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Microdialysis combined with liquid chromatography–tandem mass spectrometry for the determination of 6-aminobutylphthalide and its main metabolite in the brains of awake freely-moving rats

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Abstract

6-Aminobutylphthalide (ABP) is a new drug candidate which is currently being developed for the treatment of cerebral ischemia. The pharmacokinetics and metabolism of ABP were studied using in situ microdialysis sampling in the brains of awake freely-moving rats. Two LC-MS/MS methods were used for the quantitative and qualitative analysis of microdialysate. For comparison and confirmation, brain tissue samples were also analyzed by LC-MS/MS and GC/MS. The results described provide more authentic information in pharmacokinetics and metabolism at the site of action by using the coupling of microdialysis to LC-MS/MS technique than the traditional sampling methods.

Keyword: 6-Aminobutylphthalide

1. Introduction

The technique of microdialysis is based on the kinetic dialysis principle that compounds will diffuse down their concentration gradient, over a semipermeable membrane, into or out of the perfusion medium that flows through the microdialysis probe [1]. This method is relatively useful for monitoring an analyte dynamically because endogenous and exogenous substances can be sampled directly and continuously from the target organ [2–4].

In contrast to other sampling methods, microdialysis techniques provide several advantages for in vivo sampling of drugs [5]. No fluid is actually removed, so continuous sampling can be performed with minimal perturbation to the physiological system. Samples are relatively clean because they are protein-free, which makes it possible to directly inject into an analytical system. In addition, the membrane acts as an effective enzyme inhibitor because it excludes enzymes that could cause degradation of the analyte. Lastly, since proteins can not pass through the membrane, only the free, unbound portion of drug is measured, which is important to pharmacodynamics research.

The main disadvantage of microdialysis sampling is that the small volume of the dialysate, normally combined with low concentration of analytes, becomes a challenging factor in chemical analysis [6]. Thus, the analytical detection method must be sensitive enough to determine the substance of interest at low concentrations. The liquid chromatography/tandem mass spectrometry (LC-MS/MS) method is an attractive choice because of the high selectivity and the ability to conclusively identify the analytes. LC-MS/MS is also capable of handling the analysis of the small sample volumes with low concentrations of analytes frequently present in microdialysis samples [7]. The main drawback of the method is that the detection sensitivity was affected by the high salt level of microdialysis samples and severe signal suppression was evident [8].

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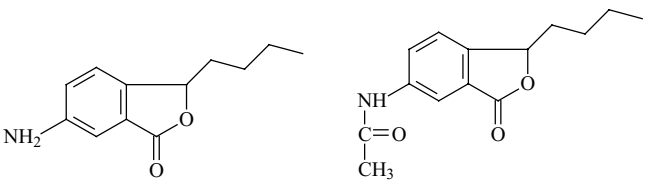

ABP **M1**

Fig. 1. The structures of ABP and the *N*-acetylated metabolite (M1).

Additionally, the technique is not equally suitable for all analytes, because most microdialysis membranes are permeable to hydrophilic compounds of low molecular weight. Analytes that are extremely hydrophobic, highly protein bound or with molecular weights beyond the membrane cut-off limit are not suitable for the microdialysis technique.

Because of these limitations, although microdialysis has gained wide recognition as an important tool for assessing the pharmacokinetics and metabolism of drugs in a target organ [9–11], a number of technical issues have to be overcome in the development of this novel application of the microdialysis sampling.

ABP is a new drug candidate under preclinical development which is derived from a lead compound isolated from Apium graveolens L. seeds. The compound has promising protective action against cerebral ischemia. During the development of a new drug, it is significant to obtain early information on the metabolism of this compound as fast as possible. In the present study, in vivo detection of ABP and its metabolites was performed using in situ microdialysis sampling in the brain of awake freely-moving rats. Two LC-MS/MS methods were used for the quantitative and qualitative analysis of the microdialysate. The time course of the appearance and disappearance of ABP after oral administration was determined, and the structure of the main metabolite was identified. For comparative purpose, tissue samples were analyzed using LC-MS/MS and gas chromatography/mass spectrometry (GC/MS), the structure of the metabolite was validated. The results showed that ABP can cross the blood brain barrier (BBB) and be detected in rat brain by utilizing in vivo microdialysis and LC-MS/MS method. Fig. 1 shows the structures of ABP and *N*-acetylated metabolite (M1).

2. Experimental

2.1. Chemicals

ABP and M1 were provided by Professor Guo ZongRu (Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China). The dialysis perfusate used was Ringer solution and consisted of 2.5 mM KCl, 1.18 mM $MgCl_2$, 1.26 mM $CaCl_2$ and 125 mM NaCl [12]. All reagents used in the preparation of buffer solutions were of analytical reagent grade or better.

Acetonitrile and methanol were HPLC grade, all other chemicals were of analytical reagent grade or better and were obtained from commercial suppliers.

2.2. Animals

Male Wistar rats of 6–7 weeks of age (270–330 g) were obtained from Institute of Laboratory Animals Sciences, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China). Rats were maintained in a clean room (Animal Center for Pharmaceutical Research, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) at a temperature between 20 and 23 °C, with a 12 h light–dark cycle and a relative humidity of 50%. Rats were housed in metabolic cages under the supply of filtered pathogen-free air with access to food and water ad libitum. The experimental procedures were performed in accordance with the principles of animal care outlined by the Chinese Academy of Medical Sciences and Peking Union Medical College.

2.3. Surgical and microdialysis procedures

Rats were anesthetized with an intraperitoneal (i.p.) injection of chloral hydrate (10%, 3.5 ml kg^{-1}) and mounted in a stereotaxic frame (JiangWan-I, Shanghai, China) with a homemade heating pad maintaining the body temperature at 37 °C. An incision was made in the scalp, the skull exposed and a small hole drilled. Anterior–posterior (AP) +3.8 mm, medial–lateral (ML) +3.25 mm, measurements were made with reference to bregma and the sagittal suture, respectively, and depth (dorsal–ventral, DV) −5.0 mm was measured from the surface of the cortex. The coordinates were chosen according to the atlas of Pellegino et al. [13]. A homemade guide cannula was implanted into the striatum and secured permanently in position with three skull screws and dental cement. After surgery, penicillin solution was applied topically to the wound and the rats were injected i.p. with saline (5 ml kg^{-1}).

After rats have recovered from surgery, a concentric design microdialysis probe (o.d. = 0.2 mm, molecular cut-off 9 kDa, 4 mm dialyzing membrane, BAS, USA) was slowly implanted via the guide cannula into the striatum area of the rat. The microdialysis probe was perfused with Ringer solution via FEP tubing (BAS, Indiana, USA) connected to a CMA/100 pump, at a flow rate of 1.0 μl min^{-1}. Following a 30 min equilibrium period, the rats were given a p.o. administration of 80 mg kg^{-1} ABP and the dialysates were collected every 15 min for 2.0 h, subsequently the time intervals were altered to 30 min for another 3.5 h.

When microdialysis sampling is conducted in an awake animal, its movement must be constrained with a Rodent Residence for in vivo Bioanalytical Sampling (BAS, USA) which prevents tangling of the fluid lines.

In two rats given a p.o. administration of 80 mg kg^{-1} ABP, a second p.o. administration of 80 mg kg^{-1} was given after 6 h and samples were collected every 60 min for another 4 h to study the metabolism of ABP.

2.4. Microdialysis probe calibration

Microdialysis probe recovery was calibrated by triplicate measurements of in vitro recovery and delivery and in vivo delivery [14,15]. In vitro recovery was determined by placing the microdialysis membrane in a 45.8 ng ml^{-1} ABP solution in Ringer solution and perfusing with Ringer solution at 1 μl min^{-1}. Samples of the dialysate were collected at 30 min intervals and ABP concentrations were analyzed by LC-MS/MS to give the relative in vitro recovery. In vitro delivery was determined by perfusing 45.8 ng ml^{-1} ABP solution in Ringer solution into a microdialysis probe whose dialysis membrane is placed in a vial containing Ringer solution at 37 °C. Relative in vitro delivery was calculated using the difference in ABP concentration between perfusate and microdialysate. In a similar manner to the determination of the in vitro delivery, the in vivo delivery was calculated by perfusing 45.8 ng ml^{-1} ABP solution in Ringer solution into a microdialysis probe whose dialysis membrane was inserted into a rat brain.

The probe relative recovery, R, and delivery, D, are defined by the following equations:

$$R = \left(\frac{\mathrm{Cd}}{\mathrm{Cs}}\right) \times 100 \quad (1)$$

$$D = \frac{(\mathrm{Cp} - \mathrm{Cd}) \times 100}{\mathrm{Cp}} \quad (2)$$

where Cd is the concentration in the dialysate, Cs is the concentration of the sample and Cp is the concentration in the perfusate. The relative in vitro recovery, in vitro delivery and in vivo delivery were 25.9, 27.3 and 16.2%, respectively. The in vivo recovery, therefore, was calculated according to Eq. (3) to be 15.4%.

$$\left(\frac{R}{D}\right) \text{ in vivo} = \left(\frac{R}{D}\right) \text{ in vitro} \quad (3)$$

The concentration of ABP in rat cerebrospinal fluid (CSF, ng ml^{-1}) was calculated using the in vivo recovery by multiplying the concentration determined in the microdialysate by a factor of 100/15.4.

2.5. Determination of ABP in the microdialysis samples by LC-MS/MS

The LC-MS/MS system consisted of an Agilent 1100 series liquid chromatograph (including a quaternary pump, and a Rheodyne 8125 manual injector) coupled to an Applied Biosystems API3000 triple–quadrupole mass spectrometer equipped with a Turbo IonSpray interface (Foster City, CA, USA). The positive ion mode was performed, the capillary voltage was 4.5 kV and the source temperature was 320 °C. The nebulizer and curtain gases (nitrogen) were set to 7 and 11 (arbitrary units), respectively. The multiple reaction monitoring (MRM) mode was performed by monitoring the transition between m/z 206 and m/z 150 (collision energy 30 eV, the collision gas was nitrogen).

A reversed-phase C_{18} column (YMC, 5 μm, 150 mm × 3.0 mm i.d.) was used. The mobile phase consisted of acetonitrile–methanol–water–acetic acid (10:50:40:1, v/v/v/v) at the flow rate of 0.3 ml min^{-1}.

The calibration graph was generated from MRM of increasing amounts of ABP standard solution. A calibration curve was constructed by external standard method, the concentrations in test samples were then interpolated from this calibration line.

The microdialysis samples in the present study were analyzed without prior sample purification except that samples from the first 2.0 h were diluted 1:2 with Ringer solution. A 20 μl aliquot of the sample was injected into the LC-MS/MS system.

2.6. Analysis of metabolites in dialysates by LC-MS/MS

The LC-MS/MS system for identifying the metabolite of ABP consisted of an LCQ (Thermo Finnigan) ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source, a Surveyor HPLC system and a Surveyor autosampler (ThermoQuest). Experiments were performed in positive ion mode. The capillary temperature was set at 300 °C and the spray voltage at 4.25 kV. The fluid was nebulized using nitrogen (N_2) as both the sheath gas and the auxiliary gas.

The HPLC conditions were the same as described above.

2.7. Analysis of metabolites in tissues by LC-MS/MS and GC/MS

For comparative purpose, the brain tissue sample was prepared. Briefly, tissue was homogenized in appropriate volumes of saline, the sample was extracted three times with 20 ml ethyl acetate. The organic layer was collected and evaporated to dryness under reduced pressure. The residue was reconstituted in 1.0 ml of methanol. The tissue samples were analyzed in the same manner as the microdialytes by LC-MS/MS on the LCQ mass spectrometer.

ABP and its main metabolite in brain tissue were also analyzed by VG MD800 GC/MS system. The samples were separated on a DB-5MS capillary column (25 m × 0.25 mm × 0.25 μm) using a temperature gradient. Initial column temperature was 120 °C for 1 min, raised to 180 °C at 7 °C min^{-1}, held for 3 min, increasing to 250 °C at 5 °C min^{-1} and a final hold 2 min. The EI source temperature was 230 °C and the impact voltage was 70 eV. Identification runs were made at m/z 50–500 (full scan acquisition mode). A 1 μl aliquot of the brain tissue sample was injected into the GC/MS system.

3. Results

3.1. Determination of ABP in microdialysates by LC-MS/MS

The concentrations of ABP in the brain microdialysates were determined by LC-MS/MS on the API3000 triple–quadrupole mass spectrometer. The protonated molecule $[M + H]^+$ at *m/z* 206 of ABP was chosen as the precursor ion, the product ion spectrum is shown in Fig. 2. The most prominent product ion was found at *m/z* 150, which resulted from the loss of C_4H_8 group from the side chain of the precursor ion at *m/z* 206. The product ion was used for quantitation of ABP, the MRM mode was performed by monitoring the transition between *m/z* 206 and *m/z* 150. Fig. 3 displays the MRM chromatograms of microdialysis samples, a background spectrum (i.e. biological microdialysate prior to drug administration) is shown in Fig. 3(a) and (d) is a spectrum from an in vivo microdialysate. The chromatographic retention time of ABP was 7.5 min, the limit of detection (LOD) was 2.0 ng ml^{-1} for ABP. The chromatogram of a curve sample at the LOD is displayed in Fig. 3(b).

The calibration graph for ABP was generated using a linear regression of the peak areas (*Y*) versus the nominal concentration of ABP (*X*, ng ml^{-1}) with weighting by $1/X^2$. A typical regression equations was $Y = 31000X + 18600$ ($r = 0.9990$). A good linear response over the range of 2.0–200.0 ng ml^{-1} was demonstrated. For the determination of intra- and inter-assay precision and accuracy, three QC samples were analyzed in replicates of five on three different days. The summary results of the accuracy and precision are presented in Table 1. The inter- and intra-day precision (CV%) was less than 6% and accuracy lay between 101 and 108%.

Table 1
Precision and accuracy for the analysis of ABP in the Ringer solution

Nominal concentration (ng ml^{-1})	Mean determined concentration	CV%		Accuracy (%)
		Intra-day	Inter-day	
2.0	2.1	5.9	5.6	106
20.0	21.6	3.0	4.9	108
200.0	202.1	3.4	1.7	101

Number of runs = 3, with five replicates of each level of QC in each run.

The plot of the concentrations of ABP with time following the administration (80 mg kg^{-1} p.o.) to the rats is depicted in Fig. 4.

3.2. Identification of the metabolite by LC-MS/MS

For structural identification of the main metabolite in rat brain microdialysates, the LC-MS/MS spectra were obtained using an LCQ instrument. The peaks observed in the chromatograms in Fig. 5(a) represent ABP and its metabolite, respectively. The peak eluting at 6.86 min is the parent drug (ABP), the MS/MS spectrum of $[M + H]^+$ ion at *m/z* 206 is shown in Fig. 5(b). The peak at retention time 8.09 min in the total ion chromatogram (TIC) corresponds to a metabolite with a molecular mass of 247 Da, and the MS/MS spectrum of the $[M + H]^+$ ion at *m/z* 248 is shown in Fig. 5(c). The mass difference of the two peaks is 42 Da, the structure characterization of the metabolite was analyzed by comparing its product ion spectrum with that of the parent drug and by the detailed interpretation of the fragmentation routes. It could be postulated that the peak is the *N*-acetylated metabolite of the parent drug. A match between the MS/MS spectrum of a synthesized standard compound (Fig. 5(d)) and dialysate sample confirms that the postulation is reasonable. In addition, brain tissue samples were also analyzed by LC-MS/MS

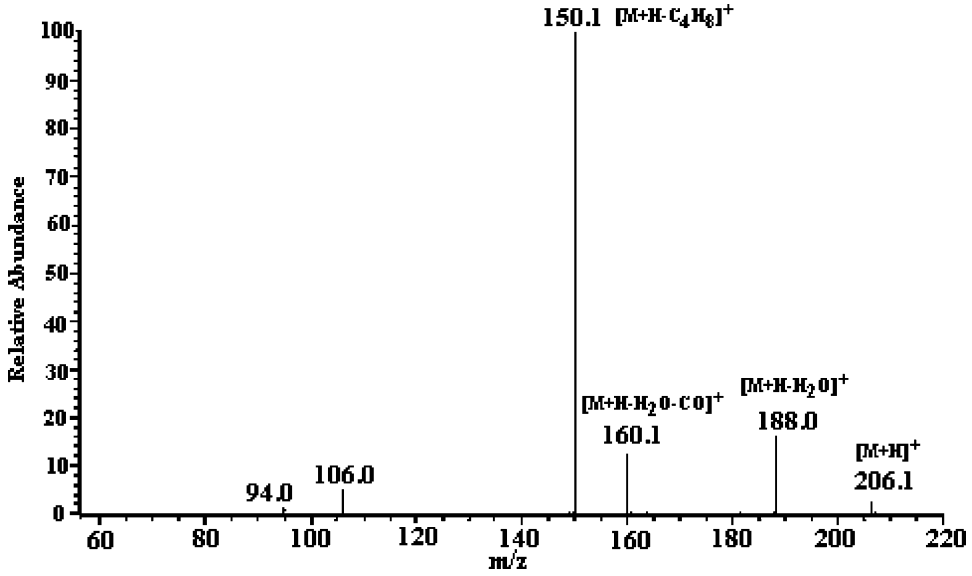

Fig. 2. The MS/MS spectrum of $[M + H]^+$ ion at *m/z* 206 of the parent drug by LC-MS/MS on an API3000 tandem mass spectrometer.

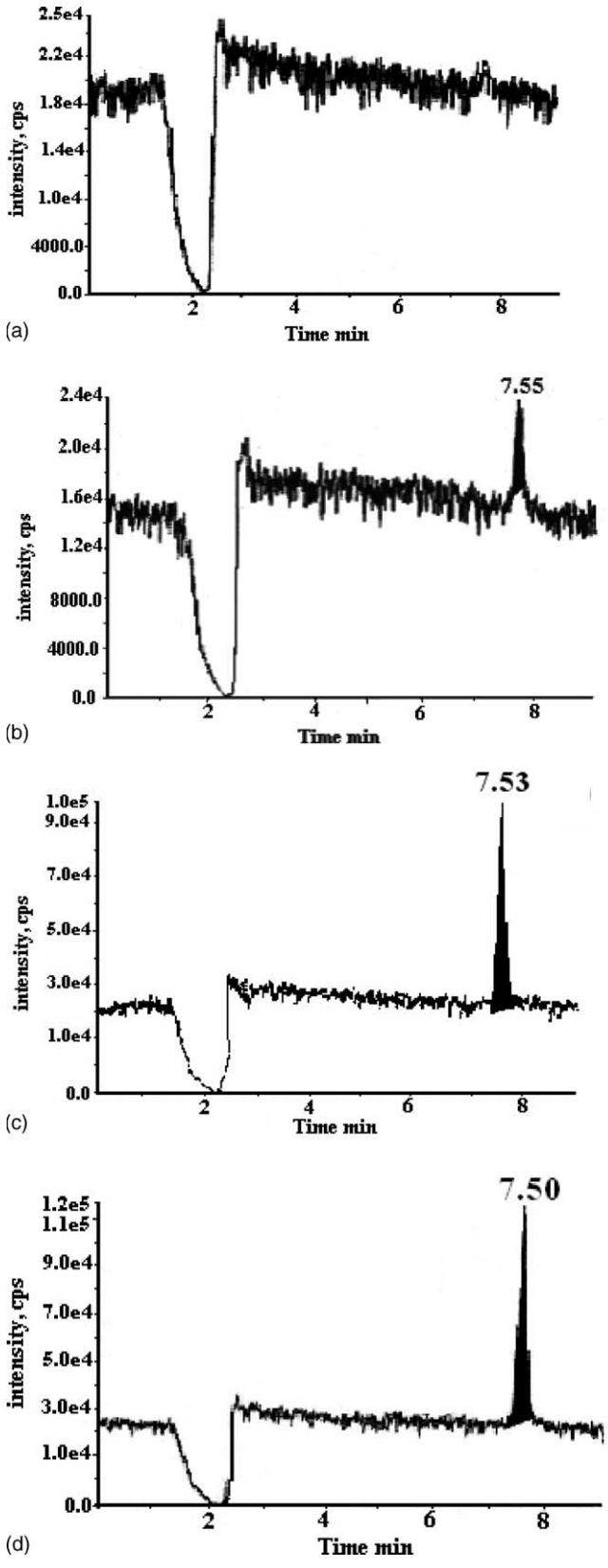

Fig. 3. The representative MRM chromatograms obtained on an API3000 tandem mass spectrometer: (a) a typical blank microdialysis sample collected just before administration of ABP; (b) a standard curve sample at the LOD (2.0 ng ml^{-1}); (c) a sample spiked with ABP (40 ng ml^{-1}) in the Ringer solution; (d) a microdialysis sample from a rat 135 min after an oral administration of 80 mg kg^{-1} ABP.

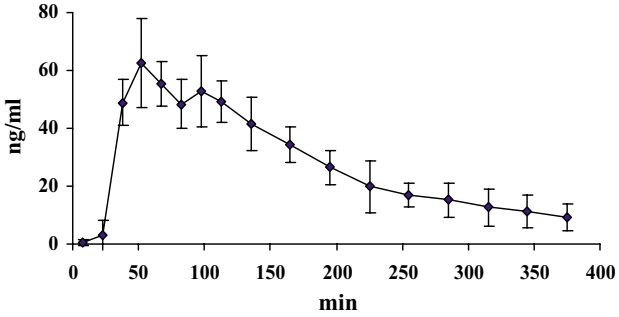

Fig. 4. The plot of the concentrations of ABP with time in the brain striatum following the administration (80 mg kg^{-1} p.o.) to the rats (mean ± S.D., $n = 5$).

under the same conditions, and identical results were obtained.

3.3. GC/MS analysis of M1 in tissue

For comparison and confirmation, a GC/MS experiment was performed. Fig. 6(a) represents a TIC chromatogram of the brain tissue sample, Fig. 6(b) and (c) show the EI-MS spectra of the peaks whose retention times are 13.1 and 15.4 min, respectively. By comparing their EI-MS spectra, it is found that their molecular weight are 205 and 247 Da, respectively, and the fragmentation behaviors are similar, so the peak eluting at 13.1 min is the parent drug and the peak at retention time 15.4 min corresponds to the *N*-acetylated metabolite of ABP, which are proved by comparisons of their mass spectra with the synthesized reference compounds (Fig. 6(d)).

4. Discussion

Previously, brain concentration-time profiles have been determined after decapitation of many animals at different time points after drug administration [16]. By using microdialysis in combination with LC-MS/MS, we studied the dynamic concentration-time course and the metabolism of ABP in rat brain. The samples were obtained repeatedly and continuously for 5.5 h from a single animal with no fluid loss and without killing the animal. The full time course for the appearance and disappearance of ABP in the brain could be acquired from a single animal.

Because the microdialysis sample volumes are quite small and the concentrations of analytes are often low, sample preparation procedures are complicated and difficult. Best results were obtained with direct injection of samples

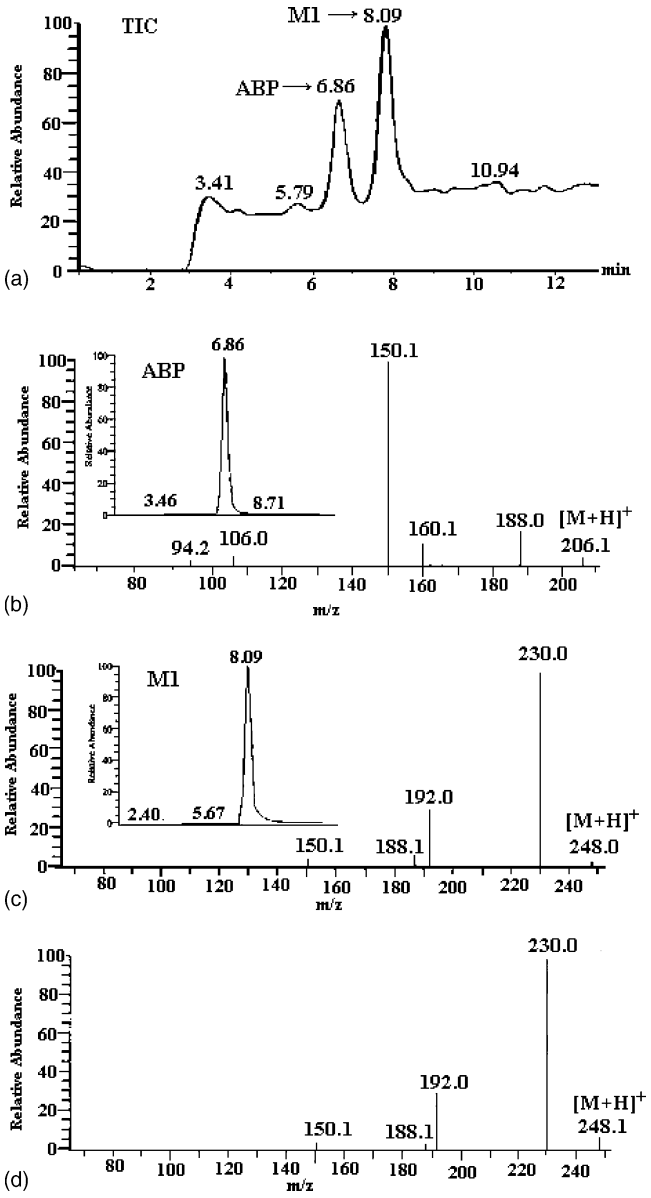

Fig. 5. The typical LC-MS/MS spectra of a microdialysis sample acquired with an LCQ mass spectrometer: (a) the total ion chromatogram (TIC); (b) the MS/MS spectrum of the peak at 6.86 min, and the inset shows the selected ion monitoring chromatogram of *m/z* 206; (c) the MS/MS spectrum of the peak at 8.08 min, and the inset shows the selected ion monitoring chromatogram of *m/z* 248; (d) the MS/MS spectrum of the synthesized M1 metabolite standard.

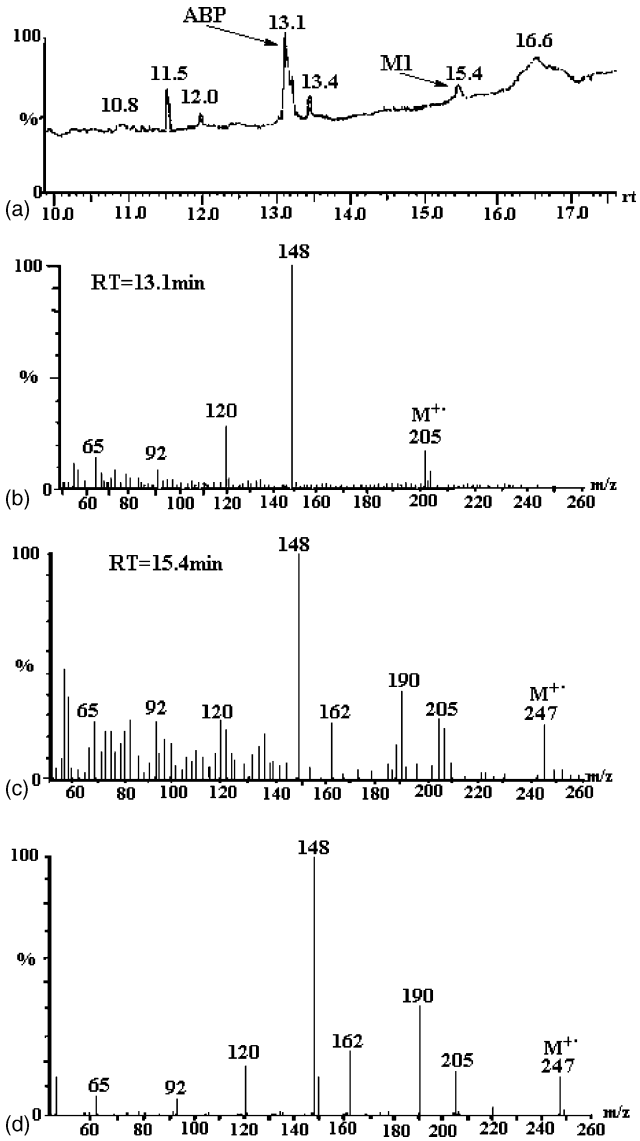

Fig. 6. The typical GC/MS spectra of a brain tissue sample acquired with a VG MD800 GC/MS system: (a) the total ion chromatogram (TIC); (b) the EI-MS spectrum of the peak at 13.1 min; (c) the EI-MS spectrum of the peak at 15.4 min; (d) the EI-MS spectrum of the synthesized M1 metabolite standard.

into the LC-MS/MS system. However, the microdialysis samples contain non-volatile salt which might interfere with the analysis. In the present study, the microdialysis samples were analyzed directly by LC-MS/MS without prior sample purification. To exclude interferences from the biological matrix, the high-quality analytical data were achieved by selecting the mobile phase and prolonging the retention time of ABP to 7.5 min.

At the beginning of the study, the LCQ mass spectrometer was applied to determine ABP concentrations in the dialysates quantitatively and identify the metabolite, the satisfied quantitative results were not obtained, and the quantitative analysis was finished by using an API3000 mass spectrometer at last. In addition, external calibration graphs [9,17,18] were used for the quantitation of ABP in dialysates, which is mainly based on two reasons, one is that the samples in the present study were analyzed without any sample preparation procedures except dilution, the other is that the API3000 mass spectrometer provided excellent linearity and stability for analysis.

Although in vivo microdialysis is a valuable methodological tool for pharmacokinetics and/or pharmacodynamics research, there are so many limitations that the coupling

of microdialysis to LC-MS/MS is not always straightforward. ABP and its main metabolite have been successfully detected in the target organ by this method, which is related to its special physico-chemical properties and chemical structure, ABP not only can penetrate the BBB, but also is suitable for microdialysis and mass spectrometry analysis.

5. Conclusions

In this paper, microdialysis in combination with LC-MS/MS method was developed to monitor the dynamic time course and metabolism of a new drug candidate in the brain of a single animal. This method can target specific tissues and determine the analytes rapidly thus providing assesses to pharmacokinetics and/or pharmacodynamics at the site of action. This method provides a number of analytical advantages including excellent selectivity, specificity and without time-consuming sample procedures.

Acknowledgements

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References

[1] R.D. Johnson, J.B. Justice, Brain Res. Bull. 10 (1983) 567.
[2] C.E. Lunte, D.C. Scott, P.T. Kissinger, Anal. Chem. 63 (1991) 773A.
[3] O. Darbin, M. Lonjon, M.H. Quentien, J.F. Michiels, P. Grellier, J. Negrin, J.C. Rostain, J.J. Risso, Brain Res. 881 (2000) 121.
[4] J.C. Day, T.J. Kornecook, R. Quirrion, Method 23 (2001) 21.
[5] L.J. Deterding, K. Dix, L.T. Burka, K.B. Tomer, Anal. Chem. 64 (1992) 2636.
[6] M.I. Davies, J.D. Cooper, S.S. Desmond, C.E. Lunte, S.M. Lunte, Adv. Drug Deliv. Rev. 45 (2000) 169.
[7] L. Romanyshyn, P.R. Tiller, C.E. Hop, Rapid Commun. Mass Spectrom. 14 (2000) 1662.
[8] Z. Wang, C.E. Hop, K.H. Leung, J.M. Pang, J. Mass Spectrum. 35 (2000) 71.
[9] E.C.M. de Lange, A.G. de Boer, D.D. Breimer, Adv. Drug Deliv. Rev. 45 (2000) 125.
[10] K.E. Garrison, S.A. Pasas, J.D. Cooper, M.I. Davies, Eur. J. Pharm. Sci. 17 (2002) 1.
[11] M.I. Davies, Anal. Chim. Acta 379 (1999) 227.
[12] U.U. Ungerstedt, in: Marsden, C.A. (Ed.), Measurement of Neurotransmitter Release, Wiley, New York, 1984, p. 81.
[13] L.J. Pellegino, A.S. Pellegino, A.J. Cushman, A stereo-taxic Altas of the rat brain, Plenum Press, New York, 1979.
[14] K. Nakashima, K. Yamamoto, O.Y. Al-Dirbashi, M.N. Nakashims, Biomed. Chromatogr. 16 (2002) 219.
[15] Y.J. Mano, S. Higuchi, H. Kamimura, Biopharm. Drug Dispos. 23 (2002) 351.
[16] H.U. Margareta, Adv. Drug Deliv. Rev. 45 (2000) 283.
[17] P.S.H. Wong, K. Yoshioka, F. Xie, P.T. Kissinger, Rapid Commun. Mass Spectrom. 13 (1999) 407.
[18] R. Kostiainen, T. Kotiaho, T. Kuranne, S. Auriola, J. Mass Spectrum. 38 (2003) 357.