An Oral-Diffusion-Sink Device for Extended Sampling of Multiple Steroid Hormones from Saliva
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Performance of a new oral-diffusion-sink (ODS) device for sampling the steroid hormones cortisol, progesterone, estradiol, and testosterone from human saliva was tested in vitro. The tested device differed from an earlier ODS sampler by using a polymeric composition of β-cyclodextrin rather than an antiserum to bind analytes diffusing into the device. The capacity of the cyclodextrin-driven device to accumulate the steroid hormones from a simple buffer, human saliva, and saliva fortified with high concentrations of glucocorticosteroids was evaluated. Further, the ability of this device to accurately register the average analyte concentration resulting from treatments simulating physiological episodic secretion events was tested. Each steroid showed a characteristic rate of uptake that was unaffected by saliva or high physiological concentrations of competing steroids, and ODS uptake resulting from temporally varied concentrations of the hormone in the medium accurately reflected the time-integrated average concentration. This ODS provides a potential noninvasive and unobtrusive means of sampling the tissue-available concentrations of multiple steroid hormones, with averaging over physiological secretion events.

Additional Keyphrases: noninvasive technology · cycloextrin · glucocorticoids

A sampling device designed to facilitate the noninvasive assessment of adrenocortical activity was recently described (1). This device, termed an oral diffusion sink (ODS), is worn in the mouth and continuously accumulates the compounds of interest as they diffuse into the device along a concentration gradient. A concentration gradient for a particular analyte is maintained by containing inside the device a composition that binds the analyte and thus maintains its free concentration inside the device at a value that is negligible when compared with its concentration in saliva (see reference 2 for further discussion of theory of operation). An antiserum having suitable specificity and binding capacity was used to maintain the concentration gradient for corticosteroids in the device already described (1).

The ODS can be used to obtain a sample that reflects the availability of hormones in saliva over a defined time interval rather than at a particular moment of sampling; it may thus prove especially useful in studies of compounds that are secreted episodically and rapidly cleared. Results obtained with the ODS are in general highly correlated with more traditional time-integrating methodologies (3, 4). The devices are well tolerated by patients and require no attention between installation and recovery; they have been used to monitor glucocorticosteroids in the workplace (5) and during sleep (J.E. Shipley, unpublished observations, 1991). Because ODS devices accumulate only analytes that can diffuse across a dialysis membrane and interact with a specific binder, they are able to selectively accumulate the free form of an analyte, even in the presence of substantial concentrations of protein-associated analyte. Consequently, blood plasma contamination of saliva (even though this can greatly increase the total saliva concentration of some hormones by introducing protein-bound hormones ordinarily excluded from saliva) does not affect the accumulation of hormones by an ODS device (1).

These properties suggest that ODS devices may provide significant advantages in the noninvasive measurement of compounds that are well represented in saliva. The practical application of such devices may be limited by costs and stability concerns attending the use of a substantial quantity of a specific antiserum to maintain the diffusion gradient for each analyte. Antisera are not the only binders suitable for use in this technique. I report here in vitro results suggesting that a polymeric composition of β-cyclodextrin can be used to build an ODS that is able to accumulate from human saliva not only the corticosteroids, but also the sex hormones estradiol (E2), progesterone (P4), and testosterone (T). β-Cyclodextrin is a toroidal or ring-like polymer of seven glucose molecules. A variety of small molecules, including the steroid hormones named above, are able to form stable inclusion complexes involving entry of some part of the small molecule into the space enclosed by the ring of β-cyclodextrin (6). Insoluble copolymers of β-cyclodextrin having similar complex-forming properties have been prepared, and the work reported here was done with one of these insoluble polymers.

The experiments described here addressed the adequacy of the cyclodextrin composition for the purpose of enabling time-integrated uptake of the glucocorticoids and sex hormones from saliva by an ODS device. I did not address the physical chemistry of binding of the various hormonal steroids to the insoluble polymer, or the actual use of cyclodextrin-driven ODS devices in humans, or the validation of data derived from such use by comparison with more commonly used methods. My
objective was to test the cyclodextrin-driven ODS configuration rigorously in vitro and determine whether further in vivo experimentation might be worthwhile. In particular, the relatively broad binding specificity of cyclodextins made it likely that binding of the desired analytes to the polymer might be impaired by competition from other more abundant species present in saliva. Also, it was not clear that binding of the analytes to the polymer would be adequate to maintain the desired concentration gradients, particularly when physiological analyte concentrations were temporally varying. Both concerns were addressed directly by the experiments described. Indeed, the cyclodextrin-driven ODS device described here apparently preserves all the advantages of the antiserum-driven ODS for measurement of cortisol (F) and cortisone (E) described earlier (I) and extends these advantages to the measurement of the sex steroids. This device may be of use in monitoring an even broader range of compounds, corresponding to the binding specificity of the \( \beta \)-cyclodextrin molecule.

**Materials and Methods**

**ODS Devices**

ODS devices were manufactured by Hammersmith Laboratories (Steamboat Springs, CO). Each finished device consisted of a rigid polycarbonate tube with closed ends (\( 2 \times 11 \) mm) presenting 12 diffusion ports, each port comprising a thin membrane of cellulose acetate attached to the inner surface of the tube and sealing a perforation in the wall of the tube. The diffusion ports collectively serve as a rate-limiting barrier to diffusion of substances between the environment of the device and the internal compartment defined by the walls of the device. Each device contained \( \sim 2 \) mg (dry mass) of \( \beta \)-cyclodextrin/epichlorhydrin copolymer (BCD-CP), fully hydrated and enclosed in a volume of \( \sim 20 \) \( \mu \)L. BCD-CP was generously provided by its manufacturer, American Maize Products (Hammond, IN).

Before use, the BCD-CP was washed thoroughly in methanol, dried under reduced pressure, ground to a fine powder in a mortar, and rehydrated with water.

**Reagents**

The steroids F, E, P4, E2, and T were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Tritiated analogs of F, P4, E2, and T were purchased from Amersham (Arlington Heights, IL) and were repurified by HPLC (mobile phase: \( \mathrm{H}_2\mathrm{O} / \mathrm{methanol/acetoni trile}, \) 50/40/10 by vol; stationary phase: \( 2 \times 150 \) mm C\( \beta \), detection by absorbance at \( 240 \) nm) \( \leq \) 4 weeks before performance of these experiments (7).

**Uptake-Rate Comparisons**

The rates of uptake of tritiated F, P4, E2, and T into ODS devices loaded with BCD-CP were determined. Differences in uptake rates among the steroids and effects of the medium (i.e., simple buffer vs saliva) on uptake rates were of interest. In particular, the effect of replacing a simple saliva-like buffer with actual saliva was examined, with the expectation that binding of the steroids to the BCD-CP might be vulnerable to competition or other interference from endogenous compounds of saliva. If such interference were significant in scale, a depression of the steroid uptake rate in saliva as compared with buffer would be expected. Similarly, the effect of adding exogenous authentic E and F to saliva was examined, with the expectation that if the BCD-CP binder had only marginally adequate capacity, competition from the added corticosteroids would depress the rate of uptake of the labeled steroids.

A saliva-like carbonate buffer (buffer C, containing, per liter, 40 mmol of \( \mathrm{NaHCO}_3 \), 15 mmol of KCl, 2.5 mmol of \( \mathrm{NaH}_2\mathrm{PO}_4 \), and 0.1 g of gelatin; degassed by brief exposure to reduced pressure before addition of gelatin) was prepared immediately before each incubation. Freshly collected saliva from one subject and additional saliva collected from volunteers earlier and stored at \( -20 \) °C for \( \leq 3 \) months were used after centrifugation (10 min at 1000 \( \times g \)) to pellet debris. Roughly equal samples of saliva from three to five subjects were pooled for each study.

Solutions of tritiated P4, E2, and T at physiological concentrations (P4, 120 pmol/L; E2, 40 pmol/L; T, 350 pmol/L) were prepared in buffer C, in saliva, and in saliva with added glucocorticosteroids (F, 10 nmol/L; E, 50 nmol/L). For F, a mixture of authentic F with tritiated F was added to a concentration of 27 nmol/L in the same three media. Four 3-mL samples of each solution were placed in small polyethylene scintillation vials supported by a rack in a 37 °C water bath. After 20 min for temperature equilibration, a single ODS device was added to each vial. After 4 h the devices were recovered and briefly rinsed in water, placed in scintillation vials, and covered with 4 mL of scintillation cocktail overnight. Accumulated tritium was measured with a Packard Instrument Co., Inc. (Meriden, CT) 2000 CA liquid-scintillation analyzer. Concentrations of tritiated steroids in the media were measured by determining the activity of replicate 100-\( \mu \)L samples. The activity of each medium was measured before and after incubations to check for nonspecific losses of steroid (e.g., adsorption to vials).

**Pulse Integration Experiments**

The capacity of cyclodextrin-driven ODS devices to accurately register the effects of episodic changes in analyte concentration was tested by comparing the amounts of steroid accumulated during 200-min incubations in media having either constant or temporally varied steroid concentration. Temporal variation was provided by transferring devices among media having low, medium, or high concentrations (defined below) of each steroid, according to schedules designated A, B, and C. All three schedules had in common a start at low concentration and two 40-min exposures at high concentration, with onsets at 20 and 100 min. The schedules differed in the handling of devices at 60 min (offset of first high exposure) and 140 min (offset of second high exposure). In schedule A, devices were returned imme-
Saliva was transferred of the three devices, divided by the product of incubation duration (d, in h) and tritium concentration (x, in dpm/mL), i.e., \( k = C/(d \cdot x) \).

### Table 1. Lack of Effect of Medium on Rate of Uptake of Trittiated Steroid Hormones

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Buffer C</th>
<th>Saliva</th>
<th>Saliva + E,F*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>31.3 (1.11)</td>
<td>29.6 (0.43)</td>
<td>27.8 (1.43)</td>
</tr>
<tr>
<td>P4</td>
<td>54.6 (1.52)</td>
<td>55.7 (1.14)</td>
<td>56.2 (3.33)</td>
</tr>
<tr>
<td>E2</td>
<td>50.3 (1.06)</td>
<td>46.7 (0.76)</td>
<td>49.7 (1.05)</td>
</tr>
<tr>
<td>T</td>
<td>44.5 (2.22)</td>
<td>41.5 (0.22)</td>
<td>41.7 (1.79)</td>
</tr>
</tbody>
</table>

* Uptake constant \( (k) \) is the mean (and SE) tritium activity (C, in dpm) of four ODS devices, divided by the product of incubation duration (d, in h) and tritium concentration (x, in dpm/mL), i.e., \( k = C/(d \cdot x) \).

The results of the pulse-integration experiments are shown in Figure 1. In each case, the increased average concentration of analyte resulting from transfer schedules A, B, and C resulted in increased accumulation of analyte, and the absolute amount of analyte accumulated under these schedules agreed well with values expected on the basis of the corresponding devices incubated under constant conditions. Linear correlation coefficients \( (r) \) between amount of analyte accumulated and average analyte concentration in the buffer ranged from 0.970 to 0.997.

The resolution among schedules A, B, and C was tested by analysis of variance for each steroid. In all cases, even with analysis restricted to the three pulse-treatment groups, there was a statistically significant effect of the transfer schedule \( (F_{(2,9)} > 4.23) \). Post hoc comparison of group means (by Fisher's least-significant-difference test, with \( \alpha = 0.05 \)) indicated that in all cases, the effects of schedule A were distinct from those of schedule C; for T, the intermediate group B result was also distinct from those of A and C.

Although the pulse-integration experiments confirmed reliable registration of the effects of episodic changes in environmental analyte concentration by these ODS devices, the absolute values of the rate constants of steroid uptake in the pulse-integration experiments were uniformly lower than those in the earlier comparisons of buffer vs. saliva as media. This discrepancy was observed at the same time that a malfunction in the water bath apparatus used for temperature regulation in all these experiments was discovered. It is probable that, both because of this mechanical problem and because of the need to remove vials from the water bath during transfers, the average temperature during the pulse-integration experiments was lower than during the experiments comparing media. Consequently, although comparisons within each experiment were unaffected, uptake rates may have varied in a temperature-dependent way between experiments. This source of error potentially affects the absolute rates of steroid uptake tabulated in Table 1, as well as those that can be inferred from Figure 1, but does not affect conclusions concerning the effects of buffer C vs. saliva or the accuracy of pulse integration.
Discussion

The results reported here suggest that an ODS device incorporating a copolymer of β-cyclodextrin can accumulate a variety of hormonal steroids from saliva. The amount of steroid accumulated is a linear function of the average steroid concentration in the medium over the interval of use, even when temporal variations in concentration exceed those expected in vivo. Neither the presence in saliva of endogenous compounds nor the addition to saliva of exogenous steroids appears to affect the rate of uptake of hormonal steroids over a 4-h period. Consequently, this ODS configuration may prove useful in noninvasive measurement of episodically secreted steroid hormones in humans, just as an earlier configuration using an anti-corticosteroid antiserum (1) has proved useful in measuring E and F.

The rate of accumulation of the steroid hormones by an ODS device is small by design. Even for the fastest-diffusing steroid (P4), the amount of saliva effectively cleared of P4 in 1 h is <60 μL; in contrast, the saliva secretion rate at its nadir during sleep is ~50 μL/min (7). Consequently, the ODS is unlikely to significantly deplete the saliva hormone concentrations that it is intended to measure. The low concentrations of the sex hormones in saliva, coupled with the slow rate of uptake of the ODS device, result in accumulations of amounts of the sex hormones in the range 2–50 fmol over 2–3 h (Figure 1). Although these amounts are within the sensitivity range of reported immunoassays, validation of such assays for use with these devices is clearly prerequisite to their effective use. Accumulations of the more abundant glucocorticosteroids are also sufficient for chromatographic analyses (11, 12). A chromatographic analytical method (7) was used to confirm that
E (of which no tritiated analogue is available) was taken up the cyclodextrin-driven ODS device at a rate similar to that for F.

β-Cyclodextrin is a less-specific binder of small molecules than a typical antiserum is, yet its epichlorhydrin copolymer appears adequate for the uses described here. This is understandable in terms of the relatively greater binding capacity of β-cyclodextrin: each toroidal molecule (molecular mass 1136 Da) is able to make an inclusion complex with a molecule almost a third its size. Considered in this light, the ~2 mg quantity of BCD-CP contained in an ODS device represents micromoles of binding sites (greater precision in characterizing the number of binding sites would require data on the number of active sites per milligram after polymerization, which are not yet available for this material). Binding sites are thus present in great excess compared with the picomoles or femtomoles of analyte being considered here. Apparently, this abundance of binding sites is sufficient to simultaneously accommodate all the species in saliva that are able to bind, because adding supraphysiological concentrations of E and F (species able to bind to BCD) to saliva did not appear to impair the uptake of any labeled steroid. If the added E and F had effectively competed for a limited number of binding sites, the result would have been increased concentrations of the labeled steroids inside the ODS device and consequently shallower concentration gradients and lower net rates of uptake.

It is interesting to ask what other compounds might potentially be accumulated from saliva by a cyclodextrin-driven ODS device. In general, small cyclic or polycyclic compounds without strong polar groups tend to form cyclodextrin inclusion complexes (6, 12). As reported here, even a molecule as large and polar as F is bound adequately to enable time-integrated accumulation by an ODS. The hormone melatonin (N-acetyl-5-methoxytryptamine) might be expected to behave similarly and, moreover, can be usefully measured in saliva (8). A variety of drugs, pesticides, and other exogenous compounds have both a cyclic structure and lipophilicity compatible with binding to cyclodextrin (6) and are well represented in saliva (9, 13). Further experimentation may establish whether a cyclodextrin-driven ODS is advantageous in monitoring these compounds.

Sampling hormones with the ODS provides several advantages, among them freedom from artifacts caused by blood contamination of saliva (1), ability to sample without interrupting work (6) and sleep (J.E. Shipley, unpublished observations, 1991), and temporal integration comparable with much more invasive methods (3, 4). Along with other methods focusing on saliva, ODS provides an emphasis upon the tissue-available concentration of the hormones, even in the presence of much larger concentrations of hormones reversibly bound to specific binding globulins (9). The cyclodextrin-driven ODS configuration has not been used in vivo. However, the use of an insoluble cyclodextrin polymer rather than antisera in construction of ODS devices may provide significant advantages of lower cost and greater storage stability. This may extend the general utility of the ODS technology in a variety of experimental settings.

The author is the inventor of the described technology and holds proprietary rights to it under US patents 4594326, 4792307, and others pending.

References