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# RECENT ADVANCES IN DOPING ANALYSIS (3)

M. Donike
H. Geyer
A. Gotzmann
U. Mareck-Engelke
(Editors)

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# Potential role for saliva as a biological specimen in doping analysis[1]

Netherlands Institute for Drugs and Doping Research/ Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht, the Netherlands

## Introduction

From a historical point of view doping analysis started with saliva as biological specimen. Already in 1910 the Russian chemist Bukowski developed a method to detect alkaloids in saliva of horses. The Austrian scientist Fränkel used a similar method in Vienna to conduct 218 exams in the period 1910 to 1911 and to prove a positive case in 1912. Nowadays saliva does not play a role in doping analysis at all.

Currently, the Netherlands Institute for Drugs and Doping Research (NIDDR) is interested in the evaluation of non-invasive techniques for the collection of biological specimens in general. Logically, the NIDDR is also studying the potentials of saliva as a non-invasive technique in the field of doping analysis, especially since for several reasons there seems to be some resistance against the introduction of blood sampling in this field.

## Anatomy and physiology of the salivary gland

The salivary glands can be characterized by their structural heterogeneity and position, resulting in glands with different functions and producing saliva with different compositions. Important salivary glands are the parotid, labialar, submandibular and sublingual glands

[1] A more extensive review will appear in the thesis of K.M. Höld, which will available at the end of 1996.

(Figure 1). All these glands secrete their products into the oral cavity and the 'saliva' in the mouth is thus in fact a mixture of saliva of different origins. Ninety percent of the volume of 'saliva' is produced by the parotid and submandibular glands. In practice 'saliva' also contains gingival fluid and desquamated oral mucosa.

Salivary glands are organs consisting of numerous excretory subunits, the salivons (Figure 2). A salivon is composed of secretory canaliculi opening into the lumen. The acinar cells secrete the initial saliva. The secretion is promoted by the contraction of the myoepithelial cells. The lumen transports the initial saliva to the oral cavity and modifies its water and electrolyte concentrations. The production of saliva by adults is in the range of 500 to 1500 ml per day.

In humans the functions of saliva are: 1) to moisten the mucous membranes of the upper aerodigestive gland, necessary for speech and for controlling the bacterial flora of the mouth; 2) to supply enzymes destined to play an important role in preparing food for digestion; 3) to produce hormones and other pharmacologically active compounds.

## Mechanisms of drug transfer from blood to saliva

The possible routes which may lead to a drug being present in mixed saliva are: 1) passive transcellular diffusion; 2) ultrafiltration; 3) active transport.

Passive transcellular diffusion is the transport of lipid-soluble substances across the capillary wall in the salivon. Examples of substances for which this kind of transport is important are the unconjugated steroids such as oestradiol and the xanthines caffeine and theophylline. For these compounds the salivary concentration approximates the unbound plasma concentration.

Ultrafiltration is the transport of small polar substances through a permeability diffusion barrier. This mechanism is restricted to substances less than 300 Da, although substances of 150 Da are filtered to a limited extent already.

Active secretion has been suggested for those substances of which the ratio of the concentration in saliva to the concentration in plasma (S/P ratio) is relative high. For many electrolytes such mechanisms have been described. Besides the S/P ratio as a parameter to classify transport mechanisms, chirality may also be used as an indicator. A difference in the salivary secretion of (*R*)- and (*S*)-enantiomer may also demonstrate an active transport

mechanism, although no difference in secretion does not exclude active transport. Also interactions with competitors in a transport mechanism may demonstrate active transport.

## Collection and analysis of saliva

The collection of saliva can be achieved by non-stimulated and stimulated spitting and by the use of a collection device. Non-stimulated spitting in f.e. a test tube is for several people and/or patients either a relative uncomfortable way of collecting saliva or even an insufficient way if the person has a dry mouth. The secretion of saliva can be stimulated by chewing f.e. on Parafilm® or adding citric acid to the mouth. A drawback of the use of Parafilm® could be the potential adsorption on the film itself and thus the loss of a significant amount of the compound of interest. Therefore, a study of the extent of adsorption on collection material is always necessary. Citric acid is very effective in stimulating the secretion, but because of its influence on the pH it may change the kind of transport. At least for studying the process of drug transfer into saliva, citric acid must be applied with a correct scientific policy and the results should be interpreted in the right context. However, stimulated or not, spitting keeps to be a relative uncomfortable way of collecting saliva. A more convenient way is the use of a collection device.

Several collection devices can be constructed and some commercially available devices are described in Table 1. The devices collect different volumes of saliva. The main disadvantage of the Omni-Sal® and the OraSure® devices are the limited amounts of saliva which can be obtained in that way. Especially if salivary concentrations are low, a limited amount of saliva requires sensitive detection techniques. With the Salivette® device more saliva can be obtained (1-2 ml), although the nature of the cotton roll can cause significant adsorption of the compounds of interest [1]. This could again eliminate the advantage of a larger volume and may result in apparent low salivary concentrations. However, if necessary the nature of the cotton roll can be changed. Also a Salivette® device with citric acid is available.

The speed of collection may be of importance in studies of transport mechanisms. The shorter the collection time, the more the sample represents a one moment sample on a fixed time. If the time of collection is relative long, the sample is of a cumulative nature. The ODS device has several advantages, but the way of collection is relative slow; 1 ml of ultrafiltrate

in 5 min. Of all devices the use of the Salivette® device is the fastest way to obtain a sample (Table 1).

As a biological sample, the analysis of saliva can be compared to the analysis of serum or plasma samples, although in saliva far less proteins are present, making the analysis less complicated. Protein precipitation is not necessary, although centrifugation is advised to get a clear supernatant saliva free of cellular remains. Because relatively low to very low

Table 1 Selection of some commercially available saliva collection devices

| Collection device | Manufacturer | Description |
|---|---|---|
| Omni-Sal® | Saliva Diagnostic Systems | Special collector is placed under the tongue untill an indicator turns completely blue. Absorption of saliva takes places into cotton pad fixed on the collector. The collector is put into a special vial and ready for laboratory processing. |
| ODS‡ | Hammersmith Laboratories Inc. | Special collector consists of a semipermeable membrane (exclusion limit 12 kDa), which encloses an osmatically active cyclodextrin. After putting the collector into the mouth, an ultrafiltrate of saliva is collected by the osmotic pump function. |
| OraSure® | Epitope Inc. | Special collector is placed between cheek and gum, is rubbed back and forth several times and kept stationary for 2 min. Absorption of saliva takes places into cotton pad fixed on the collector. The collector is put into special vial and ready for laboratory processing. |
| Salivette® | Sarstedt Int. | A dental roll is chewed on for 45 s. Absorption of saliva occurs into the roll, from which the saliva can be obtained after replacement in container and centrifugation in special tube. Clear saliva can be poured out after centrifugation. |

‡ Oral Diffusion Sink

concentrations of the compound of interest can be encountered, the detection technique may be the limiting step. In the past sometimes the lack of correct analytical validation (sensitivity, accuracy and precision) has caused interpretation problems in saliva studies.

With respect to drug transport mechanism studies the NIDDR prefers the Salivette® device over other collection devices considering all advantages and disadvantages. In order to study the secretion of substances in general our research group has chosen the chiral β-blocking drugs as model compounds. These compounds consist of a large group of chiral substances with different physical chemical properties. The selection of β-blocking drugs studied was based on different partition coefficients at similar pKa values (partition

coefficient/pKa value in parentheses); (*R,S*)-atenolol (0.015/9.55), (*R,S*)-metoprolol (0.98/9.7), (*R,S*)-celiprolol (moderate/9.7), (*R,S*)-alprenolol (9.5/9.7) and (*R,S*)-propranolol (20.2/9.45). To illustrate the problem of adsorption of a substances on the cotton roll of the Salivette®, it was shown that the loss was especially significant for apolar β-blocking drugs [1]. In this particular case the loss was no problem in our study and we did not change the nature of the cotton roll [2].

Table 2 Mean S/P ratios of selected β-blocking drugs at two different time periods after administration in several subjects

| β-blocking drug | time period 1 - 2 h | 2 - 4 h |
|---|---|---|
| (*R,S*)-alprenolol | 0.92 | 0.58 |
| (*R,S*)-metoprolol | 4.78 | 2.88 |
| (*R,S*)-propranolol | 1.55 | 0.98 |

The S/P ratios for three β-blocking drugs studied by our group so far are given in Table 2. In the first period of post-administration the S/P ratios are higher than in the second period. This can be explained by a concentration difference between the arterial and venous blood compartments, before the distribution throughout the body has reached an equilibrium [2]. After all, the salivary concentration represents the arterial concentration and the plasma the venous blood concentration (Figure 2). Regarding the transport of β-blocking agents, it can be concluded that, the mean S/P ratio of (*R,S*)-metoprolol indicates a possible active transport mechanism, although no difference was observed for the separate enantiomers. For the other β-blocking drugs no signs of active transport were observed. Compared to data which are available in literature, this study is one of the more extensive examination of S/P ratios of β-blocking drugs. It shows that so-called high S/P ratios obtained by a single observation should be handled with care.

## Potential role for saliva in doping analysis

For several reasons there seems to be some resistance against the introduction of blood sampling in doping control. As a non-invasive technique saliva collection may have some potentials to replace blood sample collection.

Regarding the screening for substances in doping control analysis no complete evaluation is available so far. To many questions have to be answered: 1) what kind of

substances should be effectively controlled?; 2) How much sample is needed and is it possible to obtain an appropriate volume?; 3) What kind of saliva sample is needed? Should it be obtained by stimulation or not?; 4) How easy is it to cheat? Therefore at this stage the introduction of collection saliva is preliminary.

With respect to clinical and pharmacological evaluations of specific cases in doping control saliva sampling may have some useful possibilities. One of those applications is the ketoconazole test. This test is recognized as a powerful test in order to determine whether a high ratio of the glucuronides of testosterone (T) and epitestosterone (E) is of a endogenous or exogenous origin [3]. Part of the evaluation in this respect are the plasma or serum concentrations of some steroid and peptide hormones, which have to be determined. Examples of some steroid hormones, which can be detected in saliva are androstenedione [4,5], cortisol [6,7], cortisone [6,7] and testosterone [4,5]. Concentrations in saliva are not only determined by transport mechanisms. The salivary concentrations of the steroids mentioned are not equal to the free steroid concentrations in plasma or serum. For testosterone for instance, the concentration in saliva is lower than the total concentration in plasma, but greater than the free concentration. It has been suggested that this phenomenon is caused by the 17-hydroxysteroid dehydrogenase activity in the salivary gland [5]. A similar reason has been given to explain the salivary concentrations of cortisol and cortisone [6]. The amount of gonadotropins in saliva however, is in general below the detection limits of available techniques and is probably due to contamination with plasma or gingival fluid [8]. Eventual possibilities for saliva as a biological specimen in the ketoconazole test are thus limited to steroid hormones.

Another evaluation in doping analysis, for which it seems to be useful to collect saliva samples, is a 'xanthine metabolism' test. This test is used by the NIDDR to determine if a high caffeine concentration in urine has a mainly endogenous or exogenous cause. Athletes, who have been declared positive for caffeine and insist on further examination, are invited to the laboratory to fill in a questionnaire and to perform an excretion experiment. That procedure consists of the collection of urine and saliva samples before and after caffeine administration. The protocol is demonstrated in Table 3. The respective urine samples are analyzed for caffeine and paraxanthine and the subsequent saliva samples for caffeine. Because the caffeine concentration in saliva reflects the unbound concentration in plasma (S/P ratio of caffeine is 0.7-0.8 and the unbound fraction is 10%-30% [9]), these data can be used for pharmacokinetic evaluation. A pharmacokinetic evaluation may provide additional

information regarding the disposition of caffeine [10] and is not subjected to as many variations as does a metabolic profiling in urine. Also a large number of literature is available regarding typical pharmacokinetic data. Two examples of caffeine kinetics are given in Figure 3 and 4. At the coming 'Workshop on Doping Analysis' the xanthine metabolism test will be discussed in more details. Up to now the test has only be useful in preventing extensive discussions concerning possible xanthine doping. After offering the possibility to perform such a test two suspected athletes were less interested in further examination procedures.

Table 3 Protocol xanthine metabolism test

| day/time | caffeine administration | collection of samples urine | saliva |
|---|---|---|---|
| 1/ 8:30 | 300 mg | x | x |
| 1/ 8:45 | | | x |
| 1/ 9:00 | | x | x |
| 1/ 9:30 | | x | x |
| 1/10:00 | | x | x |
| 1/10:30 | | x | x |
| 1/11:00 | | x | x |
| 1/11:30 | | x | x |
| 1/14:00 | | x | x |
| 1/16:00 | | x | x |
| 1/20:00 | | x | x |
| 1/22:00 | | x | |
| 2/ 9:00 | | x | |

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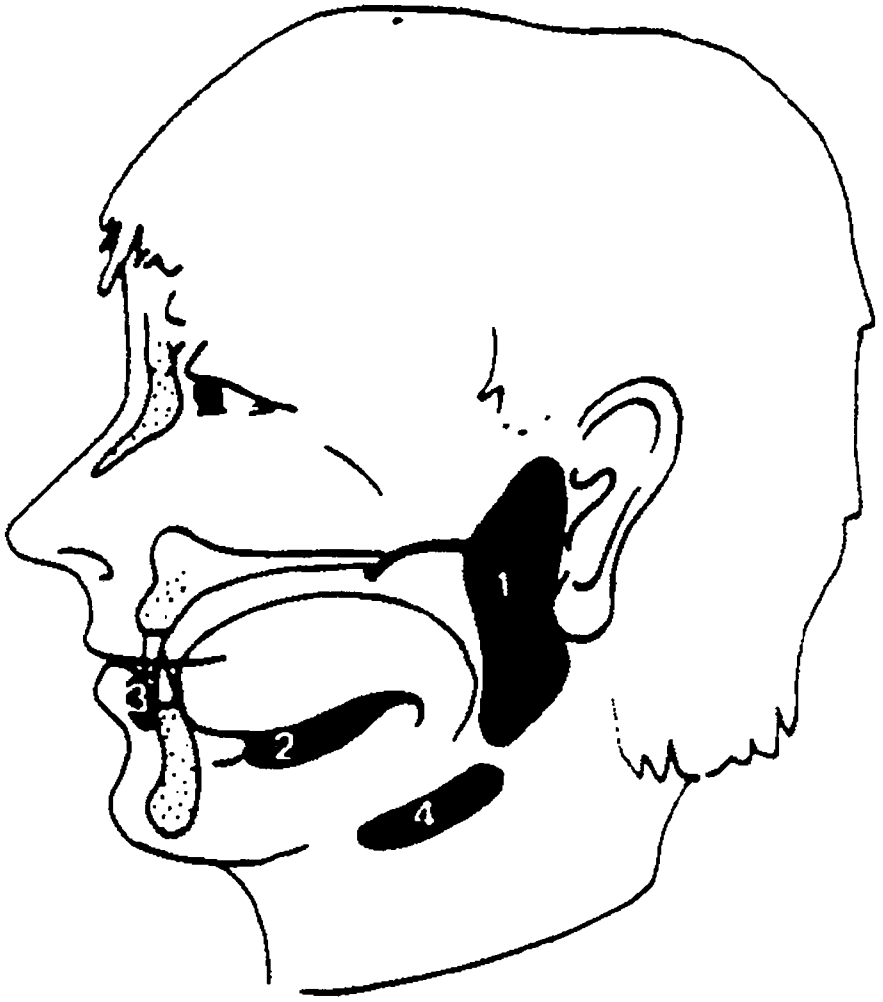



Figure 1: Topography of the salivary glands: 1. glandula parotis; 2. glandula sublingualis; 3. glandula labialis; 4. glandula submandibularis

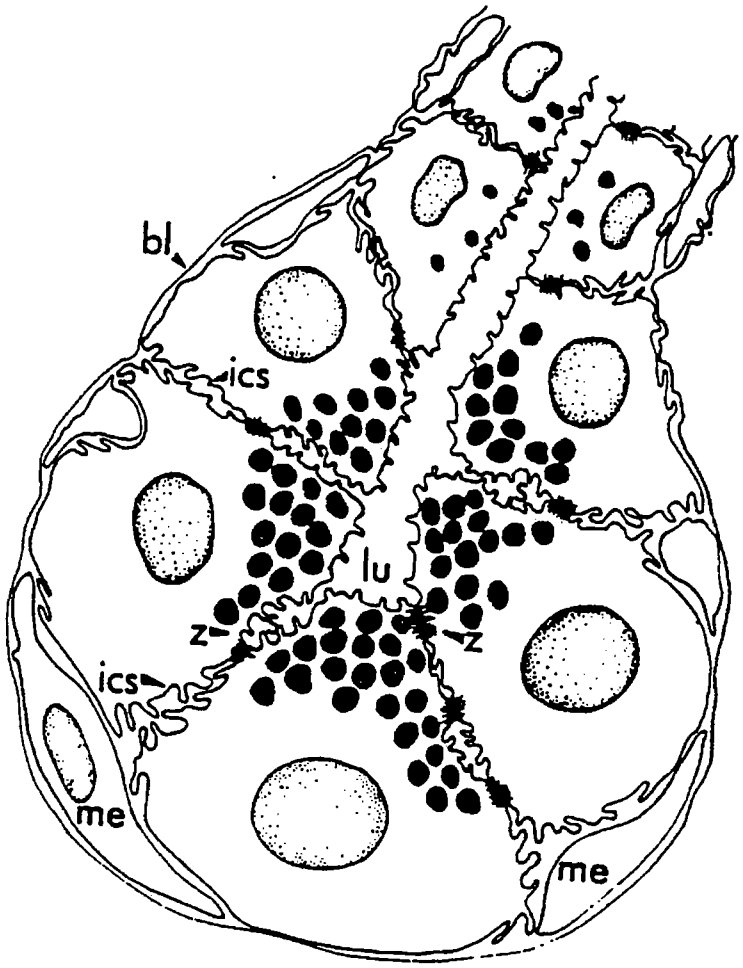
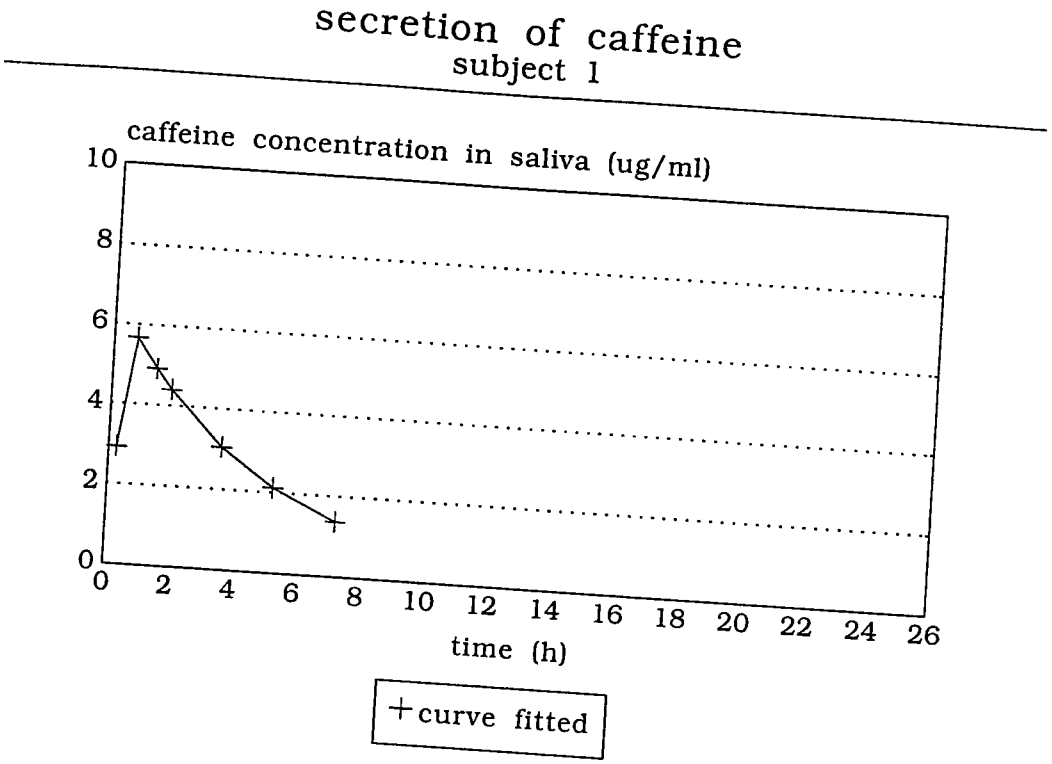



Figure 2: Schematic reconstruction of a salivon, showing secretory canaliculi opening into the lumen (lu). The canaliculi abut tight junctions (z) that separate them from the lateral intercellular spaces (ics). The canaliculi, in contrast to the intercellular spaces, do not surrond the cell on all sides and are seen only occasionally in any particular section. The adjacent acinar cells are coupled by gap junctions (not shown) that permit transcellular exchange of ions and small molecules. The functional and electrical unit is therefore the acinus rather than the individual acinar cells. In additions to the secretory cells, myoepithelial cells (me) are shown.



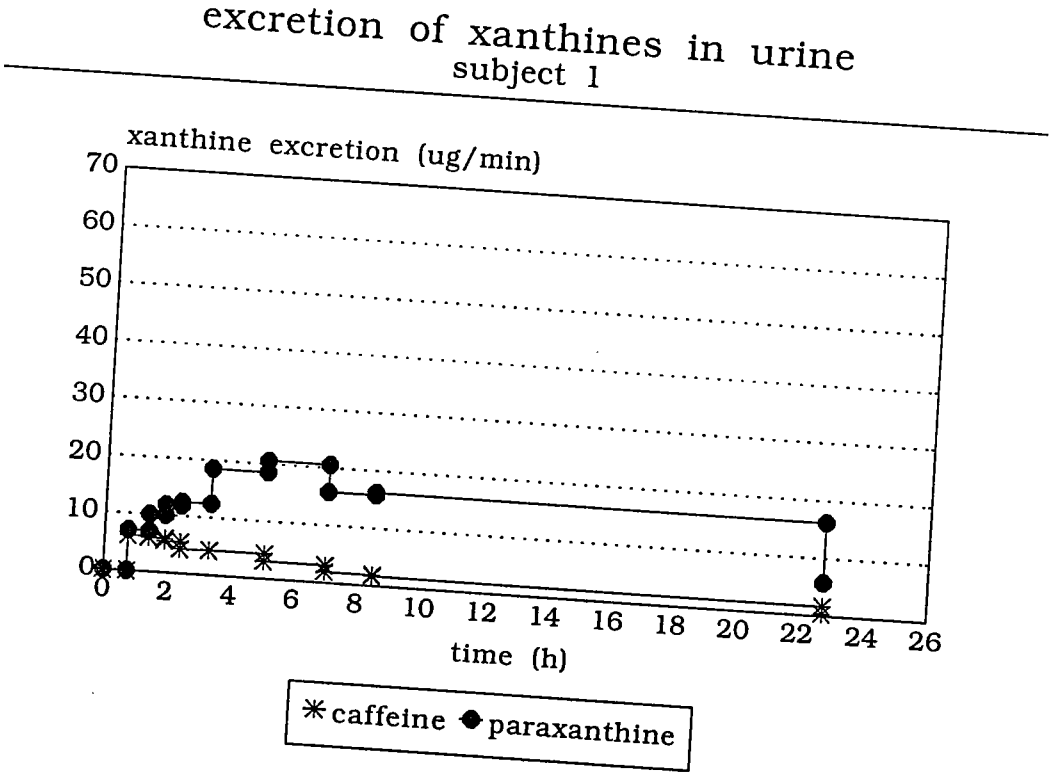
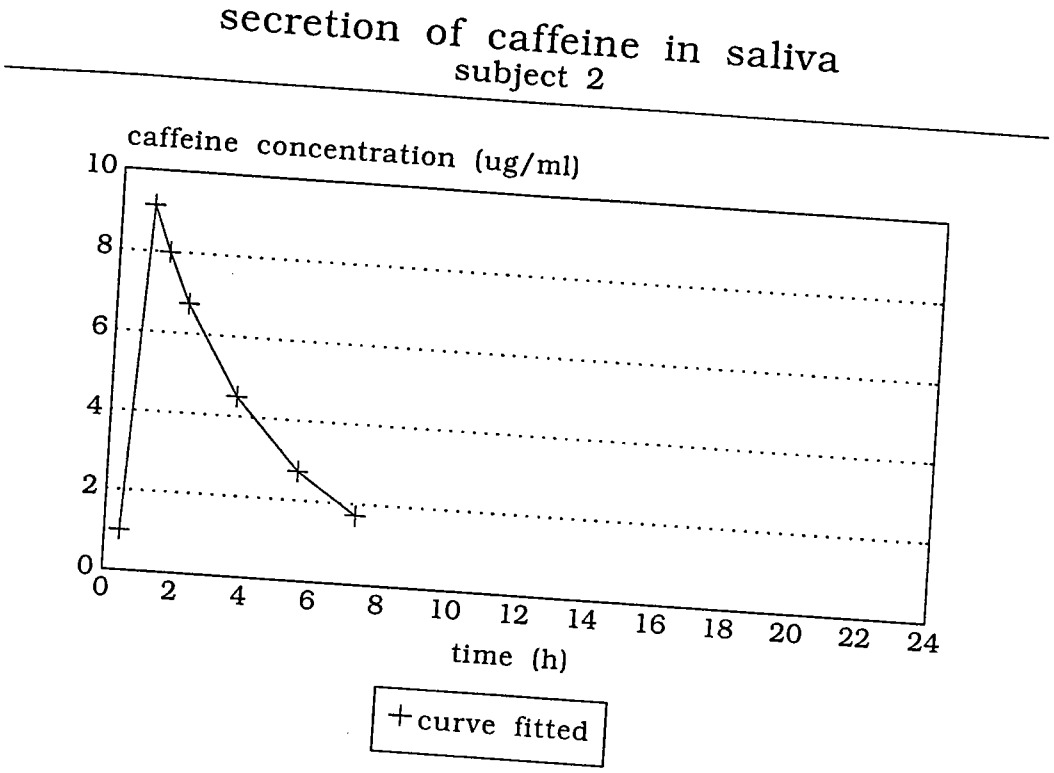




Figure 3: Xanthine metabolism test of subject 1. The secretion curve in saliva is fitted by MW\PHARM cersion 3.02 of MEDI\WARE, Groningen, the Netherlands.



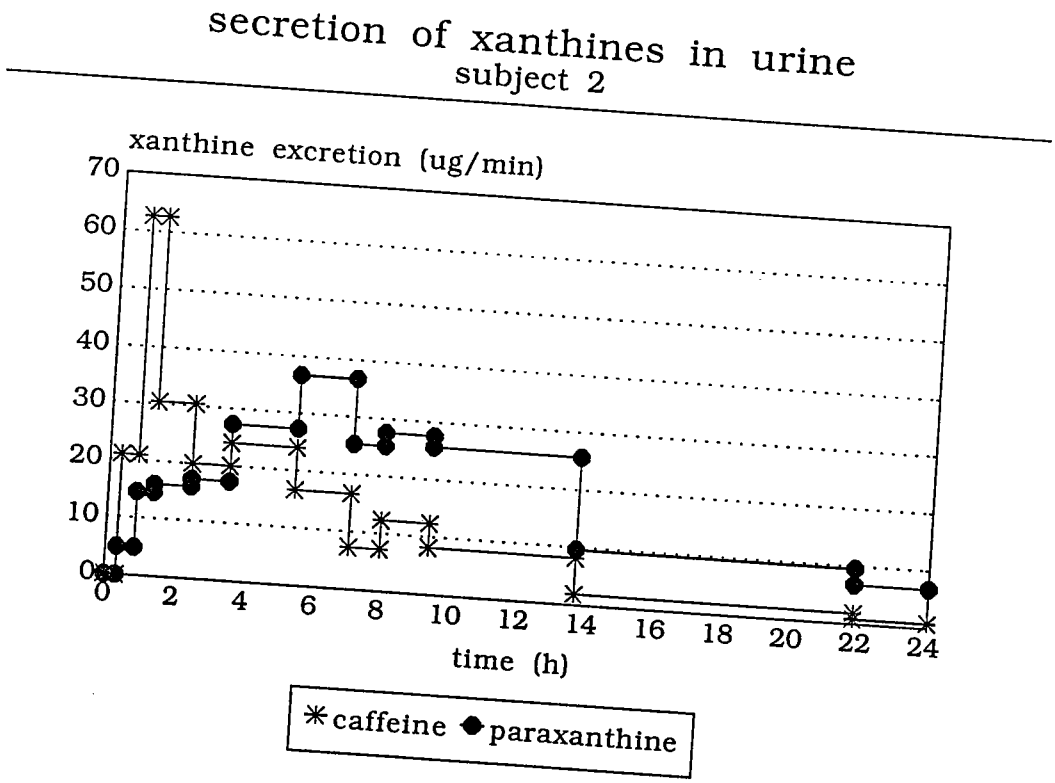




Figure 4: Xanthine metabolism test of subject 2. The secretion curve in saliva is fitted by MW\PHARM cersion 3.02 of MEDI\WARE, Groningen, the Netherlands.