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Distribution of blood–muscle for clenbuterol in rat using microdialysis

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ABSTRACT

Clenbuterol is clinically used as a bronchodilator, but it is also illegally used to increase lean meat in animal husbandry. To investigate the muscle distribution of protein-unbound clenbuterol, a microdialysis technique coupled to liquid chromatography system was applied to simultaneously monitor clenbuterol in rat blood and muscle. Two microdialysis probes were implanted into the jugular vein/right atrium and hind leg muscle of rat for sampling after clenbuterol administration (10 mg/kg) through the femoral vein. Dialysate samples of clenbuterol were separated by a reversed-phase column (250 mm × 4 mm I.D., particle size 5 μm). The results indicate that the maximum concentration of clenbuterol in muscle was found at 30–45 min after clenbuterol administration (10 mg/kg) and the area under concentration curve (AUC) of clenbuterol in blood and in muscle were 942.75 ± 101.92 and 174.81 ± 13.03 min μg/mL, respectively. The AUC_{muscle}/AUC_{blood} was 0.20 ± 0.03 representing about 20% of the clenbuterol distributing into the muscle. The elimination half-life of clenbuterol in the blood and muscle were about 2 and 6 h, respectively. These results suggest that the protein-unbound concentration of clenbuterol sustained a high level and prolonged elimination in the muscle. The accumulation of clenbuterol might result in some clinical effects when clenbuterol-contaminated meat was consumed.

1. Introduction

Clenbuterol (Fig. 1) is a β_2-agonist commonly used for bronchodilation. It has also been reported that clenbuterol has anabolic and repartition activity to improve muscle growth, reduce body fat and result in redistribution of fat in muscle (Maltin et al., 1993; Kearns et al., 2001). To increase economic profits, clenbuterol is also illegally used to produce more lean meat in animal husbandry (Maltin et al., 1993; Kearns et al., 2001). The pharmacokinetic data indicate that the elimination half-life of clenbuterol in plasma is 10.9 h and at the tissues range from 21.2 to 56.3 h in horse (Soma et al., 2004). This long accumulation in animal bodies can still be detected after they were sacrificed (Yamamoto et al., 1985; Smith, 1998). A number of acute poisoning cases of humans, who took the meat with residual clenbuterol, have been reported from Spain, France and Portugal (Salleras et al., 1995; Bilbao Garay et al., 1997; Kuiper et al., 1998; Barbosa et al., 2005). Several countries have banned the use of clenbuterol in the animal husbandry to prevent similar circumstances, but the conflict between economic effects and health concerns has led to controversy. Acute poisoning may occur when people ingest these meat, edible tissues and offal that have accumulated clenbuterol. The symptoms include tremors, palpitations, dizziness, headache and nervousness. Recent studies indicate that several methods can be detected by clenbuterol in biological samples, such as HPLC–UV (Bazylak and Nagels, 2003; Blomgren et al., 2002), LC–MS/MS (Crescenzi et al., 2001), GC–MS (Gallo et al., 2007) and capillary zone electrophoresis (Q. Chen et al., 2008). However these methods measure the total form of clenbuterol but not the protein-unbound form of clenbuterol.

The protein-unbound drug levels reflect its biological activity and so this form is considered as the therapeutic portion (Weiss et al., 2000; Tsai, 2003; Plock and Kloft, 2005). Microdialysis is a useful sampling technique for measuring both protein-unbound endogenous substances and exogenous drugs *in vivo*. Currently, microdialysis has been applied to many tissues or organs, such as liver, blood, muscle, brain, bile (Tsai et al., 1999, 2002; Wu et al., 2007; Y.J. Chen et al., 2008) for pharmadcokinetic study and cell culture application (Wu et al., 2001). Samples collected by microdialysis are in the protein-unbound form and the dialysate is cleaner than samples collected by traditional methods. Moreover, continuous and multi-site sampling of microdialysis in the same animal can decrease number of sacrificed animals, which is another advantage to microdialysis for pharmacokinetic application.

Up-to-date, there have been no reports about applying the microdialysis technique to measure protein-unbound clenbuterol in the blood and muscle simultaneously. The aim of this study is to clarify the distribution of clenbuterol in rat muscle and blood.

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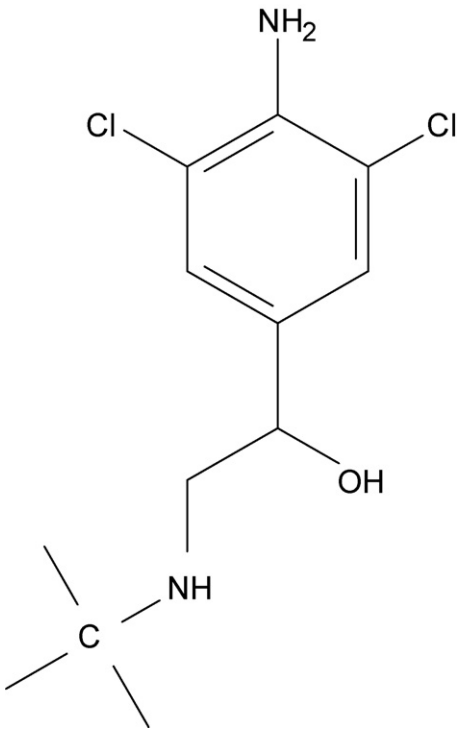

Fig. 1. Chemical structure of clenbuterol.

2. Experimental

2.1. Chemical and reagent

Clenbuterol hydrochloride, chloralose and urethane were purchased from Sigma–Aldrich Chemicals (St. Louis, MO, USA). Triply de-ionized water from Millipore (Bedford, MA, USA) was prepared for all aqueous solutions. Triethylamine (99%), orthophosphoric acid (85%) and sodium dihydrogenophosphate monohydrate were obtained from E. Merck (Darmstadt, Germany). Liquid chromatographic grade acetonitrile was purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA).

2.2. HPLC instrumentation

The HPLC method was modified from that developed by Botterblom et al. (1993), consisting of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), equipped with a 20-μL sample loop and a UV/vis detector (Varian, Walnut Creek, CA, USA). The detection wavelength was set at 211 nm. The analytical column for clenbuterol separation was used a reversed-phase column (Purospher® RP-18e, 250 mm × 4 mm, particle size 5 μm, E. Merck) and a guard column (4 mm × 4 mm, E. Merck). Samples were injected into the column by refrigerated autosampler (CMA/200, Stockholm, Sweden). The mobile phase consisted of acetonitrile–0.05 M sodium NaH_2PO_4 (pH 3.3 adjusted by H_3PO_4)–triethylamine (25:75:0.1, v/v/v). The mobile phase was filtered through a Millipore 0.45-μm filter (Bedford, MA, USA) and degassed by sonicator (BRANSON 2510) for 10 min before use. The flow-rate was set at 1 mL/min. Output data from the detector were integrated via a chromatographic data system (EZChrom, Scientific Software version 6.8, Pleasanton, CA, USA).

2.3. Experimental animals

Adult, male Sprague–Dawley rats (230–320 g) were obtained from the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). These rats were specifically pathogen-free and had freely access to food (Laboratory Rodent Diet 5001, PMI Nutrition International LLC, MO, USA) and water. The rats were

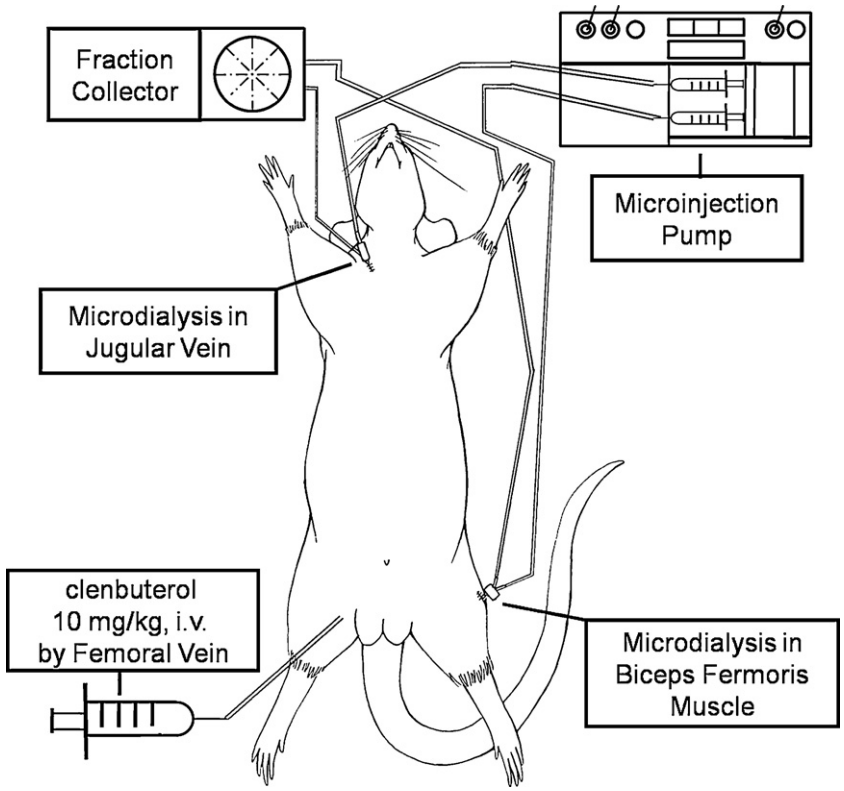

Fig. 2. Diagram of microdialysis system in rat and the probes inserted into jugular vein and biceps fermoris muscle.

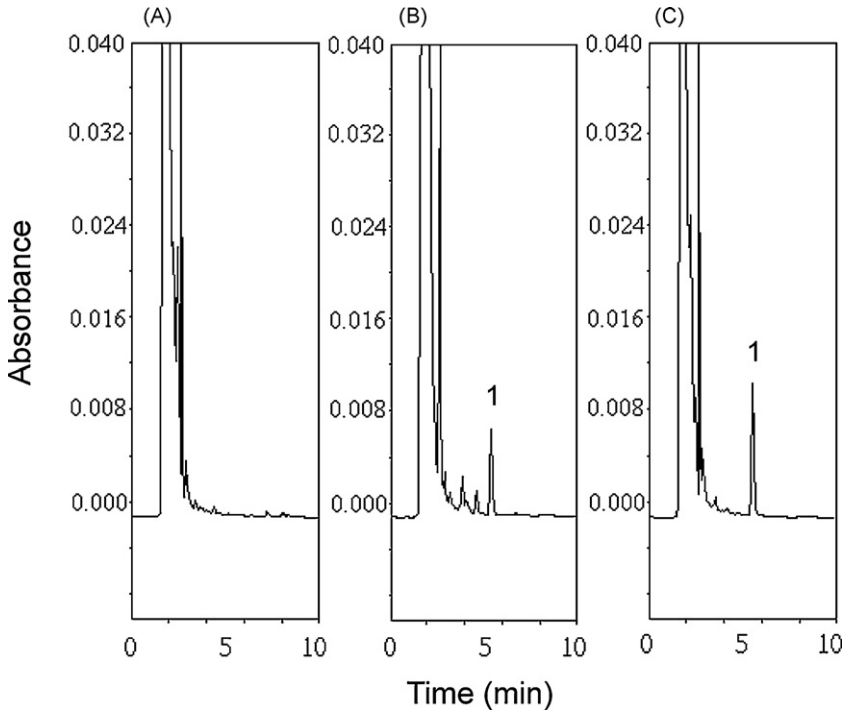

Fig. 3. Typical chromatograms of (A) blank blood dialysate, (B) blank blood dialysate spiked with clenbuterol (0.5 μg/mL), and (C) blood dialysate sample containing clenbuterol (0.79 μg/mL) after clenbuterol administration (10 mg/kg, i.v.). (1) Clenbuterol.

housed with a 12-h light and 12-h dark cycle. All experimental animal surgery procedures were reviewed and approved by the institutional animal experimentation committee of National Yang-Ming University. After rats were anesthetized by an anesthetic (1 mL/kg, i.p.) mixed with chloralose (0.1 g/mL) and urethane (1 g/mL), polyethylene tubing (PE-50; Clay Adams, NJ, USA) was implanted in the femoral vein for i.v. administration of drug. The rats remained anesthetized during the experimental period and their bodies were maintained heated through a heating pad set at 37 °C.

2.4. Microdialysis in rat blood and muscle

After rats were anesthetized, microdialysis probes were implanted into the jugular vein and muscle for blood and muscle sampling, respectively. For the blood sample, a custom-made

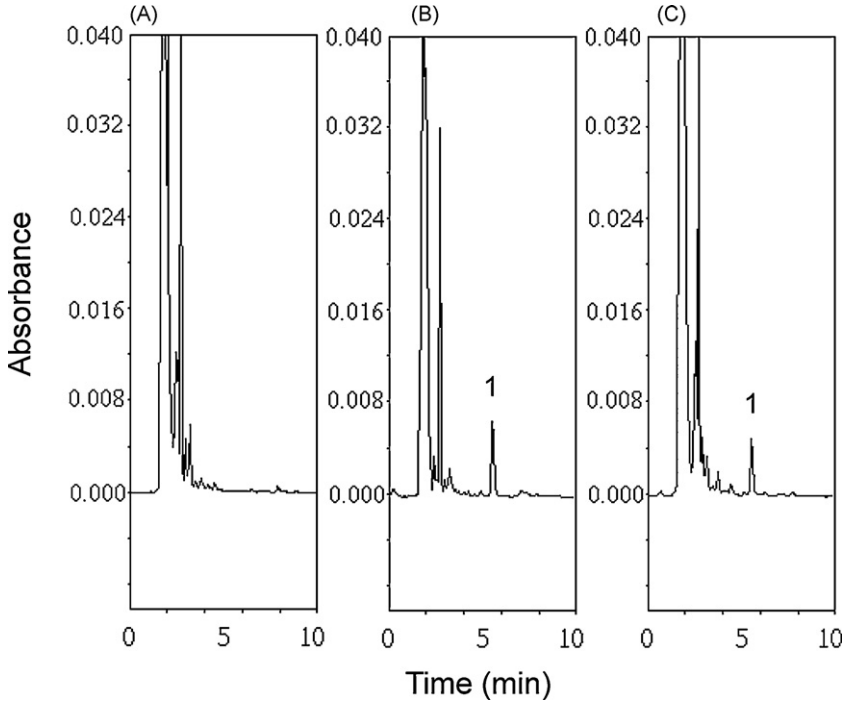

Fig. 4. Typical chromatograms of (A) a blank muscle dialysate, (B) a blank muscle dialysate spiked with clenbuterol (0.5 μg/mL), and (C) a muscle dialysate sample containing clenbuterol (0.35 μg/mL) after clenbuterol administration (10 mg/kg, i.v.). (1) Clenbuterol.

microdialysis probe was used. The length of dialyzing membrane was 10 mm and the molecular weight cut-off was 13,000 Da. The blood microdialysis probe implanted into the jugular vein/right atrium was then perfused with ACD solution (anticoagulant citrate dextrose, consisting 3.5 mM contain citric acid, 7.5 mM sodium citrate, and 13.6 mM dextrose). The microdialysis probe for muscle was commercially available (CMA/20 Elite, CMA/Microdialysis, Solna, Sweden) with a 10-mm dialyzing membrane and the molecular weight cut-off of 20,000 Da. For implantation of biceps fermoris muscle microdialysis probe (Fig. 2), the fur on the hind leg was first shaved away and then the skin was incised. A 23 gauge needle (introducer) was inserted into the incision and the splitting tubing was threaded through it. The needle was removed from the incision and the splitting tubing was left. Then, the linear microdialysis probe was implanted into the muscle by threading the split tubing. The muscle microdialysis probe was perfused with Ringer's solution (consisting of NaCl, 8.6 g; KCl, 0.3 g; KCl, 0.33 g $CaCl_2$ in 1000 mL H_2O; pH 7.0). The flow-rates of ACD and Ringer's solution in microdialysis were set at 2.0 μL/min by a microinjection pump (CMA/100) and dialysates were collected by microfraction collector (CMA/140). The collected samples were preserved at −20 °C before analysis.

2.5. Method validation

Clenbuterol hydrochloride was dissolved and stocked with a concentration of 1 mg/mL in water at room temperature. Calibration curves were calculated by blank dialysate spiked with different amounts of clenbuterol. The within-day variability was determined by quantitating six replicates at concentrations of 0.05, 0.1, 0.5, 1, 5, and 10 μg/mL on the same day, while between-day variability was determined on six consecutive days. The accuracy (Bias) was calculated from the nominal concentration (C_{nom}) and the mean value of the observed concentrations (C_{obs}) was as follows: Bias (%) = $[(C_{obs} - C_{nom})/C_{nom}] \times 100$. The precision, according to relative standard deviation (R.S.D.) was calculated from the observed concentrations as follows: R.S.D. (%) = [standard deviation (S.D.)/C_{obs}] × 100.

2.6. Recovery of microdialysis probes

In vivo recovery was calculated using a retrodialysis method. The blood and muscle microdialysis probes were implanted into the rat jugular vein and hind leg, respectively (Fig. 2). The solutions containing clenbuterol (1 and 10 μg/mL), ACD for blood, and Ringer's for muscle, were perfused through the microdialysis probes at a constant flow-rate of 2.0 μL/min by a microinjection pump. The clenbuterol concentration of the perfusate (C_{perf}) and that of the collected dialysate (C_{dial}) were then determined by HPLC. The relative *in vivo* recovery (R_{dial}) of clenbuterol across the dialysis membrane was calculated as $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$.

Table 1
Within-day and between-day precision (%R.S.D.) and accuracy (%Bias) for the determination of clenbuterol in blood.

Nominal concentration (μg/mL)	Observed concentration (μg/mL)	R.S.D. (%)	Bias (%)
Within-day			
0.05	0.052 ± 0.007	13.5	4
0.1	0.101 ± 0.009	8.9	1
0.5	0.490 ± 0.002	0.4	−2
1	1.003 ± 0.008	0.8	0.3
5	5.008 ± 0.025	0.5	0.16
10	9.996 ± 0.013	0.1	−0.04
Between-day			
0.05	0.045 ± 0.002	4.4	−10
0.1	0.103 ± 0.008	7.8	3
0.5	0.482 ± 0.003	0.6	−3.6
1	1.001 ± 0.006	0.6	0.1
5	5.036 ± 0.024	0.5	0.72
10	9.983 ± 0.011	0.1	−0.17

Data are expressed as mean ± S.D. (n = 6).

Table 2
Within-day and between-day precision (%R.S.D.) and accuracy (%Bias) for the determination of clenbuterol in muscle.

Nominal concentration (μg/mL)	Observed concentration (μg/mL)	R.S.D. (%)	Bias (%)
Within-day			
0.05	0.052 ± 0.002	3.85	4.04
0.1	0.104 ± 0.007	6.73	4.35
0.5	0.488 ± 0.003	0.61	−2.49
1	1.003 ± 0.020	1.99	0.33
5	5.005 ± 0.036	0.72	0.1
10	9.998 ± 0.016	0.16	−0.02
Between-day			
0.05	0.052 ± 0.003	5.77	3.27
0.1	0.102 ± 0.006	5.88	1.81
0.5	0.487 ± 0.008	1.64	−2.61
1	0.994 ± 0.011	1.11	−0.59
5	5.029 ± 0.031	0.62	0.57
10	9.982 ± 0.014	0.14	−0.18

Data are expressed as mean ± S.D. (n = 6).

2.7. Drug administration

Aliquots of 30 μL dialysate of blood and muscle were collected by microfraction collector every 15 min over 2-h equilibrium period. After this equilibrium period, clenbuterol (10 mg/kg) was intravenously administered via the femoral vein. The dialysate collected from the blood and muscle was injected into the HPLC system for analysis.

2.8. Pharmacokinetic calculations

The concentration of clenbuterol in dialysate (C_m) was converted to unbound concentrations (C_u) according to the following equation: $C_u = C_m/R_{dial}$. Unbound clenbuterol concentration (C_u) was applied to pharmacokinetic calculations. The midpoint of the 15-min period was used as the sampling time for clenbuterol concentration–time profiles. Each individual set of data was calculated for pharmacokinetic parameters by the non-compartmental method and the pharmacokinetic program, WinNonlin Standard Edition Version 1.1 (Scientific Consulting, Apex, NC, USA), was used. The area under the concentration–time curve (AUC) extrapolated to infinity was calculated by the log linear trapezoidal method. The clearance (Cl), mean residence time (MRT), half-life ($t_{1/2}$) and apparent volume of distribution at steady-state (V_{ss}) were calculated as following relations: Cl = dose/AUC; MRT = AUMC/AUC (AUMC is the area under the first moment–time curve); $t_{1/2} = 0.693/k$ (k is the elimination rate constant); $V_{ss} = Cl \times MRT$. The AUC ratio

Table 3
In vivo microdialysis recovery (%) of clenbuterol in rat blood and muscle.

Concentration (μg/mL)	Recovery (%)
Blood	
1	18.30 ± 3.20
10	18.52 ± 2.61
Average	18.41 ± 2.91
Muscle	
1	30.86 ± 2.73
10	31.50 ± 3.70
Average	31.18 ± 3.22

Data are expressed as means ± S.D. (n = 3).

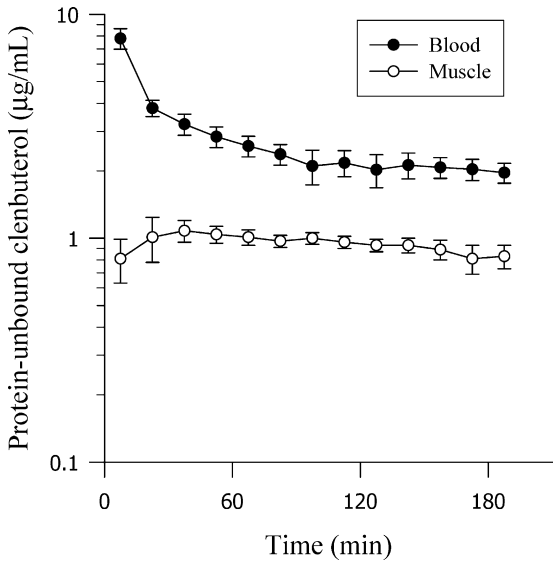

Fig. 5. Mean clenbuterol level in rat blood and muscle after administration of clenbuterol (10 mg/kg, i.v.) ($n = 6$). Data were presented as mean ± standard error of the mean (S.E.M.).

Table 4
Pharmacokinetic data of clenbuterol in the rat blood and muscle after clenbuterol administration (10 mg/kg, i.v.).

Parameter	Estimate
Blood	
C_{max} (μg/mL)	11.18 ± 1.32
$t_{1/2}$ (min)	132.3 ± 6.8
AUC (min μg/mL)	942.75 ± 101.92
Cl (mL/(min kg))	11.39 ± 1.45
MRT (min)	194.7 ± 10.7
V_{ss} (mL/kg)	2176.07 ± 207.65
Muscle	
C_{max} (μg/mL)	1.27 ± 0.17
AUC (min μg/mL)	174.81 ± 13.03
AUC_{muscle}/AUC_{blood}	0.20 ± 0.03

Data are expressed as means ± S.E.M. ($n = 6$).

of AUC_{muscle}/AUC_{blood} was defined as the blood-to-muscle distribution. All data are presented as mean ± standard error mean (S.E.M.).

3. Results and discussion

3.1. Chromatography

The HPLC condition for separation of clenbuterol from the endogenous interferences was evaluated by analyzing the drug-free (blank) blood and muscle dialysates. Figs. 3 and 4 show the chromatograms of blood and muscle dialysates, respectively. Fig. 3A represents the blank blood dialysate, Fig. 3B is the blank blood dialysate spiked with clenbuterol (0.5 μg/mL) and Fig. 3C shows the real blood sample containing clenbuterol (0.79 μg/mL) after clenbuterol administration (10 mg/kg, i.v.). Fig. 4C shows the real sample containing clenbuterol (0.35 μg/mL) in rat muscle after clenbuterol administration (10 mg/kg, i.v.). There was no detectable endogenous interference close to the retention time of clenbuterol, which was eluted at 5.6 min. The HPLC condition here was acceptable for analysis of the clenbuterol resulting from successful separation of endogenous interferences and clenbuterol.

3.2. Method validation—linearity, accuracy and precision

Method validation was established by blank dialysate spiked with various concentrations of clenbuterol. All calibration curves were considered linear with a coefficient of determination (r^2) greater than 0.995. The limit of quantitation (LOQ) was defined as the lowest concentration of clenbuterol in blood and muscle dialysate that was under the acceptable criteria (accuracy and precision were <20%). The LOQ of clenbuterol for blood and muscle dialysates were 0.05 μg/mL. The within- and between-day precision and accuracy values for blood and muscle are presented in Tables 1 and 2, respectively. Within-day precisions (%R.S.D.) ranged from 0.1% to 13.5% and accuracies (%Bias) were −2.49% to 4.33%. Between-day precision ranged from 0.1% to 7.8% and accuracy was −10% to 3.27%. In this study, the within- and between-day accuracy and precision show repeatability and reliability. The calibration curves of clenbuterol in blood and muscle dialysates were acceptable for quantification and can be applied to pharmacokinetic studies.

3.3. In vivo recovery of clenbuterol from microdialysis probe

In vivo recovery of clenbuterol in blood and muscle are presented in Table 3. The recoveries of clenbuterol in blood were 18.30% and 18.52% for 1 and 10 μg/mL, respectively, and average recovery was 18.41%. For the muscle microdialysis probe, average recovery was 31.18%. The true concentration outside the microdialysis probe in the probe-implanted site (here, blood and muscle) is needed to convert from the recovery through equation: $C_u = C_m/R_{dial}$.

Although *in vitro* and *in vivo* methods can be used to assess the probe efficiency, *in vivo* recovery is more reliable than *in vitro* (Weiss et al., 2000), since the probe-implanted site might more accurately represent the actual physiological conditions. As the result, *in vivo* retrodialysis was selected to mimic the real conditions in the body and to assess the probe efficacy, which interacted with the probe environment.

3.4. Pharmacokinetic parameters of clenbuterol in rat blood and muscle

In contrast to traditional sampling methods, which consider the combination of protein-bound and unbound form, microdialysis considers the protein-unbound form with continuous sampling with high temporal resolution. Fig. 5 shows the protein-unbound concentration of clenbuterol versus time curve in rat blood and muscle after clenbuterol administration (10 mg/kg, i.v.). The concentration of clenbuterol in the blood decreased, though the muscle level increased dramatically in the initial phase. The time to maximum concentration and elimination half-life of clenbuterol in muscle was found at 30–45 min and 6 h, respectively, a high concentration that was sustained and decreased slowly. The pharmacokinetic data indicate that the elimination half-life of clenbuterol in blood was 132.3 ± 6.8 min. The blood-to-muscle distribution ratio (AUC_{muscle}/AUC_{blood}) was 0.20 ± 0.03, represented about 20% clenbuterol distributed to the muscle (Table 4).

Using traditional sampling method, Yamamoto et al. (1985) estimated the long elimination half-life of clenbuterol in animal. However, the elimination half-life of protein-unbound clenbuterol detected in this study was about 2 h, which may due to the protein binding. The traditional sampling method combines the form of protein-bound and -unbound. Conversely, the semi-permeable membrane of the microdialysis probe blocked the substances with molecular weight greater than 13,000 (in blood) and 20,000 (in muscle) Da. It is known that the unbound form of the drug is the active form to distribute into tissue, so the unbound clen-

buterol might penetrate from the vessels into the tissues and accumulate in the muscle as the protein-bound form. According to the book of Goodman & Gilman's 'The Pharmacological Basis of Therapeutics' (Buxton, 2006), the drug binging with protein in vessel is reversible, and the only unbound drugs can across the membrane. When protein-unbound drugs across the membrane between vessel and tissue, drugs will bind with proteins in the tissue and reach other equilibrium, which is also reversible. With microdialysis sampling, the so-called therapeutic portions of the drug in probe-implanted sites could be monitored. In this study, we found that clenbuterol could penetrate from vessels into the muscle, since clenbuterol was administrated through intravenous. Combined with previous studies (Yamamoto et al., 1985; Smith, 1998), we speculated that penetrated clenbuterol may accumulate in the muscle, binding with cellular constituents and reaches a reversible equilibrium with unbound form. While unbound clenbuterol decreases, the protein binding clenbuterol will release become unbound clenbuterol and reach a new balance. The concentration of protein-unbound clenbuterol in the muscle decreased very slowly compared to that in the blood which was shown in this study.

According to the previous reports (Brambilla et al., 1997, 2000), people who consumed the clenbuterol-contaminated meat had clinical signs and symptoms, such as distal tremors, headache, neurologic, cardiovascular, etc. The clenbuterol could be detected in these patients' urine even after ingesting for over 48 h and clinical symptoms could last for 3–5 days. Our pharmacokinetic data indicated that clenbuterol could distribute to the muscle easily but excreted slowly. Combined with the previous studies, we assume that if people ingest the clenbuterol-contaminated meat, the intoxication of clenbuterol could be happened and continue for days.

4. Conclusion

In this study, we demonstrate a validated method to measure protein-unbound clenbuterol in rat blood and muscle for the pharmacokinetic study. The results indicate that clenbuterol might rapidly distribute into muscle and the distribution ratio from blood-to-muscle was estimated to be about 20%.

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