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Saliva-catalyzed hydrolysis of a ketobemidone ester prodrug: Factors influencing human salivary esterase activity

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Summary

Saliva enzyme-catalysed hydrolysis of ester prodrugs or drugs containing sensitive ester groups may be a limiting factor for the buccal absorption of such compounds. Using the isopropyl carbonate ester of ketobemidone as a model substance of a hydrolysis-sensitive prodrug the esterase activity of human saliva has been characterized as a function of various factors. The esterase activity was found to decrease rapidly upon storage of the saliva at 37°C. The activity increased with increasing pH in the range 4 5-7.4 and with increasing salivation flow rate up to a rate of 0.9 ml min^{-1} Under resting conditions, the flow rate was about 0 2 ml min^{-1} which implied a greatly decreased esterase activity. The activity was highest after fasting and decreased after intake of a meal The intraindividual variation in the saliva esterase activity was small whereas a larger interindividual variation was found

Introduction

Absorption of drugs through the mucosal epithelium of the oral cavity is gaining increasing popularity as a route of drug delivery. It is especially useful if peroral absorption is incomplete due to first-pass metabolism in the gut or liver or when more rapid absorption is desired relative to the peroral pathway (Motwani and Lipworth, 1991).

The buccal mucosa has been shown to contain a high activity of various esterases and peptidases (Garren and Repta, 1988; Zhou and Li Wan Po, 1990) which may limit the absorption of enzymatically labile drugs such as peptides or prodrugs following buccal or sublingual administration. Another absorption-limiting factor for such compounds may be degradation by enzymes in the saliva. Human saliva has been shown to contain a variety of esterases (Chauncey et al., 1954, 1957; Burstone, 1956; Chauncey, 1961; Lindqvist and Augustinsson, 1975; Tan, 1976; Lindquist et al., 1977), mainly carboxylesterases (EC 3.1.1.1) and therefore, saliva-catalyzed degradation of ester prodrugs or drugs containing susceptible ester groups may preclude an efficient buccal bioavailability. Nevertheless, only little attention has apparently hitherto been paid to this factor. Hussain et al. (1988) noted that an ester prodrug of

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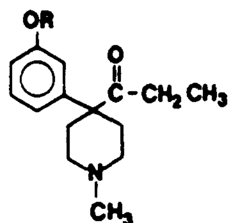
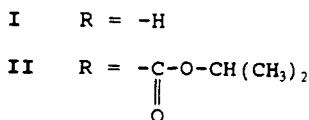
nalbuphine given buccally was tasteless initially, but bitterness developed within 1 min, presumably because of hydrolysis to the bitter tasting parent drug within the fluids of the mouth. With the aim of obtaining a ketobemidone formulation suitable for buccal or sublingual absorption we have recently described some more lipophilic carboxylic acid and carbonate ester prodrugs of this opioid analgesic (Hansen et al., 1991). Most of these ester prodrugs were shown to undergo a rapid enzymatic hydrolysis when incubated in whole human saliva, the half-lives being 3–8 min except for one sterically hindered ester which had a half-life of 5 h.

These findings inspired us to study in more detail various factors possibly influencing the esterase activity of human saliva such as the salivation flow rate, pH, intake of meals and storage of saliva. In addition intra- and interindividual variation in the saliva esterase activity was examined. Besides being a useful step in the development and evaluation of a buccally absorbable ketobemidone prodrug, the present study was believed to provide information that is of general value, e.g., in the evaluation of ester prodrugs and other hydrolysis sensitive compounds for buccal or sublingual administration. The structures of ketobemidone (I) and the isopropyl carbonate ester derivative (II) used in this study are shown in Scheme 1.





Scheme 1.

Materials and Methods

Apparatus

High-performance liquid chromatography (HPLC) was carried out using a system consisting of a Milton Roy constaMetric 3000 pump, a variable-wavelength UV detector type Milton Roy spectroMonitor 3100, a Milton Roy computing integrator model 4000 and a Hitachi Auto Sampler model 655A-40, using an injection volume of 20 μl. A deactivated reversed-phase Supelcosil LC-8-DB column (33 × 4.6 mm) (3-μm particles) equipped with a Supelguard 20 mm precolumn (both from Supelco Inc., U.S.A.) was used. Measurements of pH were performed at the temperature of study using a Metrohn type 632 pH Meter instrument.

Chemicals

Ketobemidone hydrochloride was obtained from H. Lundbeck A/S, Copenhagen, Denmark. The hydrochloric acid salt of the isopropyl carbonate ester of ketobemidone (**II**) was prepared as previously described (Hansen et al., 1991).

Collection of saliva

Whole saliva was collected by expectoration into screw-capped test tubes. Salivation was stimulated by paraffin chewing using a 1.5 g paraffin wax sample with a melting point of about 49°C. The paraffin was moved from one side of the mouth to the other in order to activate all salivary glands (Kerr, 1961). Brushing of teeth was only allowed with toothpaste containing no fluoride and was performed at least 1 h prior to saliva collection since esterases in the saliva can be inhibited by several of the commonly used dental materials (Lindquist et al., 1980).

Determination of esterase activity

The esterase activity of saliva was expressed in terms of the half-life of hydrolysis of the ketobemidone ester **II** when incubated in 80 or 100% whole saliva at 37°C or, in the cases of slow rates of hydrolysis, by the percentage amount of the ester being hydrolyzed following incubation for 20 min. The initial ester concentration was in all cases 7×10^{-5} M. The reactions were initiated by

adding 50 μl of a stock solution of the ester in acetonitrile-water to 3 ml of 80 or 100% whole saliva. The mixtures were kept in a water-bath at 37°C for up to 25 min, and at 3–5-min intervals, samples of 250 μl were withdrawn and added to 500 μl of a 2% solution of zinc sulfate in acetonitrile-water (1 : 1 v/v) in order to stop the reaction and deproteinize the saliva. After mixing and centrifugation for 5 min at 13 000 rpm, 20 μl of the clear supernatant was analyzed for intact ester or ketobemidone by HPLC as described below.

HPLC analysis

A reversed-phase HPLC procedure was used for the quantitative determination of the ketobemidone ester **II** and the parent drug. A deactivated Supelcosil column was eluted with a mobile phase consisting of a mixture of methanol-acetonitrile-0.1% phosphoric acid containing triethylamine (10^{-3} M) in order to improve peak shape. For the analysis of ketobemidone, the composition of the mixture was 5 : 15 : 80 (v/v) whereas it was 5 : 30 : 65 (v/v) for the carbonate ester (**II**). The flow rate was 1–1.5 ml min^{-1} and the column effluent was monitored at 215 nm. Quantitation of the compounds was done by measurement of peak heights in relation to those of standards chromatographed under the same conditions.

Influence of storage of saliva on the esterase activity

Whole saliva (15 ml) was collected from one female subject (29 years) after paraffin chewing before breakfast (salivary flow 1.1 ml/min) and was divided into four equal portions of 3 ml. The samples were kept at 37°C in screw-capped test tubes and at appropriate intervals (1, 30, 60 and 128 min) ester **II** was added and the esterase activity determined as described above.

Influence of salivary flow rate on the esterase activity

Whole saliva (5 ml) was collected each day from one female subject after paraffin chewing before breakfast. The saliva flow rate was varied from one day to another by chewing at different frequencies. Immediately after saliva collection, the esterase activity was determined as described above.

Influence of pH on the esterase activity

The influence of pH on the esterase activity was studied with human whole saliva diluted to 80% with water, 0.05 M acetate buffer of pH 4.5 or 0.05 M phosphate buffer of pH 6.0, 6.8 or 7.4. The saliva (15 ml) was collected from one female subject after paraffin chewing before breakfast, the salivary flow rate being about 1.0 ml/min, and was divided into five equal portions of 2.6 ml. The experiments were carried out in quadruplicate.

Intra- and interindividual variation in saliva esterase activity

Whole saliva was collected from eight individuals (one male and seven females, age 23–36 years) at the same day after paraffin chewing before breakfast, the saliva flow rate being about 1.0 ml/min. The esterase activity was determined immediately after collection of the saliva as described above. Saliva from one of the subjects was obtained in a similar way on nine different days in order to examine the intraindividual variation in esterase activity.

Variation in saliva esterase activity through the day

Whole saliva was collected from two female subjects (age 29 and 36 years) after paraffin chewing before and after breakfast, lunch and dinner. The salivary flow rate was in the range of 1.0–1.2 ml/min. Between the meals the subjects were only allowed to drink water. The standardized meals were given at 9 a.m., 1 p.m. and 6 p.m. The esterase activity was determined immediately after saliva collection as described above. The experiments were carried out in triplicate.

Results and Discussion

Hydrolysis in saliva

The isopropyl carbonate ester of ketobemidone (**II**) was rapidly and quantitatively hydrolyzed to the parent ketobemidone upon incuba-

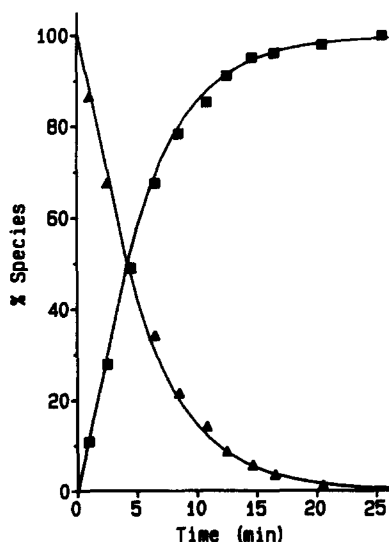


Fig. 1. Time courses for *o*-isopropyloxycarbonyl ketobemidone II (▲) and ketobemidone (■) during hydrolysis at 37°C of the ester in 100% whole human paraffin chewing-stimulated saliva, used immediately after collection. The initial ester concentration was 7×10^{-5} M

tion in fresh whole human saliva at 37°C (Fig. 1). As seen from Fig. 2, the rate of hydrolysis proceeded according to strict first-order kinetics under the conditions used. The great catalytic effect of saliva on the hydrolysis of compound **II** is evident by comparing the half-life in fresh saliva (5.0 min) with that in a pH 7.4 buffer solution (35.6 h at 60°C (Hansen et al., 1991)).

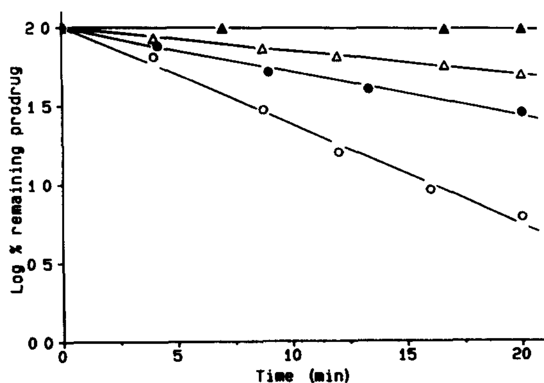


Fig. 2. First-order kinetic plots for the hydrolysis of *o*-isopropyloxycarbonyl ketobemidone **II** in 100% whole human saliva stored at 37°C for 1 min (○), 30 min (●), 60 min (△) and 128 min (▲) before addition of the ester.

TABLE 1

*Influence of the time of storage (at 37°C) of whole human saliva on its esterase activity as expressed in terms of half-lives ($t_{1/2}$) of hydrolysis of the ketobemidone ester **II** or percent hydrolyzed ester upon incubation in saliva for 20 min*

Time of storage (min)	$t_{1/2}$ (min)	% hydrolyzed ester after 20 min
1	5.0	98 8
30	11 3	71 7
60	20.1	50.5
128	~ 250	5.5

As shown by the data in Fig. 1 and Table 1, the esterase activity in human saliva decreases quite rapidly following storage at 37°C. Therefore, in order to imitate the in vivo situation as much as possible all further hydrolysis experiments were performed with saliva immediately after its collection.

Influence of salivary flow on the esterase activity

The influence of the salivary flow on the esterase activity has apparently not been reported before but it is known that the kallikrein activity of parotid saliva is inversely related to the flow rate (Heidland et al., 1979). To obtain information on esterase activity as a function of salivary flow, the stability of the ester **II** was examined in human whole saliva collected from the same subject at varying saliva flow rates at different days. Saliva was collected before breakfast and only one experiment was performed per day since initial experiments showed that the esterase activity varied between two saliva samples collected immediately after each other, the first sample having the highest activity.

The results obtained are depicted in Fig. 3. It is seen that the esterase activity of saliva increases markedly with increasing salivary flow rate up to a rate of 0.9 ml min^{-1} whereas further increase in the salivary flow does not result in changes in the esterase activity. Without stimulation (i.e., under resting conditions) salivation was found to occur at a rate of 0.2–0.25 ml min^{-1}. When sucking movements were made by the tongue and cheeks, salivary flow rates of 0.4–0.6 ml min^{-1} were found. Slow paraffin chewing

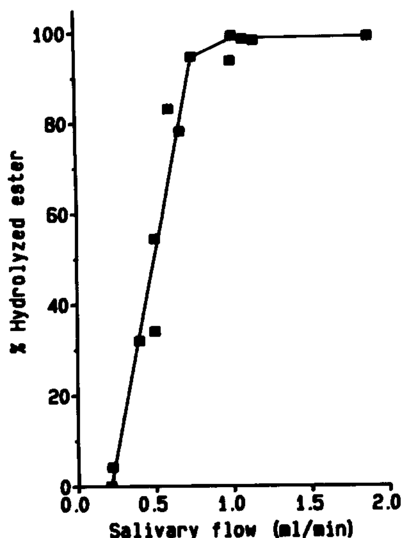


Fig. 3. Influence of the salivary flow rate on the saliva esterase activity as expressed in terms of % hydrolyzed ester **II** following incubation for 20 min in the saliva at 37°C The saliva was obtained from subject 1 and was used immediately after collection

gave flow rates of 0.7–0.9 ml min^{-1} whereas normal paraffin chewing resulted in flow rates of 1.0–1.2 ml min^{-1}. The subject who participated in the experiments in Fig. 3 had a normal saliva secretion rate which is defined as a rate of ≥ 1.0 ml min^{-1} with normal frequent paraffin chewing (Zickert, 1974). It can be concluded that normal frequent paraffin chewing results in high saliva esterase activity (i.e., high prodrug lability) whereas the esterase activity in saliva collected at resting salivary flow is much lower. To ensure no underestimation of prodrug stability in saliva, all further experiments were performed with saliva collected after normal frequent paraffin chewing.

Influence of pH on the esterase activity of human whole saliva

The influence of pH on the esterase activity was studied in whole saliva collected from one subject after normal frequent paraffin chewing and before breakfast. The saliva was immediately diluted to 80% with water or 0.05 M buffer of pH 4.5–7.4. The results obtained (Fig. 4) show that the esterase activity increases with pH from 4.5 to 7.4. The addition of the ester **II** to the saliva samples in the low concentration of 7×10^{-5} M did not change the pH. Freshly paraffin chewing-stimulated saliva was found to have pH values in the range 6.6–7.2. Ericsson (1958) has previously found a pH of 6.7 in saliva collected at resting salivation conditions whereas it increased up to 7.8 at high salivary flows.

Interindividual variations in saliva esterase activity

The esterase activity in freshly paraffin chewing-stimulated saliva from eight individuals was determined and the results obtained are listed in Table 2. The saliva samples tested were obtained before breakfast and after fasting for 10 h. A pronounced interindividual variation in the esterase activity is seen to occur which parallels previous findings of Lindqvist et al. (1980).

Saliva esterase activity through the day

The esterase activity of saliva was examined before and after breakfast, lunch and dinner for two individuals. The results obtained (Fig. 5) show that the activity decreases after a meal and that

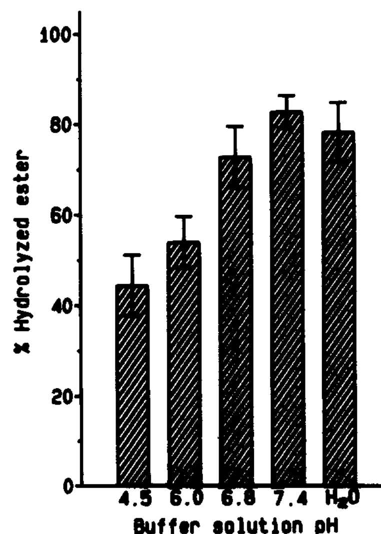


Fig. 4 Influence of pH of saliva upon the esterase activity as expressed in terms of % hydrolyzed ester **II** following incubation for 20 min in the saliva samples (diluted to 80% with a buffer or water) at 37°C. The saliva was obtained from subject 1 and was used immediately after collection. Error bars represent standard deviation (S D.) for $n = 4$.

TABLE 2

Interindividual variation in the saliva esterase activity as expressed in terms of half-lives ($t_{1/2}$) of hydrolysis of the ester II

Subject	Half-life (min)
1 (♀)	4.8
2 (♀)	9.3
3 (♀)	7.1
4 (♀)	7.3
5 (♂)	26.5
6 (♀)	2.7
7 (♀)	4.2
8 (♀)	7.8
Mean [a]	6.2
S.D. [a]	2.3

[a] The value from subject 5 was excluded as he smoked before saliva collection.

Whole saliva was collected by normal paraffin chewing stimulation before breakfast prior to 10 h fasting and used immediately after collection.

the activity for both subjects is highest after 10 h fasting, i.e., before breakfast.

The day-to-day variation of the esterase activity was studied in one subject over a period of 9 days. Saliva was collected before breakfast and the half-lives of hydrolysis of the ester **II** varied from 3.1 to 6.5 min, the mean value being 4.8 ± 1.0 min.

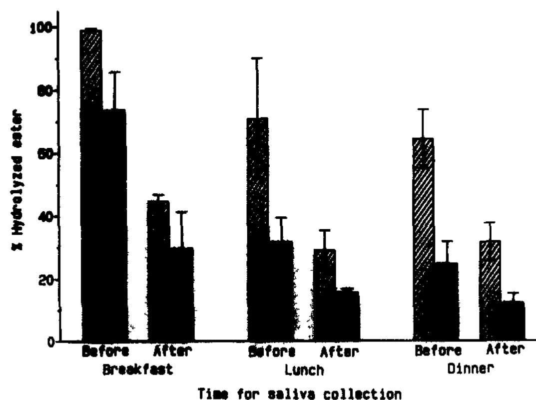


Fig. 5. Saliva esterase activity as a function of time of collection. Saliva was collected from subject 1 (hatched bars) and subject 2 (cross-hatched bars) and was used immediately after collection. Error bars represent standard deviation (S.D.) for $n = 3$.

Conclusions

The results of this study demonstrate the influence of various factors on the esterase activity of human whole saliva. For the assessment of the potential impact of saliva esterase-catalyzed hydrolysis on the buccal or sublingual delivery of hydrolysis-sensitive drugs or prodrugs conditions implying the highest esterase activity must be used. Such in vitro conditions involve the use of fresh saliva, collected before breakfast with prior fasting and by paraffin chewing-stimulated salivation.

Acknowledgements

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