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Comparison of Saliva Stimulation Methods for Noninvasive Therapeutic Drug Monitoring by Using Saliva Samples

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To find out an appropriate therapeutic drug monitoring method by using saliva, we investigated the effect of four kinds of saliva stimulation methods on salivary pH, flow rate and composition in normal male subjects. Each of methods showed significantly different pH, flow rate and compositions (total protein, K^+ and creatinine levels). It was observed that the simplest method (i.e. to move mouth and tongue) had the smallest inter-individual variation of pH and relatively small inter-individual variations of total protein and Na^+ levels. In addition, this method showed no diurnal variations of pH, flow rate and compositions (total protein, Na^+ and K^+ levels). The present results suggest that to move mouth and tongue for salivation may be the simplest and most suitable stimulation method for therapeutic drug monitoring by using saliva samples. Furthermore, this method was found to be well applicable to a pilot study on the saliva to serum concentration ratio of mexiletine hydrochloride after the oral administration in normal subject.

Keywords—saliva stimulation method; therapeutic drug monitoring; salivary drug concentration; salivary pH; salivary flow rate; salivary composition; mexiletine hydrochloride; human

Introduction

Utilization of salivary drug concentration for therapeutic drug monitoring meets the requirement of noninvasive and simple sample collection. It has been reported for many drugs that the salivary drug concentration is equal to the protein-unbound concentration in serum or plasma, which is regarded as the drug level related directly to the clinical effect^{1,2)}. Since there is relatively large variation in the saliva to serum or saliva to plasma concentration ratio for many drugs, the practical application of the salivary drug concentration to the therapeutic monitoring in place of serum or plasma is very limited¹⁻³⁾. It has been reported that saliva to serum drug concentration ratio is

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influenced by some factors such as salivary pH, flow rate and drug protein binding as well as serum protein binding of the drug³⁻⁶). Furthermore, these factors and composition of saliva have been known to be influenced by the method to stimulate salivation⁷⁻⁹).

The present study was, therefore, designed to compare the effects of four different stimulation methods for salivation on the pH, flow rate and compositions (i.e. total protein, Na⁺, K⁺, Cl⁻, creatinine levels) of mixed saliva which was collected for 5 min within the appointed one-hour. The most appropriate stimulation method among them was then tested in the diurnal variation study between 7:30 and 19:30. In addition, the proposed method was applied to a pilot study on the monitoring of salivary levels of mexiletine hydrochloride, a new antiarrhythmic drug with relatively narrow therapeutic range (0.75-2.0 µg/ml)¹⁰), in a normal subject.

Experimental

1. Materials

Bovine serum albumin (BSA) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mexiletine hydrochloride (MEX) was kindly supplied by Nippon Boehringer Ingelheim Co. Ltd. (Kawanishi, Japan). All other reagents used were of analytical grade.

2. Subjects

The present study was carried out in 10 normal healthy male volunteers (35±6 years, 64±12 kg). In pilot study with MEX, one of these volunteers (30 years, 68 kg) was selected. The pilot study protocol was approved by the Division of the Institutional Review Board.

3. Comparison among stimulation methods

a) Stimulation and collection of saliva

Salivation was stimulated by applying the following four different methods: A) to move mouth and tongue, B) to chew Sealon film (Fuji Photo Film Co. Ltd., Tokyo, Japan), C) to smell at lemon, D) to move mouth and tongue after gustatory stimulation with a drop of 10% citric acid solution on the tongue. The pre-saliva was discarded from the mouth cavity as completely as possible. All stimulations were continued for 5 min. In method D, the stimulant in the salivated fluid accumulated for the initial 10 sec was discarded. After stimulation, the salivated fluid (i.e. mixed saliva) accumulated in the mouth cavity was directly expectorated into a glass funnel set on polystyrene tube as completely as possible. The saliva supernatant obtained after centrifugation (3000 rpm, 10 min) was frozen until analysis. Each stimulation was applied to each subject in randomized order between 18:00 and 19:00.

b) Measurement of salivary pH and flow rate

The saliva pH tends to increase gradually when exposed to the atmosphere. Therefore, the saliva pH was measured with the glass electrode (Horiba 6028-10T, Horiba Ltd., Kyoto, Japan) which was inserted directly into the mouth cavity immediately after the stimulation for 5 min. The specific gravity of mixed saliva was determined according to the method of Japanese Pharmacopoeia XI, yielding the value of 1.005±0.005.

Therefore, the measured weight of saliva sample instead of the volume was used for the calculation of the salivary flow rate by assuming that the specific gravity is approximately 1.00 as reported previously in other papers^{5,11}).

c) Determination of total protein

Total protein level in mixed saliva was determined by the method of Lowry et al.¹²) using BSA as the standard.

d) Determination of Na^+ , K^+ and Cl^-

Na^+ and K^+ levels in mixed saliva were determined by flame photometer (Jasco Flame 30, Jasco Medical Instruments Inc., Tokyo, Japan). Chloride ion level was determined by chloride counter (Hiranuma CL-5, Hiranuma Sangyo Co. Ltd., Mito, Japan).

e) Determination of creatinine

Creatinine level in mixed saliva was determined by the method of Bonsnes et al.¹³).

4. Diurnal variation study

The diurnal variations of pH, flow rate and composition of mixed saliva collected by using method A were investigated. Saliva samples were collected at 90-min intervals between 7:30 and 19:30. The subjects took the same standardized meals, breakfast (758 Kcal) at 8:15 and lunch (827 Kcal) at 12:45, and were allowed to drink water freely during the experimental period.

5. Pilot study with MEX

Single dose study was carried out in one subject who received 200 mg of MEX capsule (Mexitil®) orally at 8:15 with about 200 ml of water. Blood and saliva samples were collected at 1, 2, 3, 4, 6, 8, 12 and 24h after administration. The meal was taken at 7:30, 12:30 and 19:00. Periodical blood sample (5 ml) was withdrawn from cephalic vein at the mid-point of the corresponding period of saliva collection. Saliva was collected for 5 min by using the stimulation method A. The concentrations of MEX in serum (total and free) and saliva samples were determined by high-performance liquid chromatography according to Grech-Bélanger et al.¹⁴). Serum free fraction was obtained by ultracentrifugation of the serum sample with Centrisart I (Sartorius GmbH, Göttingen, W. Germany).

Results

Comparison among stimulation methods

Comparisons of pH, flow rate and composition of mixed saliva obtained by four different stimulation methods are summarized in Table 1. Method A tended to give the lowest pH and the smallest flow rate. In contrast, method B yielded the highest pH and the largest flow rate. It was observed that the pH tended to be dependent on the flow rate. Method C, which is olfactory and visual stimulation method, exhibited the lowest levels of total protein and creatinine among methods.

The results of analysis of variance for these four stimulation methods presented in Table 1 are shown in Table 2. Each of pH, flow rate, total protein, K^+ and creatinine levels was significantly different among four methods.

Table 3 represents inter-individual variations of pH, flow rate and composition of

mixed saliva collected by using four methods. It was observed that the simplest method, A, had the smallest inter-individual variation of pH and relatively small inter-individual variations of total protein and Na^+ levels. On the other hand, method C yielded the largest inter-individual variations of pH, flow rate, Na^+ and Cl^- levels. Therefore, method A was employed as a candidate method hereafter. All methods had larger inter-individual variations of Na^+ and Cl^- levels than that of K^+ level.

Diurnal variation study

Fig.1 and 2 show the diurnal time-course of pH, flow rate and composition of mixed saliva collected by method A between 7:30 and 19:30. Only salivary pH seemed to have a tendency to rise very gradually with the flow rate. Table 4 shows the results of analysis of variance for these 9 collection time-points summarized previously in Fig.1 and 2. The diurnal variations of pH, flow rate, total protein, Na^+ and K^+ levels were not observed at all. Each of pH, flow rate and composition of mixed saliva was also compared between pre- and post-meal times. There was no significant difference between them.

Pilot study with MEX

The serum and saliva levels of MEX after the single oral dose in one subject are shown in Fig.3. Saliva MEX level was always higher than the serum level. Time-course of the salivary drug level was found to be almost parallel with that of the serum total or free level. Any time-lag between both peaks in serum and saliva were not observed. In regression analysis for saliva against serum MEX levels at post-absorption phase (4-24h), a good correlation between saliva and serum drug levels was obtained (serum total; $r=0.986$, $p<0.001$: serum free; $r=0.986$, $p<0.01$). Accordingly, it was found that saliva to serum MEX concentration ratio was almost constant in this subject at 4-24h after the dosage (serum total; 3.7 ± 0.4 : serum free; 10.0 ± 0.7).

Discussion

It has been reported that salivary stimulation method may modulate significantly salivary pH, flow rate and composition and subsequently modify the saliva to serum or saliva to plasma drug concentration ratio³⁻⁶). Therefore, more fundamental investigation for the effect of stimulation methods on salivary pH, flow rate and composition is required before saliva samples are used efficiently instead of blood samples for drug monitoring. In the first place of the present study, effect of four stimulation methods on pH, flow rate and composition of mixed saliva collected within the appointed one-hour was examined in detail.

Each of pH, flow rate and composition (total protein, K^+ and creatinine levels) of the mixed saliva was significantly different among methods. The analysis of variance for these four stimulation methods suggested the possibility that drug concentration in mixed saliva might be influenced by stimulation method. Namely, stimulation method itself would be of substantial importance for the variation of the saliva to serum (or saliva to plasma) drug concentration ratios.

It has been reported that the reliable use of salivary concentration data in the therapeutic drug monitoring may be dependent on the magnitude of the inter-individual

Table 1. pH, Flow Rate and Composition of Mixed Saliva by Four Stimulation Methods

Stimulation Method	pH	Flow Rate (ml/min)	Total Protein (mg/ml)	Na ⁺ (mEq/l)	K ⁺ (mEq/l)	Cl ⁻ (mEq/l)	Creatinine (mg/dl)
A	6.65 ± 0.19	0.51 ± 0.17	3.51 ± 0.74	7.5 ± 3.2	20.2 ± 2.7	13.7 ± 2.3	0.31 ± 0.06
B	7.01 ± 0.21	1.43 ± 0.49	2.82 ± 0.84	24.0 ± 18.2	19.3 ± 2.1	21.4 ± 9.4	0.27 ± 0.05
C	6.92 ± 0.24	1.31 ± 0.76	1.96 ± 0.49	13.9 ± 13.8	16.6 ± 2.2	13.5 ± 10.0	0.19 ± 0.03
D	6.73 ± 0.21	0.85 ± 0.27	2.32 ± 0.46	16.3 ± 11.8	19.4 ± 1.9	16.8 ± 7.6	0.40 ± 0.05

Each value represents the mean ± S.D. of 10 subjects.

A: Move mouth and tongue.

B: Chew Sealon film.

C: Smell at lemon.

D: Move mouth and tongue after gustatory stimulation with a drop of citric acid solution.

Table 2. Analysis of Variance for Four Stimulation Methods

	Sum of Squares		Mean Squares		F-Ratio
	among	within	among	within	
pH	0.84	1.63	0.28	0.05	6.15 ^{a)}
Flow Rate	5.39	8.37	1.80	0.23	7.73 ^{a)}
Total Protein	13.52	15.33	4.51	0.43	10.58 ^{a)}
Na ⁺	1394.28	6033.50	464.76	167.60	2.77
K ⁺	74.53	186.31	24.84	5.18	4.80 ^{a)}
Cl ⁻	406.33	2274.95	135.44	63.19	2.14
Creatinine	0.24	0.09	0.08	0.003	31.25 ^{a)}

Degree of freedom is 3, 36.

a) Significant at $p < 0.01$.

Table 3. Inter-Individual Variation in pH, Flow Rate and Composition of Mixed Saliva by Four Stimulation Methods

Stimulation Method	Coefficient of Variation (%)						
	pH	Flow Rate	Total Protein	Na ⁺	K ⁺	Cl ⁻	Creatinine
A	2.9	33.3	21.1	42.7	13.4	16.8	19.4
B	3.0	34.8	29.8	75.8	10.9	43.9	18.5
C	3.5	58.0	25.0	99.3	13.3	74.1	15.8
D	3.1	31.8	19.8	72.4	9.8	45.2	12.5

A: Move mouth and tongue.

B: Chew Sealon film.

C: Smell at lemon.

D: Move mouth and tongue after gustatory stimulation with a drop of citric acid solution.

Fig. 1. Diurnal Variation of pH, Flow Rate, Total Protein and Na⁺ in Mixed Saliva

Each point and vertical bar represent the mean and S.D. of 10 subjects. Arrow indicates the time of breakfast and lunch. Salivation was stimulated by method A.

Fig. 2. Diurnal Variation of K⁺, Cl⁻ and Creatinine in Mixed Saliva

Each point and vertical bar represent the mean and S.D. of 10 subjects except creatinine (7 subjects). Arrow indicates the time of breakfast and lunch. Salivation was stimulated by method A.

Table 4. Analysis of Variance for Nine Collection Times

	Sum of Squares		Mean Squares		F-Ratio
	among	within	among	within	
pH	0.33	3.98	0.04	0.05	0.85
Flow Rate	0.48	2.86	0.06	0.04	1.71
Total Protein	4.17	47.52	0.52	0.59	0.89
Na ⁺	174.51	1524.35	21.81	18.82	1.16
K ⁺	191.04	1353.54	23.88	16.71	1.43
Cl ⁻	761.02	2639.10	95.13	32.58	2.92 ^{a)}
Creatinine	0.04	0.13	0.005	0.002	2.14 ^{b)}

Salivation was stimulated by method A.

Degree of freedom is 8, 81 except creatinine (8, 54).

a) Significant at $p < 0.01$.

b) Significant at $p < 0.05$.

Fig. 3. Serum and Saliva Levels of MEX after Single Oral Administration in One Subject

Dose: 200mg as MEX. Saliva stimulation: method A.

Key: ○, serum total; ●, serum free; ▽, saliva

variation in the saliva to serum (or plasma) concentration ratio values¹⁻³). Larger inter-individual variations of salivary pH, flow rate and composition may produce larger inter-individual variation of saliva to serum (or plasma) drug concentration ratios. Among them, salivary pH is one of the most important determining factors for the salivary concentration of ionizable drugs according to Martin's equation based on the pH-partition hypothesis¹⁵). Salivary protein level might be another important factor. As shown in Table 3, method A yielded the smallest inter-individual variation of pH and relatively small inter-individual variations of total protein and Na⁺ levels. Therefore, it was proposed that method A might be the most useful method to stimulate salivation among the present four methods and this method was employed thereafter.

Dawes has reported that unstimulated whole saliva (i.e. mixed saliva) and stimulated parotid saliva showed significant circadian rhythm either in the flow rate or in the compositions (protein, Na⁺ and K⁺ and Cl⁻ levels)¹⁶). As regards method A, we then investigated the diurnal variation of these factors. The diurnal variations of pH, flow rate and major components (total protein, Na⁺ and K⁺ levels) was not observed at all. These

results suggest that method A for salivation may be the simplest and most suitable method for therapeutic drug monitoring by using saliva samples.

Therapeutic monitoring of serum (or plasma) MEX levels has been required due to relatively narrow therapeutic range of this drug¹⁰⁾. Beckett and Chidomere¹⁷⁾ have reported that the salivary concentration of MEX is higher than the corresponding plasma concentration. The results in the present pilot study were consistent with those by Beckett and Chidomere and showed almost constant ratio of saliva to serum MEX levels over the wide concentration range. As is obvious from the present results, method A was thought to be well applicable to the fundamental studies on the saliva to serum concentration ratio of MEX.

In conclusion, to move mouth and tongue was the most suitable method to stimulate salivation for use of the saliva as a biological sample. Furthermore, this method was found to be useful for the detailed investigation to predict or estimate serum MEX level from the salivary level.

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