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Measurement of unbound caffeic acid in rat blood by on-line microdialysis coupled with liquid chromatography and its application to pharmacokinetic study

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Abstract

To monitor the levels of caffeic acid in rat blood, an on-line microdialysis system coupled with liquid chromatography was developed. The microdialysis probe was inserted into the jugular vein/right atrium of male Sprague-Dawley rats. Caffeic acid (100 mg/kg, i.v.) was then administered via the femoral vein. Dialysates were automatically injected onto a liquid chromatographic system via an on-line injector. Samples were eluted with a mobile phase containing methanol–100 m*M* monosodium phosphoric acid (35:65, v/v, pH 2.5). The UV detector wavelength was set at 320 nm. The detection limit of caffeic acid was 20 ng/ml. The in vivo recoveries of the microdialysis probe for caffeic acid at 0.5 and 1 μg/ml were 48.34±2.68 and 47.64±3.43%, respectively (n=6). Intra- and inter-assay accuracy and precision of the analyses were ≤10% in the range of 0.05 to 10 μg/ml. Pharmacokinetics analysis of results obtained using such a microdialysis–chromatographic method indicated that unbound caffeic acid in the rat fitted best to a biexponential decay model.

Keywords: On-line microdialysis; Caffeic acid

1. Introduction

Caffeic acid (3,4-dihydroxycinnamic acid; Fig. 1), a natural phenolic compound, is contained in many beverages and foods [1–3]. It has been found to be pharmacologically active as an antioxidant [4,5], antimutagenic [6], anticarcinogenic agent [7,8] and as a lipoxygenase inhibitor [9]. In view of these important effects, an accurate assay method for the determination of caffeic acid in biological samples is

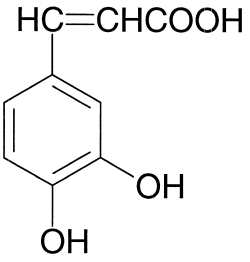

Fig. 1. Chemical structure of caffeic acid.

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warranted. The most frequently used method for the determination of caffeic acid in biological samples is high-performance liquid chromatography (HPLC) [10–12]. Several other methods for the determination of caffeic acid in natural products, employing thin-layer chromatography [13], HPLC [14,15] and gas chromatography [16] have also been developed. For the determination of protein-free drugs in biological samples, analytical methods with high sensitivities or low limits of quantitation are required. Previously reported limits of quantitation for plasma of 0.1 [10] or 1.8 μg/ml [11] are inadequate for the measurement of unbound caffeic acid in rat blood.

Caffeic acid is unstable, being subject to degradation by the non-enzymatic autoxidative phenolic browning reaction, which is temperature- and pH-dependent [5]. This reaction is slowed down under acidic conditions. To minimize the degradation of caffeic acid at physiological pH, an automatic sampling system and stable analytical conditions are required. In the present study, we used an in vivo on-line microdialysis sampling method coupled with a HPLC analytical system for the measurement of unbound caffeic acid in rat blood.

2. Experimental

2.1. Reagents

Caffeic acid was purchased from Sigma (St. Louis, MO, USA). Sodium dihydrogen phosphate and other reagents were obtained from Merck (Darmstadt, Germany). Triple deionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Liquid chromatography

The liquid chromatographic system consisted of a chromatographic pump (BAS PM-80, West Lafayette, IN, USA), an on-line injector (CMA 160, Stockholm, Sweden) equipped with a 20-μl sample loop and a UV detector (Soma S-3702 ultraviolet detector, Tokyo, Japan). Analytes were separated using a reversed-phase column (Merck, RP-18, 250×4 mm I.D.; 5 μm). Chromatography was performed at ambient temperature. The mobile phase consisted of methanol–100 m*M* monosodium phosphoric acid (35:65, v/v, pH 2.5) at a flow-rate of 1 ml/min. The mobile-phase mixture was filtered through a 0.45-μm Millipore membrane prior to use. The wavelength of the UV detector was set at 320 nm. Output data from the detector were integrated via an EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA).

2.3. Animals

Adult, male Sprague-Dawley rats (280–320 g) were obtained from the Laboratory Animal Center at National Yang-Ming University (Taipei, Taiwan). These animals were specific-pathogens-free and were allowed to acclimatize in our environmentally controlled quarters (24±1°C and 12:12 h light–dark cycle) for at least five days before being used for experiments. The rats were initially anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and remained anesthetized throughout the experimental period. The rat's body temperature was maintained at 37°C with a heating blanket.

2.4. Microdialysis experiments

The blood microdialysis system (Fig. 2) consisted of a CMA/100 microinjection pump and a CMA/160 on-line injector for the dialysate sample [17,18]. Blood dialysis probes were made of silica capillaries in a concentric design, which were covered at the tips by dialysis membranes (Spectrum, 10 mm length, 150 μm O.D., with a cut-off at a nominal molecular mass of 13 000; Laguna Hills, CA, USA). The blood microdialysis probe was inserted into the jugular vein/right atrium and perfused with ACD solution (3.5 m*M* citric acid, 7.5 m*M* sodium citrate, 13.6 m*M* dextrose) at a flow-rate of 2 μl/min using the CMA/100 microinjection pump (Fig. 2) [19]. Following a 2-h period of baseline reference collection, during which experimental conditions had become stabilized, caffeic acid (100 mg/kg) was intravenously administered via a femoral vein. Dialysis samples were collected every 10 min and 20 μl of dialysate were assayed by HPLC [20].

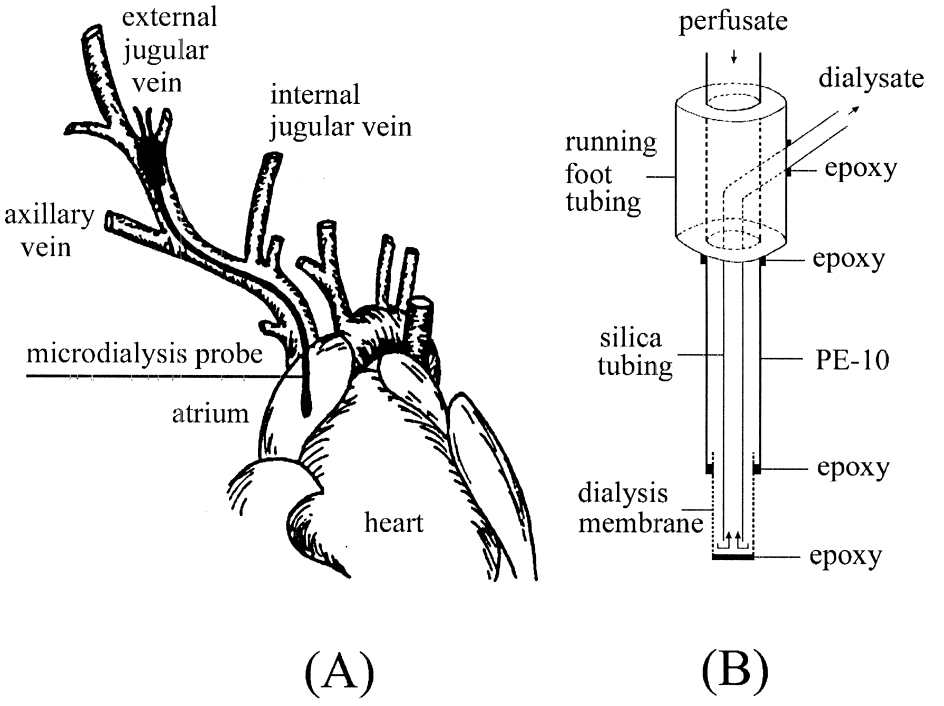

Fig. 2. Microdialysis probe used in rat blood. (A) A microdialysis probe was inserted into the rat's jugular vein/right atrium; (B) a laboratory-made microdialysis probe.

2.5. Recovery of microdialysate

In vitro recovery using the blood microdialysis probe was calibrated by inserting the probe into an Eppendorf sample vial containing 500 or 1000 ng/ml of caffeic acid. The perfusion media and pumping flow-rate were the same as described above. Recovery by the probe was calculated by dividing the concentrations in the dialysate (C_{out}) by the concentration in the tube (C_{in}) [21], that is, $\text{recovery}_{\text{in vitro}} = C_{out}/C_{in}$

For in vivo recovery, a retrograde calibration technique was used. The blood microdialysis probe was inserted into the rat's jugular vein under anesthesia with sodium pentobarbital. ACD solution containing caffeic acid (500 or 1000 ng/ml) was passed through the probe at a constant flow-rate (2 μl/min) using the infusion pump (CMA/100). After a stabilization period of 2 h, the inlet (C_{in}) and outlet (C_{out}) concentrations of caffeic acid were determined by HPLC. The in vivo recovery ratios were then calculated using the following equation [22]: $\text{Recovery}_{\text{in vivo}} = 1-(C_{out}/C_{in})$

2.6. Method validation

All calibration curves of analytes (external standards) were determined prior to the experiments, with correlation values of at least 0.995. The intra- and inter-day variabilities for caffeic acid were assayed (six replicates) at concentrations of 0.05, 0.1, 0.5, 1, 2, 5, and 10 μg/ml on the same day and on six sequential days, respectively. The accuracy (% bias) was calculated from the nominal concentration (C_{nom}) and the mean value of the observed concentration (C_{obs}), as follows: Bias (%)=$[(C_{nom}-C_{obs})/(C_{nom})]\times 100$. The precision coefficient of variation (CV) was calculated from the observed concentrations, as follows: % CV=[standard deviation (SD)/C_{obs}]×100. Accuracy (% bias) and precision (% CV) values of ±15%, covering the range of

actual experimental concentrations, were considered to be acceptable [23].

2.7. Pharmacokinetic study

Calibration curves were constructed based on HPLC analyses of various concentrations of caffeic acid. The concentrations of caffeic acid in rat blood dialysates were determined from the calibration curves. Following a 2-h period of stabilization, caffeic acid (100 mg/kg, i.v.) was administered. Dialysates were injected every 10 min by an on-line injector (CMA/160) for an additional 90 min following caffeic acid administration. Absolute concentrations in extracellular fluid were calculated from the concentrations in dialysates using the following equation: concentration=dialysate/recovery.

Pharmacokinetic calculations were performed on each individual set of data. Blood data were fitted to a biexponential decay given by the following formula: $C=A\ e^{-\alpha t}+B\ e^{-\beta t}$. The distribution and elimination rate constants, α and β were calculated using the equation: α (or β)=(ln C_2−ln C_1)/(t_2-t_1), where C_1 is the value of C at time t_1 and C_2 is the value of C at time t_2. Formation rate constants were calculated by extrapolation of the formation slope determined by the residuals method. The areas under the concentration curves (AUCs) were calculated by the trapezoid method. Half-life ($t_{1/2}$) values were calculated using the equations: $t_{1/2,\alpha}=0.693/\alpha$ and $t_{1/2,\beta}=0.693/\beta$ for the distribution and elimination half-life, respectively.

3. Results and discussion

3.1. Specificity of caffeic acid in blood microdialysate

Chromatograms for standard caffeic acid, blank blood dialysate and for a typical chromatogram obtained from a rat sample following intravenous administration of caffeic acid are shown in Fig. 3. The detection limit for caffeic acid was 20 ng/ml at a signal-to-noise ratio of 3:1. Fig. 3A shows the lower limit of quantitation of caffeic acid (50 ng/ml). The blank sample (Fig. 3B) shows that the chromatographic conditions revealed no biological substances that would significantly interfere with the accurate determination of the drug. Fig. 3C depicts a chromatogram of actual unbound caffeic acid in rat blood. The dialysate sample contains caffeic acid (0.39 μg/ml) collected from the jugular vein 30 min after the administration of caffeic acid (100 mg/kg, i.v.).

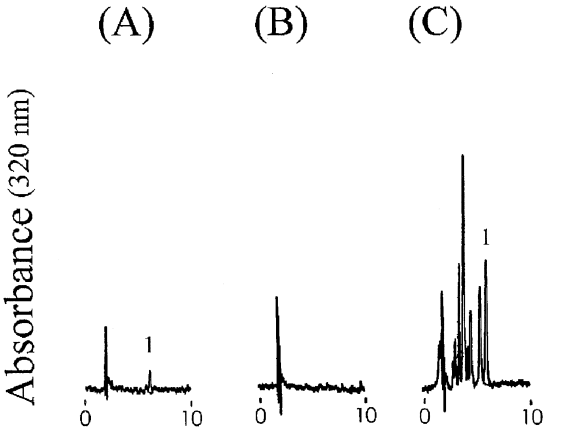

Fig. 3. Typical chromatogram following injection of (A) standard caffeic acid (1; at 50 ng/ml), (B) a blank blood dialysate and (C) a blood dialysate sample containing caffeic acid (0.39 μg/ml), collected from the jugular vein 30 min after caffeic acid administration (100 mg/kg, i.v.). 1, caffeic acid.

3.2. Linearity, precision and accuracy

The calibration curve was measured by linear regression ($r^2>0.995$) over a concentration range of 0.05–10 μg/ml of caffeic acid. Intra- and inter-day precision and accuracy for caffeic acid (Table 1) fell well within predefined limits of acceptability. All % bias and % CV values were within±10%.

3.3. In vitro and in vivo recoveries

Based on responses using 0.5 and 1 μg/ml standard caffeic acid, both the in vitro and in vivo recoveries from blood were examined (Table 2). In vivo recoveries were higher than the corresponding in vitro recoveries (Table 2). The efficiency of dialysis (recovery) can be affected by certain factors, which are mostly physical in nature, such as temperature, perfusion rate, and the nature and dimensions of the probe. Thus, the probes need to be

Table 1
Intra- and inter-day accuracy and precision of the HPLC method for the determination of caffeic acid

Nominal concentration (μg/ml)	Observed concentration (μg/ml)[a]	CV (%)	Accuracy (% bias)
Intra-assay (n=6)			
0.05	0.051±0.0024	4.7	2.0
0.1	0.099±0.0011	1.1	−0.1
0.5	0.51±0.0079	1.5	2.0
1	1.02±0.0038	0.4	2.0
2	1.96±0.022	1.1	−2.0
5	5.01±0.052	1.0	0.2
10	10.01±0.022	0.2	0.1
Inter-assay (n=6)			
0.05	0.051±0.0023	4.5	2.0
0.1	0.104±0.002	1.9	4.0
0.5	0.49±0.0066	1.3	−2.2
1	1.003±0.0074	0.7	0.3
2	1.97±0.025	1.3	−1.5
5	5.002±0.012	0.2	0.04
10	9.99±0.028	0.3	−0.1

[a] Observed concentration data are expressed as rounded means±S.D.

calibrated and the physical constants in a study kept constant as much as possible. In general, in vitro calibration allows optimization of physical parameters for consistency, reliability and maximal yield, while in vivo calibration strives to simulate in vivo conditions so that determined concentrations can be converted to absolute values. The combination of the heterogeneity of the tissue matrix, variability of fluid movements and the fact that a bioactive compound applied exogenously may induce physiological responses makes in vivo calibration very difficult, or virtually impossible, and many workers simply report changes relative to the basal levels, particularly in cases where changes in levels of endogenous compounds, such as neurotransmitters, are monitored, assuming that the factors that may affect the recovery remain essentially constant throughout the course of the experiments.

Table 2
In vivo and in vitro microdialysis recoveries (%) of caffeic acid in rat blood[a]

Concentration (μg/ml)	In vivo	In vitro
0.5	48.34±2.68	15.86±1.09
1	47.64±3.43	15.87±0.79

[a] Data are expressed as mean±S.E.M. (n=6).

Controversial comparisons of in vitro (recovery by gain or loss) and in vivo recoveries led to the reporting of a non-statistically significant difference for flurbiprofen and naproxen [24] and a statistically significant difference for topotecan [25]. In the present study, we attempted both in vitro calibration, which characterized mainly the physical constants, and in vivo calibration, which we hoped would yield further information that would be helpful in the correction for in vivo recovery. The fact that our results indicated three-fold higher in vivo than in vitro recoveries from blood (Table 2) was not unusual. Menachery et al. [26] systematically compared in vitro and in vivo probe recoveries for exogenously administered compounds in the brain and found higher in vivo recoveries for each of the two perfusion rates used. They attributed this higher recovery to the higher body temperature of 37°C, tortuosity (λ) or the increased path length through which the tortuous tissue matrix molecules must travel to reach the probe as well as volume fraction (α) or restriction of the analytes to the extracellular fluid, which comprised about 20% of the total volume. Tortuosity and volume fraction may change depending on the ease with which a compound penetrates a cell membrane. In the present study, in vitro calibration served only as a rough guide to the acceptability and uniformity of the probes, as they were laboratory-made.

3.4. Microdialysis and pharmacokinetics

The concentrations of caffeic acid in rat blood dialysate after caffeic acid (100 mg/kg, i.v.) administration are shown in Fig. 4. The samples were collected at 10 min intervals during the entire experimental period. The results, as derived from microdialysis studies and corrected for in vivo recoveries, suggested that the pharmacokinetics of unbound caffeic acid appeared to best fit the kinetics of a two-compartment model in rat blood. The volume of distribution (VOL) and clearance (Cl) were 1.14±0.35 l and 0.14±0.027 (l/min/kg), respectively. Other pharmacokinetic parameters are shown in Table 3.

By injecting the samples automatically onto a

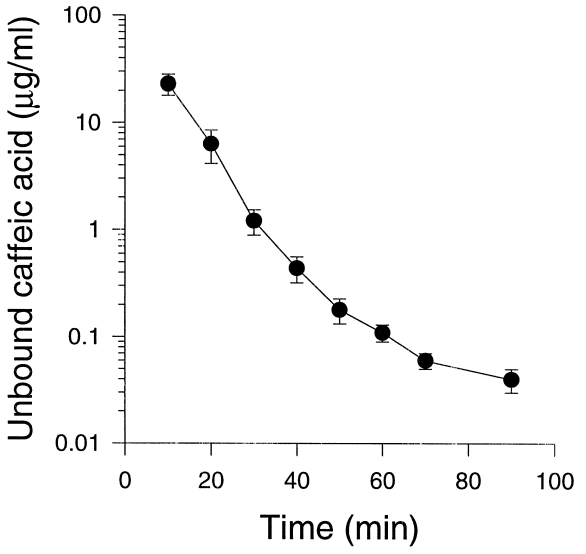

Fig. 4. Mean unbound levels of caffeic acid (●) in rat blood after caffeic acid administration (100 mg/kg, i.v.).

liquid chromatographic system for analysis, the present on-line microdialysis technique provides a convenient means for the continuous in vivo monitoring of unbound drugs in blood, particularly for relatively unstable compounds. Furthermore, because microdialysis involves virtually no loss of body fluids, it is particularly suitable for pharmacokinetics studies as it eliminates possible effects on hemodynamics due to blood loss compared to the conventional blood-withdrawing approach [27]. In addition, microdialysis is relatively inexpensive and easy to set up. Laboratory-made probes further reduce expenses and allow customization for special needs, such as sampling in blood. With certain precautions, such as allowing time for stabilization and partial recovery from surgical trauma and careful recovery calibration, the disadvantages can be minimized. The dialysates can be analyzed using any appropriate analytical methods. However, a finite volume of dialysate is required for the analysis and the sensitivity of the method dictates the amount of dialysate required and, therefore, the sampling time; hence, a disadvantage is that the values actually represent mean values over the period of dialysate collection. In the present case, HPLC was again relatively accessible and the method had been optimized so that only 10 min of collection time was required.

Table 3
Estimate pharmacokinetic parameters following caffeic acid administration (100 mg/kg, i.v.)[a]

Parameter	Estimate
A (μg/ml)	174.01±67.77
B (μg/ml)	3.30±2.87
α (1/min)	0.18±0.034
β (1/min)	0.043±0.011
$t_{1/2,\,\alpha}$ (min)	4.91±1.12
$t_{1/2,\,\beta}$ (min)	17.89±3.16
AUC (μg min/ml)	904.87±255.94
VOL (l)	1.14±0.35
Cl (l/min/kg)	0.14±0.027

[a] Data are expressed as means±S.E.M. (n=6). $P<0.05$. Details of the abbreviations are given in the text.

4. Conclusion

In conclusion, this study represents a first attempt in which in vivo protein unbound caffeic acid was determined in rat blood by microdialysis, demonstrating that it is feasible to monitor caffeic acid from blood using a microdialysis probe. Compared with other in vivo methods for pharmacokinetics study in the blood, microdialysis offers the advantages of being able to continuously monitor drug concentrations in the extracellular compartment in the same animal, it causes virtually no biological fluid loss and, therefore, exerts only minimal strain on hemodynamics. The results indicated that the disposition of protein-free caffeic acid in the blood appeared to fit a two-compartment model.

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