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Evaluation of the Salivette as sampling device for monitoring β-adrenoceptor blocking drugs in saliva

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Abstract

The Salivette® was evaluated with a range of racemic β-adrenoceptor blocking drugs with different lipophilicity. Recovery from the Salivette appeared to be independent of the stereochemical configuration of the drugs but a significant loss of drug due to the Salivette was observed for all tested drugs. The performance of the method, in terms of accuracy and precision, fitted well within the generally accepted criteria for validation, except near the limit of quantification. The Salivette is successfully used for quantitating salivary β-blocking drugs.

1. Introduction

Over the last few years, a lot of research has been done to develop a methodology that could solve the problems that occur when saliva is used for non-invasive qualitative and quantitative drug monitoring. Several aspects of saliva drug monitoring have recently been summarised in a review [1].

Being readily accessible and collectable, saliva shows many advantages over classical biological fluids such as blood and urine [1]. Pretreatment of saliva in analytical procedures is often easier and faster than pretreatment of blood, serum or plasma. Detection of drugs in urine is possible roughly 12 h after use of the drug. In saliva low levels of drugs may already be detected within one hour after use, depending on the dose and kinetics of the drug used. Therefore, saliva is more amenable to pharmacokinetic interpretations, monitoring of drug abuse and therapeutic drug monitoring.

Modern and easy to handle equipment for the collection of saliva has been developed in the past decade. The Oral-Diffusion-Sink® device is an example. This device is worn in the mouth and continuously accumulates the compounds of interest as they diffuse into the device along a concentration gradient [2]. Thus, with the Oral-Diffusion-Sink device samples are collected over a defined time-interval, whereas with devices capable of rapidly collecting the compounds of interest, such as the Salivette described in the present paper, samples are taken at a certain moment in time. Cooper et al. [3] and May et al. [4] were the first to use a dental cotton roll to

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collect saliva for monitoring desipramine. Over the years their method has undergone improvements and the dental cotton roll has been developed into what is nowadays called the Salivette [5].

The collection devices have proven to be useful for monitoring of several substances. Ethanol, theophylline and cortisol have been collected with the Salivette [5–7]. Corticosteroids have been collected over a defined time-interval with the Oral-Diffusion-Sink device [8]. Progesterone, phenytoin, carbamazepine and testosterone have been collected with the ultrafiltrate collector [9–12]. The OraSure®, mainly developed for the collection of immunoglobulines, was also used for the collection of cotinine in oral samples [13].

Until now, no study is available evaluating the Salivette for monitoring β-adrenoceptor blocking drugs. In this study the recovery from this collection device was studied with β-adrenoceptor blocking drugs as model compounds. Because β-blocking drugs are available as racemic mixtures and because they differ markedly in lipophilicity and only slightly in pK_a and molecular mass, the effect of lipophilicity and stereochemical configuration on the recovery from the Salivette could be examined. To prove that concentrations of β-blocking drugs can be adequately measured with the Salivette method validation was started with propranolol even despite its low recovery from the Salivette.

2. Experimental

2.1. Materials and reagents

All enantiomers of the β-blocking drugs and their racemates were kindly supplied by their corresponding manufacturers: atenolol and propranolol hydrochloride (ICI Holland, Ridderkerk, Netherlands), metoprolol tartrate and alprenolol hydrochloride (AB Hässle, Mölndal, Sweden), nadolol (Bristol-Myers Squibb, Woerden, Netherlands), pindolol (Sandoz, Uden, Netherlands) and tertatolol hydrochloride (Servier, Leiden, Netherlands). Standard solutions of racemic β-blocking drugs and their separate enantiomers were prepared (0.1, 1.0, or 10.0 mg/l in methanol) and kept at 4°C.

N-Hexane, diethylamine, 2-propranol and methanol (Baker analysed HPLC-reagent) were purchased from JT Baker Chemicals (Deventer, Netherlands). Ethanol absolute (Grade Reagent) and borate buffer pH 9.0 were obtained from Merck (Darmstadt, Germany). Acidified methanol was prepared by mixing 0.3 ml of 96% acetic acid (Merck, Darmstadt, Germany) with 50 ml of methanol. Acetonitrile (HPLC grade) was obtained from Biosolve (Barneveld, Netherlands).

Bond-Elut® C_2 solid-phase extraction (SPE) columns (100 mg/1.0 ml) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). The vacuum manifold was purchased from JT Baker Chemicals. Neutral Salivettes were obtained from Sarstedt (Etten-Leur, Netherlands).

2.2. HPLC-equipment and chromatography

The HPLC-equipment consisted of the following components: pump Model 6000A (Waters, Etten-Leur, Netherlands), a Kratos fluorescence detector, Model Spectroflow 980 (Separations, Hendrik-Ido-Ambacht, Netherlands), an injector Model U6K (Waters) with a 2000-μl loop and an LCI-100 integrator Model (Perkin-Elmer, Gouda, Netherlands). The injection volume for the HPLC-analysis was 40 μl. The excitation wavelength was 225 nm, and a cut-off filter of 320 nm was used for the detection of metoprolol, propranolol and alprenolol; a cut-off filter of 290 nm was used for the detection of atenolol, nadolol, tertatolol and pindolol. The stainless steel column used was a Chiralcel OD-H (250 × 4.6 mm I.D.) from Daicel Industries (JT Baker Chemicals). The column temperature was ambient. A RCSS silica guard-pack pre-column (Waters) was used.

The mobile phase consisted of ethanol–*n*-hexane–diethylamine (9:91:0.1, v/v), for propranolol, a mixture of 2-propanol–*n*-hexane–diethylamine (8:91:1, v/v), for metoprolol and alprenolol, and a mixture of 2-propanol–*n*-hexane–diethylamine (50:50:1, v/v), for atenolol,

nadolol, tertatolol and pindolol. The flow-rate was 1.0 ml/min. Prior to use, the mobile phase was filtered and degassed, and the system was allowed to reach equilibrium for at least 12 h.

(*S*)-Alprenolol (10 mg/l) was used as an internal or external standard for the analysis of (*R,S*)-metoprolol, (*R,S*)-propranolol and (*R,S*)-nadolol. (*R,S*)-Metoprolol (10 mg/l) was used as an internal or external standard for the analysis of (*R,S*)-alprenolol. Only the peak of (*S*)-metoprolol was used to calculate the peak height. (*R,S*)-Tertatolol (100 mg/l) was used as an internal or external standard for the analysis of (*R,S*)-atenolol and (*R,S*)-pindolol. Because no separate enantiomers of tertatolol were available the elution order of the two peaks of this drug is uncertain. The second peak was used to calculate the peak height. The peak heights of each enantiomer were divided by those of the internal or external standard and the ratios were plotted as a function of the concentration of the enantiomer added.

2.3. Preparation of drug-free saliva with the Salivette

Drug-free saliva was obtained from healthy subjects with the Salivette. After chewing on a dental cotton roll for about 45 s, it was placed in a container, which was closed with a plastic stopper. The container fitted into a polystyrol tube which was centrifuged for 5 min at about 1000 *g*. During centrifugation about 1.5 ml of nonviscous, easily pipettable saliva passed from the cotton roll into the lower part of the tube. Cellular particles were retained at the bottom of the tube in a small sink compartment. The container was removed from the tube.

2.4. Preparation of quality control and calibration samples

Duplicate samples were prepared in 1.5 ml of saliva, by adding aliquots of the stock solution of (*R,S*)-propranolol to drug-free saliva at three different concentrations: 12.8, 25.7, and 38.6 n*M* of each enantiomer to determine the accuracy and precision of the method. These samples are named quality control (QC) samples. The spiked saliva was allowed to soak into the neutral cotton roll, which was then placed in the container, and centrifuged for 5 min at about 1000 *g*. The duplicate QC-samples were mixed and frozen at −20°C.

Calibration samples containing 12.8, 19.3, 25.7, 32.1, 38.6 n*M* of each enantiomer of propranolol were prepared. The calibration samples were treated in the same manner as the QC samples. For each validation run QC samples were thawed and extracted.

2.5. Extraction procedure for saliva

Samples of 1 ml of the saliva were extracted after the addition of 50 μl of the internal standard solution according to a solid-phase extraction (SPE) procedure. SPE was performed according to the manufacturer's recommendations. The columns were conditioned by eluting twice with 1 ml of methanol, once with 1 ml of demineralized water and once with 1 ml of borate buffer. The saliva samples were added to the columns and suction was applied. The columns were washed once with 500 μl of demineralized water and once with 500 μl of acetonitrile. Elution of the analytes was carried out using twice 500 μl of acidified methanol. The aliquots were evaporated to dryness under a gentle stream of nitrogen at 60°C and the residues reconstituted in 50 μl of the mobile phase by mixing for 15 s.

2.6. Recovery experiments

Saliva was spiked by adding a known amount of racemic propranolol to drug-free saliva to obtain a total volume of 500 μl. Samples were prepared in duplicate. One of the two samples was allowed to soak into the cotton roll followed by centrifugation and extraction. The other sample was directly extracted.

Control samples were prepared by adding a known amount of propranolol to acidified methanol to obtain a total volume of 1000 μl. These control samples were not extracted, but directly evaporated to dryness under a gentle

stream of nitrogen at 60°C and the residues reconstituted in 50 μl of the mobile phase by mixing for 15 s. An external standard instead of the internal standard was added to all of the samples before the samples were evaporated to dryness.

Recoveries were determined by comparison of the SPE fraction of each enantiomer with a control (SPE recovery) and by comparison of the fraction, recovered from the salivette with a control (SPE + Sal recovery).

2.7. Method validation

The accuracy of the method was determined by injection of the propranolol calibration samples and the three different QC samples after extraction on six separate days. All calibration curves were required to have a correlation value of at least 0.990. The accuracy was calculated as a percentage of the nominal concentration: accuracy = $(\text{conc.}_{obs}/\text{conc.}_{nominal}) \cdot 100\%$.

The same data used in the accuracy determinations were used for the calculation of the between-run percentage relative standard deviation (%R.S.D.): R.S.D. = (S.D./mean) · 100%.

The within-run %R.S.D. resulted from analysis of six QC samples at each concentration with injection on the same day.

The detection limit of the HPLC assay (LOD) after extraction was estimated as the drug quantity in saliva which corresponded to three times the baseline noise. The lower limit of quantitation (LOQ) was defined as the quantity of the sample after preparation and extraction which was quantified with a deviation and precision less than 20%.

2.8. Clinical application

One healthy female volunteer (30 years old) took a single gelatine capsule containing 10 mg of propranolol. Duplicate saliva samples were obtained with the Salivette after a written instruction. Subjects had to rinse their mouth two times with water. After the subjects had swallowed the freshly formed saliva, the Salivette was placed in their mouth. The sample was collected according to the manufacturer's recommendations. Before ingestion of the capsule, samples of saliva were obtained for assay blanks. Saliva samples were taken approximately 15 and 30 min, 1, 2, 4 and 8 h after administration. The samples were stored at −20°C until assayed.

2.9. Statistics

Statistical analysis was performed with the SPSS PC$^+$ program (SPSS, Chicago, IL, USA). Linear regression analysis was performed on the standard curves. The results of the recoveries were analyzed with a pooled t-test. The significance level was set at 0.05.

3. Results

The ethanol–*n*-hexane–diethylamine mobile phase allowed the enantiomers of propranolol to be well resolved from each other (mean R_s = 3.46, S.E.M. = 0.16, n = 5). Mean capacity factors for (*S*)-alprenolol, (*R*)-propranolol, and (*S*)-propranolol were 0.84, 1.77, and 2.69, respectively, with a mean hold-up time of 3.83 min. Representative chromatograms shown in Fig. 1 demonstrate that there were no endogenous interferences to any of the peaks of interest with

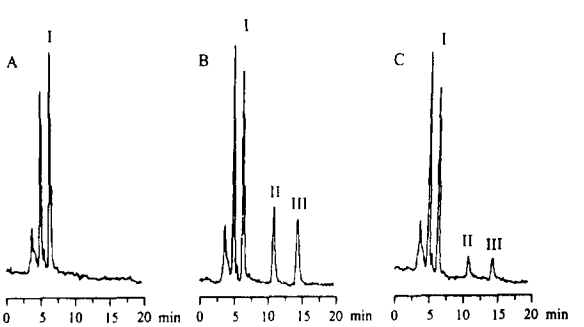

Fig. 1. Representative chromatograms of (A) extracted Salivette sample, (B) extracted Salivette sample spiked with 25.7 n*M* of each enantiomers of propranolol and (C) extracted subject sample. Concentrations in the subject sample were: 16.2 n*M* (*R*)-propranolol, 20.4 n*M* (*S*)-propranolol. Peaks: I = (*S*)-alprenolol (internal standard); II = (*R*)-propranolol; III = (*S*)-propranolol.

drug-free saliva under the detection conditions chosen.

3.1. Recovery experiments

No significant differences ($p > 0.05$) were found between the recoveries of the (*R*)- and (*S*)-enantiomers of each compound. For all tested drugs, however, significant differences ($p < 0.05$) were observed between the SPE recoveries and the SPE + Sal recoveries (mean ± S.E.M.) (Fig. 2).

The recovery from the Salivette appeared to be correlated to the log of the partition coefficient of the drug [14] (Fig. 3). The more lipophilic a drug, the lower the recovery from the Salivette.

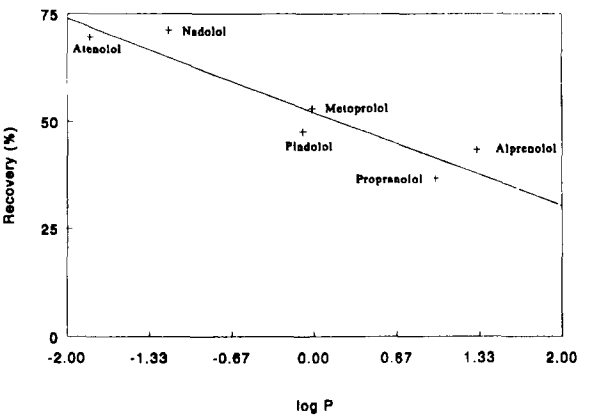

Fig. 3. Salivette recovery as a function of log *P* of different β-blocking drugs. The Sal recoveries were calculated by the equation: Sal recovery = 100%-[SPE recovery-(SPE + Sal) recovery].

3.2. Calibration curves and method validation

The calibration curves of propranolol were linear over the concentration range used, with high correlation coefficients (0.994). The slope and intercept values for (*R*)-propranolol were 0.019 and 0.0079, respectively. The slope and intercept values for (*S*)-propranolol were 0.018 and 0.0078, respectively.

Table 1 summarizes the results of the accuracy and the within- and between-run precision for the enantiomers of propranolol. Except for the lowest QC sample, the between-run precision for the QC samples was less than 20%. The LOD of the method was determined to be 1.33 ng base for each enantiomer of propranolol. The LOQ of the method was approximately 3.00 ng base of each enantiomer of propranolol.

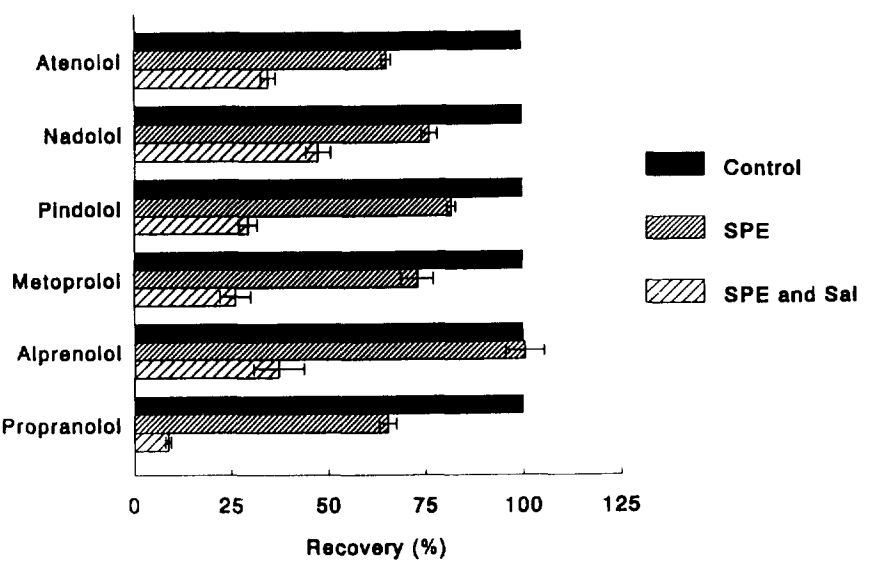

Fig. 2. Mean recoveries ± S.E.M. for the different β-blocking drugs ($n = 4$).

Table 1

Precision and accuracy of the determination of propranolol enantiomers from spiked saliva samples

Compound	Concentration[a] (n*M*)	Accuracy (%)	Between-run precision (%)	Within-run precision (%)	*n*
(*R*)-Propranolol	12.8	114.0	24.1	7.1	6
(*S*)-Propranolol	12.8	99.4	28.3	5.3	6
(*R*)-Propranolol	25.7	95.6	10.8	13.5	6
(*S*)-Propranolol	25.7	99.2	9.7	7.3	6
(*R*)-Propranolol	38.6	102.2	13.5	7.6	5
(*S*)-Propranolol	38.6	107.6	12.1	8.9	5

[a]All concentrations in terms of base.

3.3. Clinical application

The saliva samples from the subject taking racemic propranolol were analyzed using this procedure. The 30-min sample is shown in Fig. 1. The pharmacokinetic disposition of salivary propranolol enantiomers over the 7-h dosing interval is shown in Fig. 4. The concentrations in saliva reached a peak of 6.66 ng/ml and 9.34 ng/ml, respectively, for (*R*)-propranolol and (*S*)-propranolol after 1 h 18 min.

4. Discussion

Salivette sampling devices are promising for easy saliva sample handling. The recovery of drugs or xenobiotics, the influence of lipophilicity or stereochemical configuration and the method validation are important factors in the evaluation of this product not yet published. β-Blocking drugs are relevant as model drugs in this respect. They are an important class of drugs, marketed as racemic mixtures and they can be detected after systemic administration, not only in blood but also in urine, cerebrospinal fluid and in saliva [15–17]. Differences in kinetic and dynamic behaviour between the enantiomers of the β-blocking drugs may potentially be of clinical importance. For instance, it was found that the renal clearance of metoprolol was stereoselective [18].

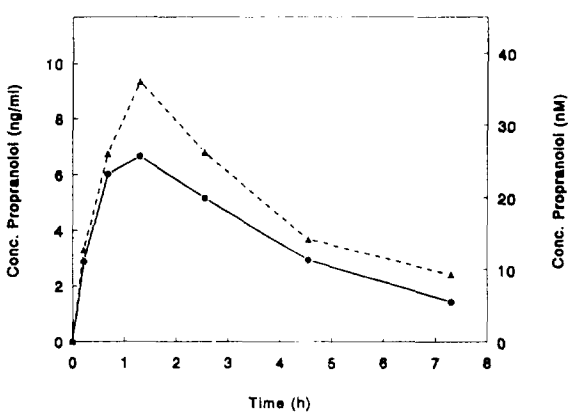

Fig. 4. Saliva concentrations of (*R*)-propranolol (●) and (*S*)-propranolol (▲) from one subject receiving a single gelatine capsule containing 10 mg of propranolol.

A significant loss of drug due to the Salivette was observed for all β-blocking drugs as shown by the recovery experiments. The recovery of the drugs appeared to be independent of the stereochemical configuration of the model compounds but it appeared to be correlated with the partition coefficient of the drug. Atenolol is the most hydrophilic β-blocking drug; the highest recovery from the Salivette was found for this drug. Propranolol and alprenolol are more lipophilic. For these compounds recovery from the Salivette was lower than for atenolol. Low recoveries may cause difficulties in the measurement of very low concentrations of drugs as may be found in the case of lipophilic drugs. However, most lipophilic drugs are transported better from the blood into the saliva than hydrophilic drugs [1]. Therefore, the loss of lipophilic drugs caused by the cotton may often be partly compensated by the higher saliva concentrations. In

line with these findings ethanol, being relatively hydrophilic, shows no loss due to absorption to the cotton of the Salivette [5]. Slightly higher recoveries of propranolol were obtained after extracting the Salivette with hexane or methanol. However, more interfering peaks were found near the peaks of interest.

The Salivette does not influence the linearity of the calibration curves. The calibration curves for propranolol were linear with high correlation coefficients. The results of the method validation showed that, except for the lowest QC sample, the accuracy and between-run and within-run precision are according to the validation procedures presented at the conference on 'Analytical Methods Validation: Bioavailability, Bioequivalence, and Pharmacokinetic Studies' held in 1990 [19]. The between-run precision for the lowest QC samples was above 20.0%, which is too high for data acceptance criteria. Therefore, if the propranolol concentrations of samples are near those of the lowest QC sample, interpretation of the results should be done very carefully. Converting the LOD from amount to concentration revealed a LOD of 6.43 n*M* for each enantiomer of propranolol under the conditions applied. The LOQ of the method expressed as the concentration was approximately 14.7 n*M* of each enantiomer of propranolol. Most of the salivary samples from the volunteer had a propranolol concentration greater than the LOQ (Fig. 4). However, three samples–at 15 min, 4 h 33 min and 7 h 18 min–have concentrations that are below the LOQ and therefore are unreliable, although the sensitivity is sufficient for therapeutic drug monitoring. In a clinical situation the propranolol doses and concentrations are much higher than in this study with a used non-therapeutic dose of only 10 mg.

The advantage of the Salivette over many other sampling devices, is that it absorbs reproducibly a relatively large volume of saliva (1.5 ml) in a short time. The OraSure absorbs only 1.0 ml and, moreover, collects a mixture of gingival crevicular fluid and saliva, rather than saliva alone, since the pad is placed between the cheek and the gums. The term "oral sample" is used rather than saliva [20]. The sampling time of the Salivette is very short in comparison with that of the ultrafiltrate collector (45 s vs. 8 min). This is attractive for frequent sample collection. An advantage of the ultrafiltrate collector is that it eliminates the problem of blood contamination since protein-bound molecules are excluded with this sampling technique [12]. However, a major disadvantage of this device is that the density of the liquid after collection has to be determined because the ultrafiltrate contains a high concentration of sucrose. Sucrose is used as the osmotic driving force. To correct for the actual concentration of analytes in the ultrafiltrate, a correction factor, derived from the density of the solution, was calculated [10].

The results found in a representative volunteer, as shown in Fig. 1, demonstrate that the method is useful for quantitation of the enantiomers of propranolol in saliva.

4. Conclusions

The use of the Salivette as a non-invasive technique simplifies the collection of a body fluid by being readily accessible and lessening the risk of infection. It allows rapid sampling, is well tolerated, it reproducibly soaks sufficient amounts of saliva, it does not interfere with the linearity of the HPLC calibration for the model compound, does not show a dependency on the stereochemical configuration, although it might result in reproducibly lower recoveries of the compounds of interest, depending on their lipophilicity. However, it was demonstrated that even with a low dose of propranolol and despite the low extraction recoveries the enantiomers of propranolol could still be adequately measured with the Salivette. This device provides new opportunities for diagnostic purposes and drug monitoring in saliva.

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