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Quantitative determination of dihydroetorphine in rat plasma and brain by liquid chromatography–tandem mass spectrometry

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Abstract

The extraordinarily strong analgesic dihydroetorphine (DHE) was registered as one of the most strictly controlled narcotic drugs by the United Nations in 1999. However, an effective detection method for DHE in biological samples has not yet been established. We developed a quantitative method for assay of DHE in rat plasma and brain by liquid chromatography–tandem mass spectrometry equipped with an ionspray interface. A 0.5-ml volume of plasma and brain homogenate spiked with buprenorphine (internal standard) was purified by the solid-phase extraction column Bond Elute Certify. DHE produced numerous weak fragment ions by collision induced dissociation. Therefore, collision energy was utilized to decompose the interferences, and the protonated molecular ion was used for both precursor and product ion monitoring. As a result of the method validation, the dynamic concentration range was determined as 0.05–10 ng/ml. DHE in these samples was stable for 2 months at −4°C and for 24 h at ambient temperatures. Using the present method, DHE was detected in rat plasma and brain tissue after intravenous injection (0.5 μg/kg).

Keywords: Dihydroetorphine

1. Introduction

Dihydroetorphine, 7,8-dihydro-7α-[1-(*R*)-hydroxy-1 - methylbutyl] - 6,14 - *endo* - ethanotetrahydrooripavine (DHE, Fig. 1a), was synthesized by Bentley and Hardy in 1967, as the strongest analgesic opioid [1]. It was reported that DHE produces an extremely strong analgesic effect, at least 1000-times more potent than morphine [2,3]. DHE began to be clinically used for pain relief in 1981 and was registered as an analgesic for acute severe pain in 1992 in China. Unexpectedly, the abuse of DHE increased rapidly soon after it was marketed, therefore the government of China restricted use of DHE in 1993 [4]. Epidemiological studies show that many of the abusers took DHE to avoid the withdrawal syndrome from heroin or other opiates, because of its psychological dependence producing properties, cheap market prices and less restricted control [5,6]. In March 1999, the United Nations decided that DHE should be included in Schedule I of the Single Convention on Narcotic Drugs of 1961 [7] and the Convention as amended by the 1972 Protocol [8,9]. Although DHE should be used for appropriate therapies under restricted control, the relation between its pharmacological effects, toxicity and disposition are not clear.

Although there are some reports regarding the

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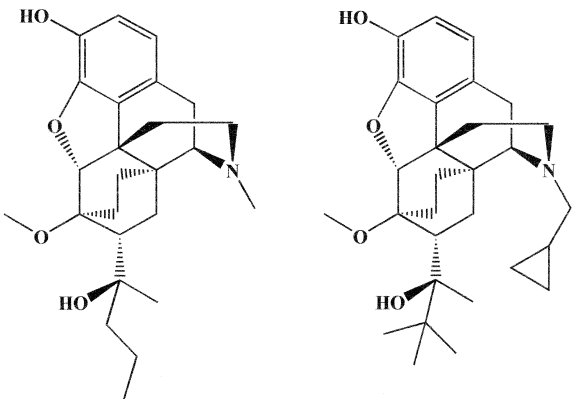

Fig. 1. Chemical structures of dihydroetorphine and buprenorphine.

analgesic effect and dependence potential after administration of DHE, the disposition of DHE is not well understood. Huang et al. investigated 14,15-H^3-DHE administered sublingually or subcutaneously to mice and measured the radioactively labeled DHE in blood and brain [10]. Radioactivity in blood and brain showed the total amount of the unchanged form and metabolites of DHE. When morphine was administered to experimental animals, the concentration of the glucuronide conjugate was higher than that of unchanged morphine in blood [11]. Concentrations of the unchanged DHE, which is the active form in the body, must therefore be measured to understand its profiles of pharmacological effect and adverse effect. However, there is no quantitative method which has sufficient sensitivity to detect unchanged DHE in biological samples.

Although quantitative methods for DHE by phosphorimetric assay [12,13] and gas chromatography–mass spectrometry (GC–MS) [14,15] have been reported, the sensitivity and selectivity of both methods are not sufficient. The GC–MS method reported by Liu et al. [15] is the most sensitive method for DHE. The limit of quantitation (LOQ) of their method was 2.5 ng/ml in human plasma and 1 ng/ml in human urine. They reported that DHE could be detected in plasma and urine from a DHE-intoxicated patient [14]. However, DHE in plasma and urine after the 20 μg sublingual administration, which is a clinically effective dose of DHE, could not be detected. A more sensitive method is necessary to estimate the concentration range of DHE in order to reveal the pharmacological effects.

Recently, liquid chromatography–tandem mass spectrometry (LC–MS–MS) has come to be recognized as a highly selective and sensitive method to detect small amounts of drug in biological samples [16,17]. The purpose of this study was to develop a sensitive method for DHE by LC–MS–MS. We developed a quantitative method of detecting DHE in rat plasma and brain tissue to evaluate the pharmacokinetics and pharmacodynamics of DHE in the rat [18].

2. Experimental

2.1. Chemicals

DHE was synthesized from codeine by reported procedures [1,19]. Codeine was first converted to thebaine in accordance with the method of Barber and Rapoport [19]. DHE was subsequently synthesized from thebaine by the method of Bentley and Hardy [1]. Buprenorphine (BPN, Fig. 1b) hydrochloride which was used as an internal standard (I.S.) was kindly supplied by Otsuka Pharmaceuticals (Tokyo, Japan). Acetonitrile, methanol, ethyl acetate and water were of high-performance liquid chromatography (HPLC) grade. All other reagents were of analytical grade.

2.2. Preparation of rat plasma and brain homogenates

Blood was withdrawn from the male hairless rats (WBN/ILA-Ht, Life Science Research Center, Josai University, Saitama, Japan) under diethyl ether anesthesia. Plasma was separated from blood by centrifugation. Brain tissue was excised from rats and weighed. Two volumes of methanol were added to the brain tissue, and the mixture was rapidly homogenized. A supernatant of the homogenate was separated by centrifugation, which was used as the sample.

2.3. Preparation of standard and quality control samples

Stock solutions of DHE and BPN were prepared with methanol. Standard samples for constructing a calibration curve and quality control samples for validating the present method were prepared in the concentration range of 0.05–10 ng/ml of DHE. Stock solutions of DHE were placed in a test tube, and the solvent was evaporated under a nitrogen stream. Then, 50 m*M* phosphate buffer (pH 6.0), rat plasma or brain homogenate were added, and completely mixed.

2.4. Extraction procedure

Bond Elut Certify cartridges (3 ml/130 mg, Varian, Harbor City, CA, USA) were washed sequentially with 3 ml of methanol and 3 ml of 50 m*M* phosphate buffer (pH 6.0). Each solvent was slowly passed through the column under atmospheric pressure. The solvent was stopped at the top of the sorbent bed to prevent the column drying. Then, 0.5 ml of the standard sample, quality control sample or unknown samples was placed in a test tube, and 3 ml of 50 m*M* phosphate buffer (pH 6.0) and 0.1 ml of the BPN stock solution (10 ng/ml) were mixed together. These mixtures were applied to the Bond Elut Certify cartridge. The column was washed with 3 ml of 100 m*M* acetic acid and 2 ml of methanol sequentially. After air was fully passed through the column, 4 ml of 2% (v/v) ammonium hydroxide in ethyl acetate was poured into the column, and the eluents were collected in a test tube. The eluent was dried under a nitrogen stream at 50°C. The residue was successively dissolved in 0.1 ml of acetonitrile–water (80:20, v/v) by vortex and sonication. An aliquot (20 μl) of the final mixture was applied to LC–MS–MS analysis.

2.5. LC–MS–MS instrumentation and conditions

The LC–MS–MS system consisted of a triple-stage quadrupole type mass spectrometer, API-300 (Perkin-Elmer Sciex, Foster City, CA, USA) equipped with an ionspray interface, TurboIonSpray [20]. The liquid chromatograph was a LC-10A system (Shimadzu, Tokyo, Japan).

For LC separation, a semi-micro LC column, Inertsil ODS-2 (5 μm, 150 mm×2.1 mm I.D., GL Science, Tokyo, Japan) which was connected to a guard column was used. Acetonitrile–50 m*M* ammonium acetate (95:5, v/v) as a mobile phase was delivered at a flow-rate of 0.3 ml/min. The column oven was maintained at 40°C.

The analysis conditions of API-300 were established as follows. The ionspray voltage and temperature were set at 4 kV and 300°C on the positive ion detection mode. The voltages of the orifice and focusing ring were 40 and 150 V, respectively. Nitrogen gas was used as a heating, nebulizing and curtain gas at a flow-rate of 8, 1.53 and 0.95 l/min, respectively. A collision energy for fragmentation of molecule was chosen at 31.5 eV under nitrogen gas ($2 \cdot 10^{-5}$ Torr; 1 Torr=133.322 Pa). The mass/charge ratio (m/z) of the precursor and product ions on the multiple reaction monitoring (MRM) were 414 u for DHE and 468 u for BPN.

Calibration curves using the peak area ratio of DHE-to-BPN and DHE concentration in the quality control samples with appropriate weighting functions were calculated using the computer software, MacQuan (Perkin-Elmer Sciex).

2.6. Method validation procedure

Validation of the quantitative method was carried out in accordance with the summary report of the conference on ''Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies'' [21].

(1) The specificity of the method was evaluated by comparison of LC–MS–MS chromatograms of plasma and brain which were obtained from six individual rats.

(2) The calibration curve was constructed with six standard samples in the range of 0.05–10 ng/ml. The linearity and the reproducibility of the curves was examined for five repeated measurements on separate days.

(3) The accuracy and the precision of the method were determined with five repeated measurements per concentration at four concentrations (0.05, 0.1, 1 and 10 ng/ml) of the quality control samples (buffer, plasma and brain homogenate) on separate days (inter-day) and within a day (intra-day). The accept-

ance criteria for the accuracy is as follows: the mean value of the repeated measurements should be within ±15% of the actual values, except for the LOQ concentrations, where it should be within ±20% of the actual value. The acceptance criteria for the precision is as follows: the relative standard deviation (RSD) of the repeated measurements should not exceed 15%, except for LOQ concentrations, where it should not exceed 20%.

(4) The stability of DHE at 1 and 10 ng/ml (buffer, plasma and brain homogenate) was established at storage temperatures of −20°C for 2 months and at the ambient temperature (25°C) for 24 h, and in addition through two freeze–thaw cycles.

2.7. Animal study

Male hairless rats were cannulated with polyethylene tubing into a femoral vein for injection of DHE and into a femoral artery for blood sampling under diethyl ether anesthesia. Blood was withdrawn after injection of DHE (0.5 μg/kg). Brain tissues

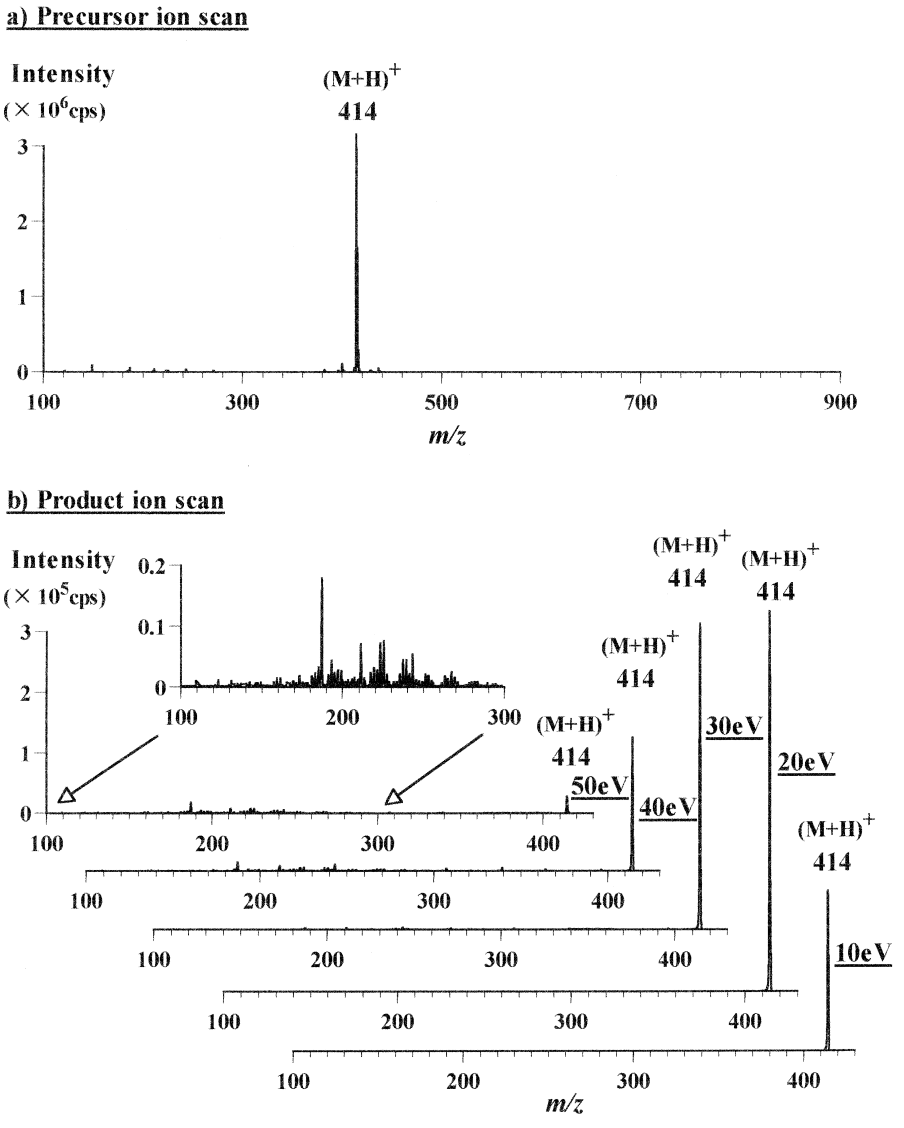

Fig. 2. Mass spectra of dihydroetorphine on collision induced dissociation.

were excised after decapitation. DHE concentrations in plasma and brain tissue were measured by the method described above.

3. Results

3.1. Mass spectrometry

Mass spectra of DHE are shown in Fig. 2. A protonated molecular ion of DHE (m/z 414) was detected clearly by precursor ion scan (Fig. 2a). No other ions such as ammonium additional molecular ion or dimer molecular ion were detected in the mass spectra. Then, we observed the dissociation of molecular ion induced by collision with nitrogen gas. The intensity of the molecular ion decreased with increasing the collision energy (Fig. 2b). DHE produced numerous weak fragment ions above 40 eV. In consideration of the intensity of the molecular ion and background interference, we chose 31.5 eV for the collision energy to decompose the unknown interferences and m/z 414 for both precursor and product ion of DHE on the MRM scan.

BPN formed a distinct molecular ion, m/z 468, and many fragment ions by the collision energy in the same way as DHE (data not shown). For the same reason as described above, 31.5 eV for the collision energy and m/z 468 for both precursor and product ion of BPN were chosen.

3.2. Chromatography

Typical LC–MS–MS chromatograms of DHE and BPN in plasma and brain are shown in Fig. 3. Retention times of DHE and BPN were 2.2 and 3.2

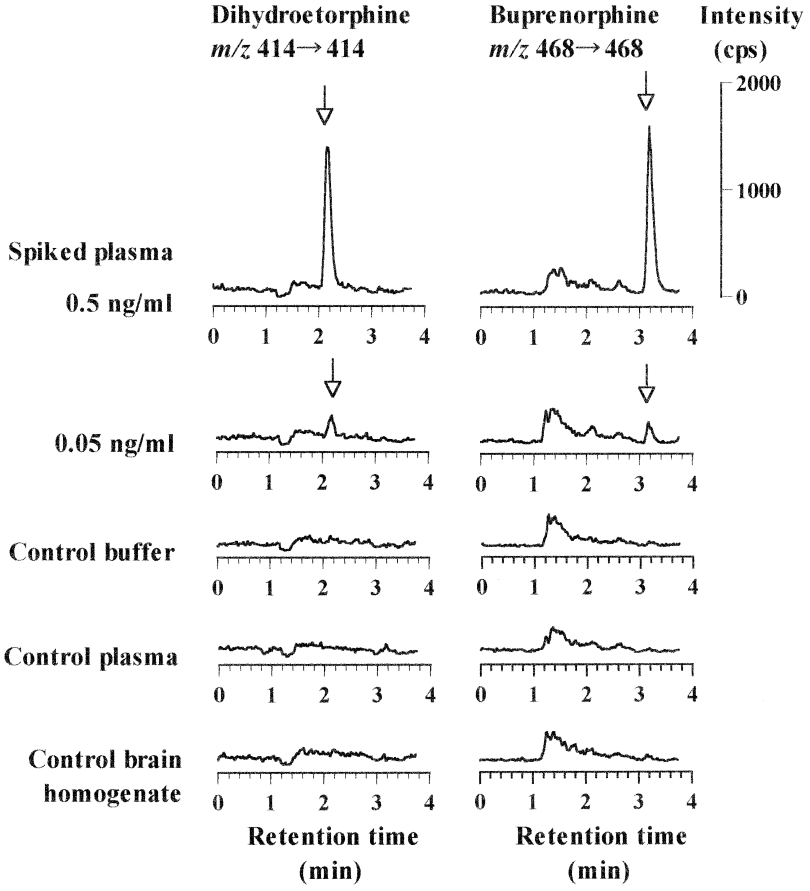

Fig. 3. Typical LC–MS–MS chromatograms of dihydroetorphine and buprenorphine (internal standard).

min, respectively. The time for the measurement of one sample was 4 min. The peaks of DHE and BPN were observed clearly up to 0.05 ng/ml (signal-to-noise ratio of 5–10) on chromatograms of spiked plasma. There was no interference peak against DHE and BPN on chromatograms of control samples which were collected from six individual rats (typical results from one rat are shown).

To verify the specificity of LC–MS–MS, we compared chromatograms of LC–MS–MS (Fig. 3) and LC–MS (Fig. 4). In the chromatogram of LC–MS (single ion monitoring on *m/z* 414 for DHE and 468 for BPN), there were many interference peaks derived from unknown elements included in the biological samples, which were not observed in the chromatogram of LC–MS–MS. DHE on LC–MS was less sensitive (about 1/10) than that on LC–MS–MS. The LC–MS–MS conditions used in this paper effectively improved the selectivity and the sensitivity.

3.3. Extraction recovery

The extraction recoveries of DHE from buffer (pH 6.0), plasma and brain homogenate in the solid-phase extraction are shown in Fig. 5. DHE in buffer, plasma and brain tissue homogenized with 100 m*M* phosphate or methanol were completely extracted. When the brain tissue was homogenized with saline (0.9% sodium chloride), the extraction ratio of DHE

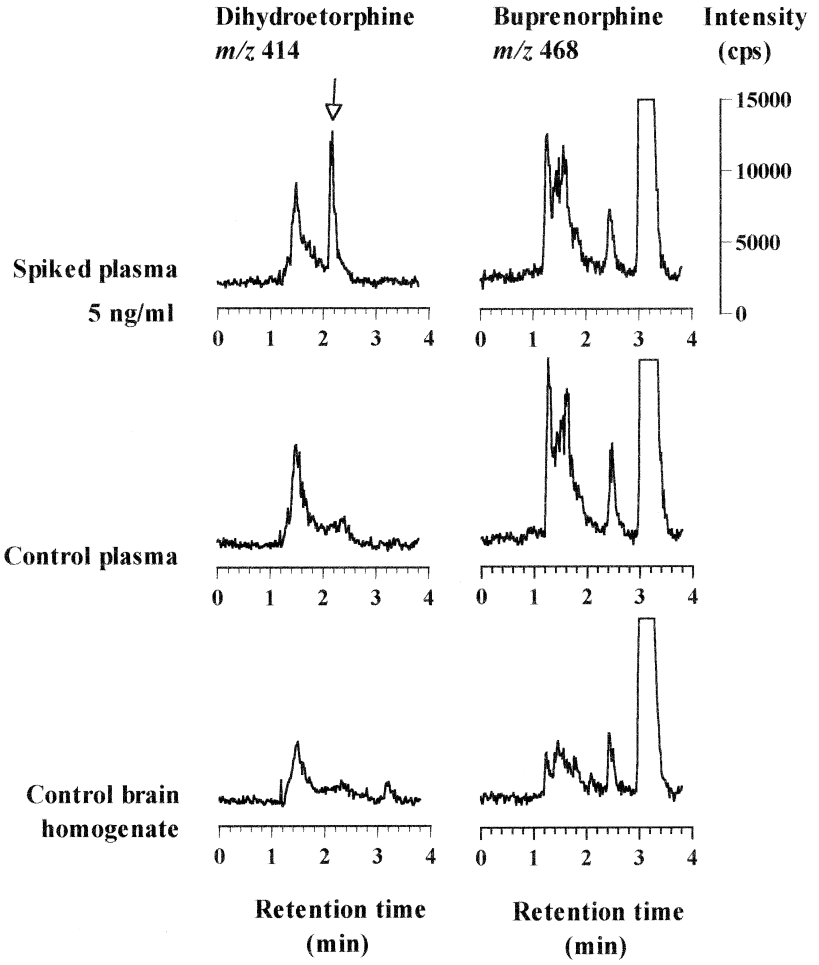

Fig. 4. Typical LC–MS chromatograms of dihydroetorphine and buprenorphine.

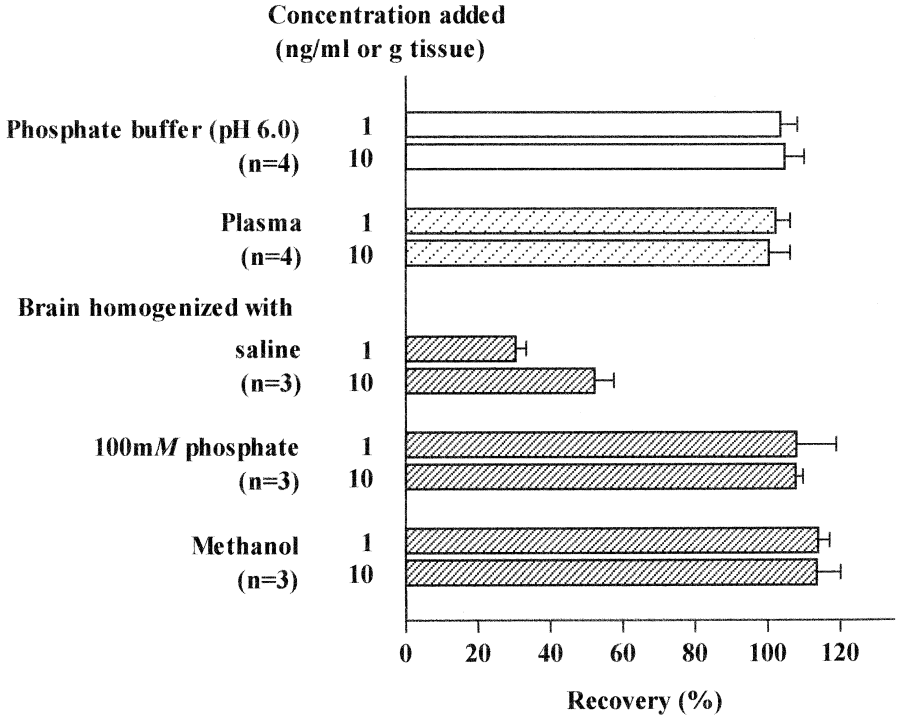

Fig. 5. Extraction recovery of dihydroetorphine in various matrices. Mean±SD.

was relatively low, 30.3% for 1 ng/g tissue and 52.2% for 10 ng/g tissue.

3.4. Calibration curve

The linearity and reproducibility of the calibration curve was examined for 5 days. The regression line with weighting of inverse concentration ($1/x$) throughout the range of 0.05 to 10 ng/ml was linear ($r>0.993$). The slope of the line was reproducible (0.425–0.511, RSD 7.9%) and the intercept on the y-axis was nearly zero (0.002–0.053).

3.5. Accuracy and precision

Intra- and inter-day variations are given in Tables 1 and 2, respectively. The accuracy and precision were within ±15% at 0.1, 1 and 10 ng/ml of the quality control samples, and within ±20% at 0.05 ng/ml for both intra-day replicates (Table 1) and in inter-day replicates (Table 2). Regardless of the kind of source (buffer, plasma or brain homogenate), the value was within this range.

3.6. Stability

The recoveries of DHE in the buffer, plasma and brain homogenate at storage conditions are shown in Fig. 6. DHE in various matrices was stable for 2 months at −20°C and for 24 h at ambient temperature (25°C). In addition, DHE was stable under two freeze–thaw cycles.

3.7. Animal study

DHE concentrations in the plasma and brain tissue after an intravenous injection of DHE (0.5 μg/kg) are shown in Fig. 7. Plasma and brain DHE concentrations were 0.30 ng/ml and 2.05 ng/g tissue at 5 min, and 0.055 ng/ml and 0.74 ng/g tissue at 30 min after injection. DHE was not detected in both plasma and brain at 90 min after injection.

Table 1
Intra-day assay accuracy and precision of dihydroetorphine in various matrices

Matrix	Concentration (ng/ml)		Accuracy	Precision
	Added	Found[a]	(%)	(%)
Phosphate buffer (pH 6.0)	10	10.04±0.92	+0.4	9.2
	1	0.938±0.111	−6.2	11.8
	0.1	0.1025±0.0132	+2.5	12.9
	0.05	0.0453±0.0063	−9.4	13.9
Plasma	10	9.05±0.91	−9.5	10.1
	1	0.868±0.054	−13.2	6.3
	0.1	0.1127±0.0129	+12.7	11.5
	0.05	0.0508±0.0088	+1.6	17.4
Supernatant of brain homogenate	10	10.02±1.20	+0.2	12.0
	1	0.865±0.064	−13.5	7.4
	0.1	0.0982±0.0137	−1.8	13.9
	0.05	0.0413±0.0082	−17.5	19.8

[a] Mean±SD (n=5).

4. Discussion

In this report, we developed a quantitative method to determine DHE by LC–MS–MS capable of evaluating the disposition of DHE after the administration of an effective dose in rats. The accuracy and precision of the method met the recommended acceptance criteria [21] and the LOQ was 0.05 ng/ml with 0.5 ml of plasma or brain homogenate. The GC–MS method reported by Liu et al. [6] was the most sensitive technique with LOQ of 2.5 ng/ml, but they required 2 ml (a relatively large volume) of plasma. Compared with the same volume of samples, the present method was 200-times more sensitive than the GC–MS method without the derivatization of DHE. We have confirmed that the present method is applicable for the determination of DHE in rat bile and urine [18], and mice plasma and brain (data not shown). We suggest that the present method is applicable for the determination of DHE in other

Table 2
Inter-day assay accuracy and precision of dihydroetorphine in various matrices

Matrix	Concentration (ng/ml)		Accuracy	Precision
	Added	Found[a]	(%)	(%)
Phosphate buffer (pH 6.0)	10	10.43±0.45	+4.3	4.3
	1	1.044±0.040	+4.4	3.8
	0.1	0.1012±0.0041	+1.2	4.1
	0.05	0.0528±0.0057	+5.6	10.8
Plasma	10	10.52±0.28	+5.2	2.6
	1	0.967±0.062	−3.3	6.4
	0.1	0.1072±0.0060	+7.2	5.6
	0.05	0.0529±0.0055	+5.7	10.4
Supernatant of brain homogenate	10	9.87±0.61	−1.3	6.2
	1	0.987±0.074	−1.3	7.5
	0.1	0.1018±0.0119	+1.8	11.7
	0.05	0.0503±0.0050	+0.6	9.9

[a] Mean±SD (n=5).

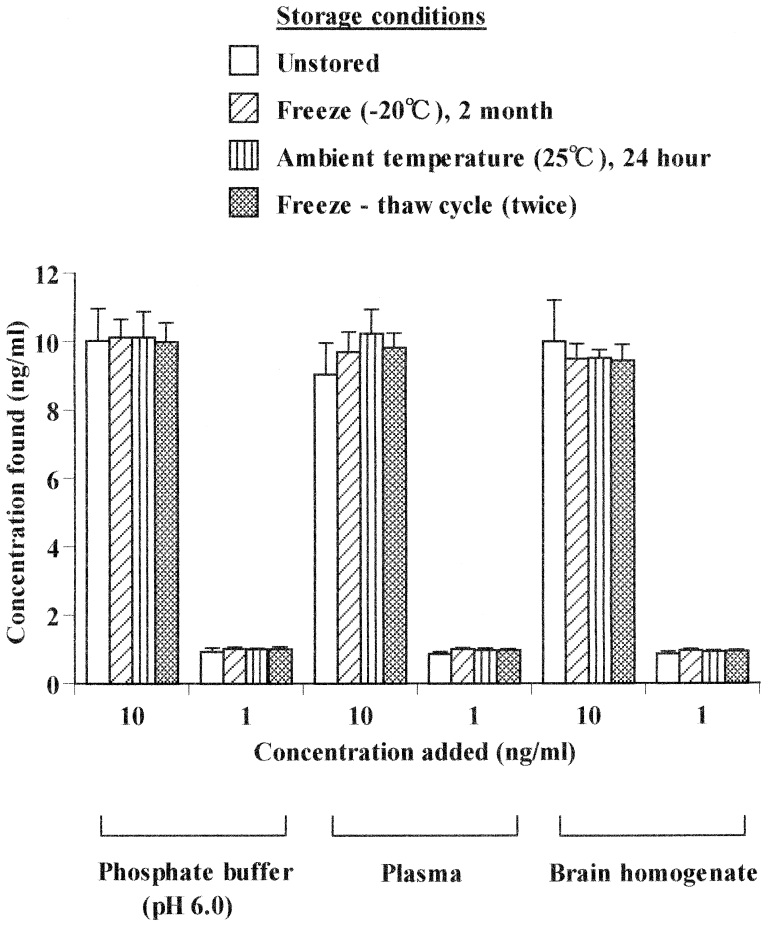

Fig. 6. Stability of dihydroetorphine in various matrices under storage conditions. Mean±SD (n=4–5).

samples such as human plasma and urine. This method will contribute to the evaluation of the relation between the pharmacological effects, toxicity and drug concentrations in experimental animals and humans.

BPN is an oripavine derivative like DHE (Fig. 1) and it has been used clinically for treatment of postoperative pain and chronic pain in cancer patients [22]. There are many reports regarding the quantitative method of BPN in human plasma, urine and hair [23]. The most sensitive methods for determination of BPN are GC with electron-capture detection [24], LC–MS and LC–MS–MS equipped with an atmospheric pressure ionization interface [25,26] for which the LOQ is 0.1 ng/ml with 1 ml of sample. We predict that the present method is able to detect not only DHE but also BPN at equivalent sensitivity.

While DHE was clinically used for a decade in China, there was no report regarding the effective DHE concentration in biological samples except one using radiolabeled DHE [10,27]. We determined the DHE concentration in plasma and brain tissue after intravenous injection, 0.5 μg/kg, in male hairless rats. Plasma DHE concentrations were 0.30 ng/ml at 5 min and then rapidly decreased to 0.055 ng/ml at 30 min after injection (Fig. 7). Brain DHE concentration was much higher (5- and 17-times) than the plasma concentration. Chen et al. [27] reported that the radioactivity in blood after intravenous injection of 14,15-H^3-DHE, 0.5 μg/200 g rat, increased to 17 ng equiv. DHE/ml, which was much

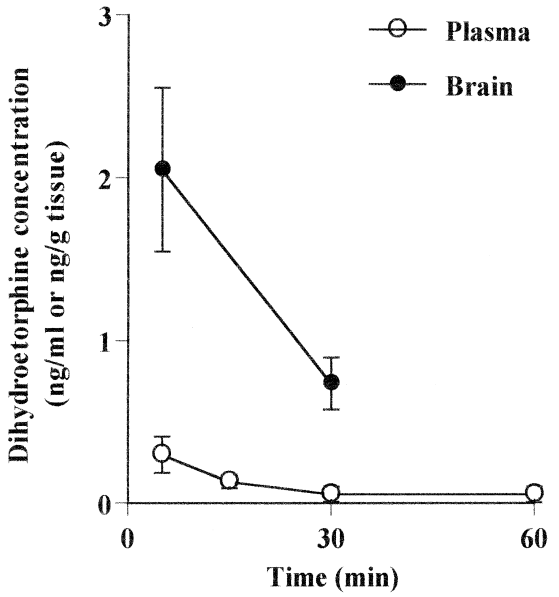

Fig. 7. Dihydroetorphine concentrations in plasma and brain after intravenous injection (0.5 μg/kg) to three male hairless rats. Mean±SD.

higher than our results correcting for the same dose. Huang et al. [10] reported that the radioactivity after sublingual administration of radiolabeled DHE were almost the same level in blood and brain. These results suggest the existence of many metabolites of DHE in rat and mice blood. We have confirmed in a pharmacokinetic study that glucuronide-conjugated DHE appeared in rat plasma at 5–200-times greater concentrations than unchanged DHE depending on the route of administration, and it was suggested that glucuronide-conjugated DHE is not pharmacologically active form [18]. Therefore, it was appreciated that a quantitative method of detecting unchanged DHE is necessary to evaluate the effective concentration of DHE during appropriate use.

5. Conclusions

Although DHE is one of the most strictly controlled narcotic drugs, a useful detection method for DHE in biological samples has not been published. In this report, we developed a quantitative method of DHE determination by LC–MS–MS to evaluate the disposition of DHE after the administration of an effective dose. The LOQ of this method was 0.05 ng/ml with 0.5 ml of plasma or brain homogenate, which was 200-times more sensitive than the previously reported GC–MS method. DHE in rat plasma and brain tissue after intravenous injection (0.5 μg/kg) could be detected by the present method. This method will contribute to the appropriate use of DHE through the evaluation of the relation between the pharmacological effects, toxicity and drug concentration in experimental animals and humans.

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