased while the abundance of $[M+1]^+$ remained the same. However, the intensity of $[M+1]^+$ decreased significantly as the abundance of fragmented ion (m/z 203) rose to a higher level. In this study, CID voltage was set at 125 V and the selective ion monitoring (m/z M+1 and 203) was used for quantitative analysis.

A liquid chromatography method utilizing a C_{18} column and isocratic elution for the analysis of FZ in rat brain has been developed by our group [11]. The same separation conditions were adopted for this application. The LC–ES-MS chromatograms of FZ in acetonitrile–water mixture and rat brain extract are shown in Fig. 3. FZ eluted at approximately 6.3 min and the separation completed in less than 8 min. Although some substances were detected in the extract of rat brain, none of them interfered with FZ. The addition of ammonia water in mobile phase will reduce the sensitivity of ES-MS due to the diminishing of protonation of FZ in the solution [17].

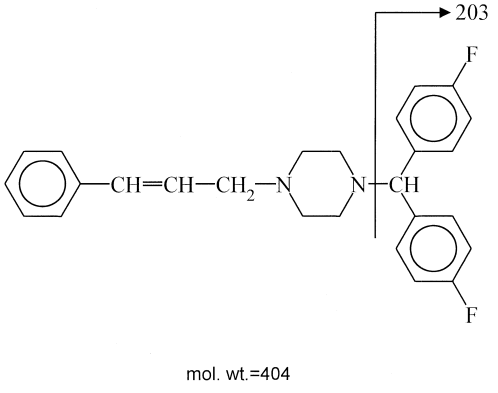

Fig. 1. Molecular structure of FZ.

However, ammonia is needed for the separation of FZ in biological fluid sample. The addition of acetic acid would increase the abundance of $[M+1]^+$ ion by 12%, approximately; however, the presence of acetic acid in mobile phase would reduce the resolution of LC separation.

The flow-rate of sample introduction is also an important parameter affecting the sensitivity of LC–ES-MS detection [18]. Although the dimension of separation column will determine the flow-rate of sample introduction of LC–ES-MS, post-column splitting could be used to reduce the amount of elute being introduced into the ES-MS system. We placed a splitting-T between the LC column and the ES/MS system to manipulate sample flow-rate and examined the effect of flow-rate on the LC–ES-MS detection. The sensitivity of LC–ES-MS detection remained the same when the splitting ratio was set at 64% (0.32 ml/min of effluent was introduced into the ES-MS system) comparing to no splitting. Nevertheless, the sensitivity of LC–ES-MS detection decreased substantially as the splitting ratio decreased further. This might attribute to high organic content of mobile phase and off-axis design of electrospray apparatus [19]. In this study we chose to utilize a 3.0 mm I.D. LC column whose flow-rate was set at 0.5 ml/min and the splitting ratio was adjusted to 64%. The implementation of splitting-T to regulate effluent introduced to the ES-MS system does not affect the sensitivity of this method; however, it would reduce possible contamination of the ES-MS system from rat brain extract sample.

The linearity of this newly developed method was evaluated by analyzing a series of FZ standards. The linearity was good from 0.05 to 5.0 μM ($r^2=0.99$). The detection limit, based on a signal-to-noise ratio of 3, was about 0.8 nM. Comparing with previously published reports, the detection limit of this newly developed method is approximately 250 times better.

Inter- and intra-day precision of this method was evaluated by replicated analysis of FZ spiked samples. Calibration standards were prepared and analyzed each day. A total of three series of samples were analyzed over a week-long period and each sample was measured in triplicate. The results of precision study are summarized in Table 1. The intra- and inter-day precisions showed relative standard deviations (RSDs) ranging from 1.9% to 2.1%

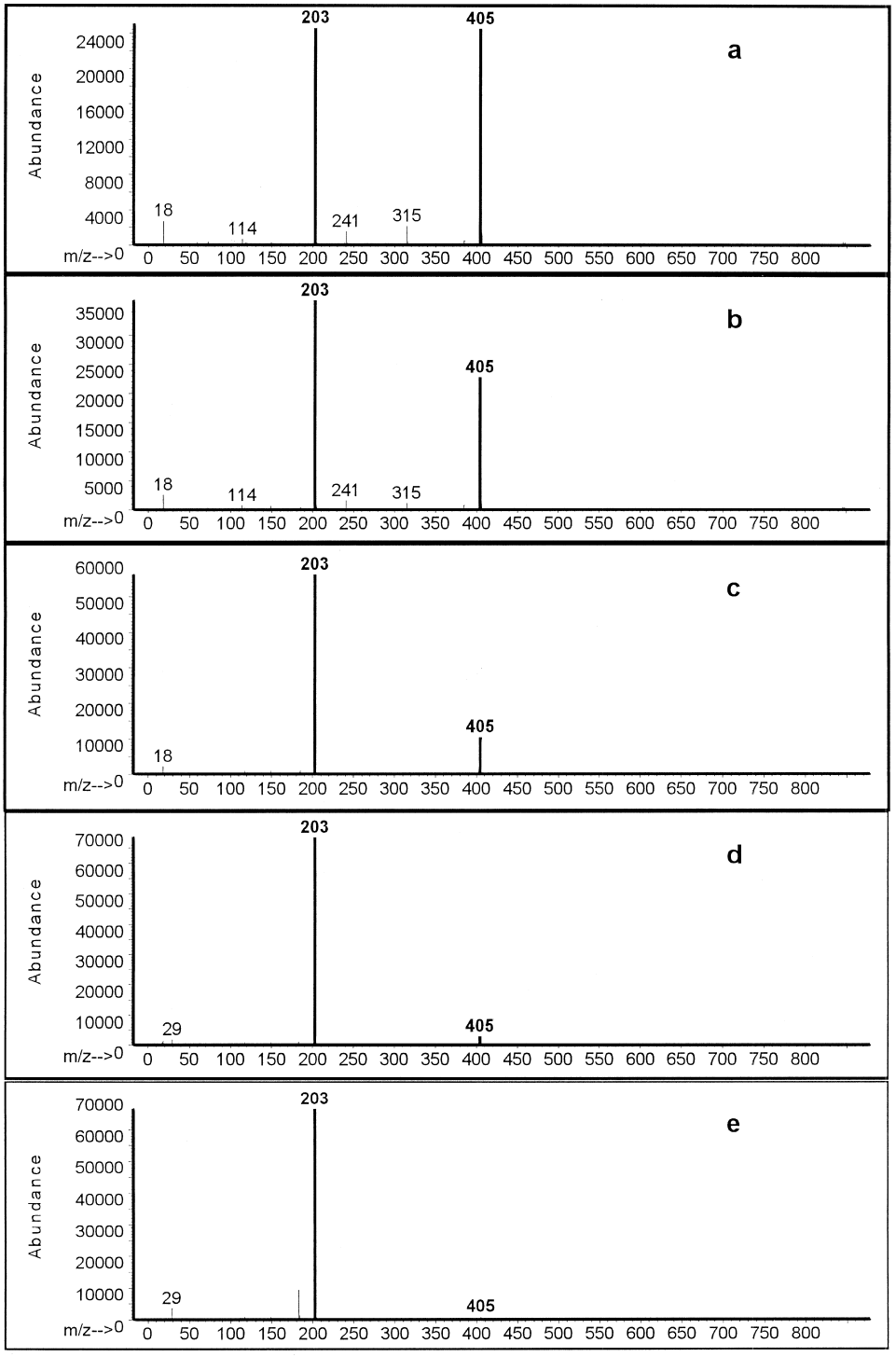

Fig. 2. Positive ESI mass spectra of FZ at different CID voltages: (a) 100 V, (b) 125 V, (c) 150 V, (d) 175 V, (e) 200 V.

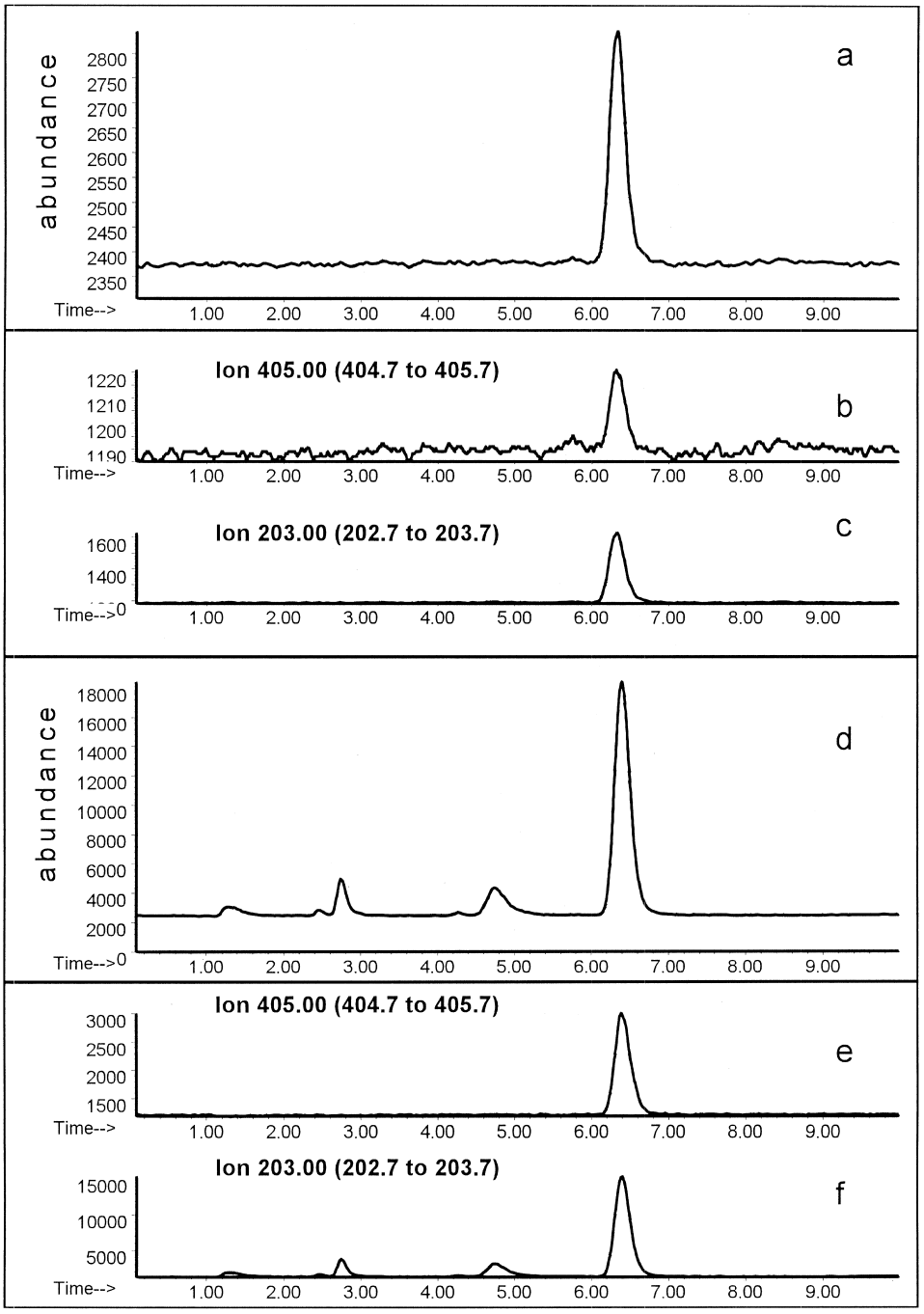

Fig. 3. Total ion chromatogram (TIC) and extracted ion chromatogram (EIC) of FZ standard (1 μ*M*) and rat brain extract. (a) TIC of FZ in acetonitrile–water (80:20, v/v), (b) EIC (*m*/*z* 405) of a, (c) EIC (*m*/*z* 203) of a, (d) TIC of rat brain extract after FZ administration, (e) EIC (*m*/*z* 405) of d, (f) EIC (*m*/*z* 203) of d.

Table 1
Inter- and intra-day precision of flunarizine in extraction solvent

Added concentration (μM)		Intra-day		Inter-day	
		Measured concentration (μM)	Accuracy (%)	Measured concentration (μM)	Accuracy (%)
0.50	Mean[a]	0.589	118	0.597	119
	SD[a]	0.011		0.010	
	RSD	1.9		1.7	
1.00	Mean[a]	1.150	115	1.150	115
	SD[a]	0.024		0.017	
	RSD	2.1		1.5	
3.00	Mean[a]	3.070	102	3.060	102
	SD[a]	0.064		0.039	
	RSD	2.1		1.3	
5.00	Mean[a]	4.850	97	4.840	97
	SD[a]	0.095		0.023	
	RSD	2.0		1.7	

[a] Mean and SD represent three different samples for each concentration.

and 1.3% to 1.7%, respectively. The accuracy (97% to 119%) of this newly developed method was determined.

A microporous filter was utilized to pretreat rat brain extract sample prior to LC analysis. This procedure is to remove the residue particle in extract so as to prolong the lifetime of the LC column. We have found some residual compounds on the filter that would interfere with FZ analysis; however, they could be effectively removed by acetonitrile–water (50:50, v/v). We examined the recovery of this filtration procedure to ensure the accuracy of this assay and the results. Table 2 is the summary of this study. Good recoveries ranging from 95.7 to 101.5% with an average of 98.0% were determined.

Table 2
Recovery of filtration

Added concentration (μM)	Measured concentration[a] (μM)	Recovery[a] (%)
5.00	4.64	92.8
5.00	4.68	93.6
5.00	5.03	100.6
1.00	0.99	99.0
1.00	0.99	99.0
1.00	1.05	105.0
0.50	0.48	96.0
0.50	0.48	96.0
0.50	0.50	100.0

[a] Average of three measurements.

The stability of FZ in the sample extract was examined. Samples were stored in a refrigerator (4°C) and then determined after 24 and 48 h. No significant decrease in response was observed. However, in order to minimize the deterioration of sample, all samples were analyzed immediately after extraction and pretreatment.

The application of this newly developed LC–ES-MS method was demonstrated by evaluating FZ concentration in rat brain. The time course of FZ concentration ($n=5$) in mouse brain which received 20 mg/kg i.p. is shown in Fig. 4. FZ concentration quickly increased in the brain and reached a maximum concentration of 3.61 ± 0.50 μg/g of tissue from 60 to 80 min after drug administration.

A non-linear curve-fitting computer program, Minsq, written by MicroMath Scientific Software (Salt Lake City, UT, USA) was used to fit an equation consisting of the difference of two first-order kinetic processes for the appearance and disappearance of FZ in the brain to experimental FZ data:

$$\mathrm{FZ} = A\left[\exp\left(-k_1 t\right) - \exp\left(-k_2 t\right)\right]$$

where

$$A = \left[k_1/(k_1 + k_2)\right]C_0$$

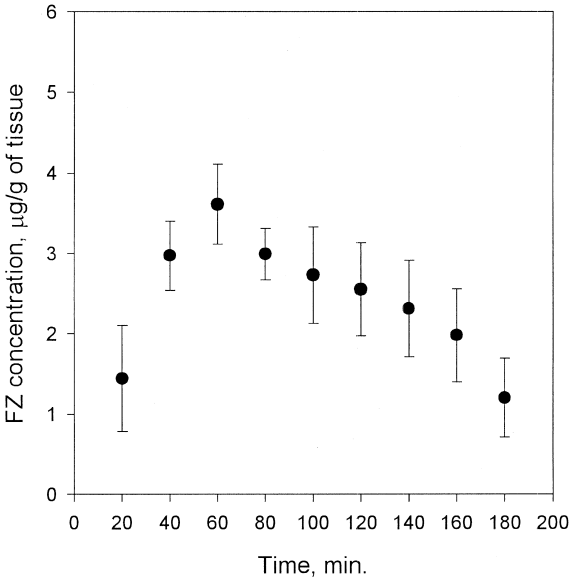

Fig. 4. Time course of FZ in rat brain ($n=5$) following a 20 mg/kg FZ i.p. injection.

Three parameters fit into the data: A, k_1 and k_2. A is a concentration and absorption efficiency factor, k_1 is the first-order rate constant for the appearance of FZ, and k_2 is the first-order rate constant for the disappearance of FZ. Both rate constants have the units of min^{-1}. After this non-linear fit, k_1 was 0.0135 ± 0.0001 and k_2 was 0.0173 ± 0.0001. The value obtained for the concentration factor, A, was 35.08 ± 0.18 μg/g of brain tissue.

In summary, the results of the work described here demonstrate the potential of LC–ES-MS for quantitative determination of FZ in the extract of rat brain. This assay proved to be sensitive and precise. Currently, we are applying this newly developed method to evaluate free-form FZ in rat brain.

Acknowledgements

This work was supported by National Science Council of Taiwan. We thank Dr. Wynn Pan of National Yang-Ming University for helpful comments.

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