Direct assessment of peripheral pharmacokinetics in humans: comparison between cantharides blister fluid sampling, *in vivo* microdialysis and saliva sampling

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Aims Skin blister fluid sampling, *in vivo* microdialysis and saliva sampling are commonly employed as surrogates for the measurement of drug concentrations in peripheral compartments. Although expected to exhibit comparable results, data derived from these techniques have never been directly compared. Thus, the aim of the present study was to evaluate the comparability of these techniques.

Methods Paracetamol, a model drug with low protein binding, was administered to seven healthy volunteers at an oral dose of 2000 mg. Subsequently, tissue kinetics were measured simultaneously in cantharides induced skin blisters, microdialysates of subcutaneous- and skeletal muscle-tissue and saliva and compared to serum concentrations.

Results Mean ratio ($AUC_{\text{blister}}/AUC_{\text{serum}}$) was 0.88 (95% CI, 0.50–1.26), mean ratio ($AUC_{\text{muscle}}/AUC_{\text{serum}}$) was 1.08 (0.67–1.49), mean ratio ($AUC_{\text{subcutaneous}}/AUC_{\text{serum}}$) was 0.96 (0.41–1.51) and mean ratio ($AUC_{\text{saliva}}/AUC_{\text{serum}}$) was 1.83 (1.39–2.27). In this study the concentration profiles after single oral administration differed among the three methods. The time course of the concentration_{peripheral compartment}/concentration_{serum}-ratios showed that cantharides blister and microdialysate concentrations closely paralleled serum levels. An equilibration period of less than 2 h had to be taken into account for blister measurements. In contrast, saliva concentrations were significantly higher than corresponding serum concentrations.

Conclusions Skin blister sampling and microdialysis closely mirrored corresponding serum concentrations and, thus, proved to be suitable techniques for the assessment of peripheral compartment pharmacokinetics. In contrast, saliva data overestimated the corresponding serum concentrations.

Keywords: skin blister, saliva, microdialysis, human, paracetamol

Introduction

In many cases, a pharmacological effect is dependent on the free, unbound drug concentration reached in the interstitial space of a peripheral target organ [1–3]. Thus, the interstitial space is frequently regarded as the true effect compartment [4].

To describe interstitial compartment pharmacokinetics and to relate interstitial pharmacokinetics to pharmacodynamics [4–6], a limited number of techniques, such as the skin blister fluid method [7, 8], microdialysis [5] and saliva sampling [9–11], have been developed. These techniques are expected to provide a surrogate for estimating the free, non-protein bound drug fraction in the interstitial space fluid. Moreover, they share the advantage of being easy to perform, minimally invasive and cheap and they are believed to yield comparable results. However, these techniques have never been directly compared.

The aim of the present study was to evaluate the different methods by measuring paracetamol concentrations following single dose administration in cantharides induced skin blisters, microdialysates of muscle- and subcutaneous tissue and saliva and comparing these concentrations with serum levels. Paracetamol was chosen as a model drug, because it was previously shown to be freely diffusible into the interstitial space fluid *in vivo* [5] and, at therapeutic concentrations, to exhibit low protein binding [12]. Thus, given unrestricted diffusion, the free concentration reached in the interstitial compartment is almost identical to corresponding serum concentrations [5].

Methods

Subjects

Seven healthy, normal weight (64–94 kg), drug free, male volunteers, 20–37 years old, were studied. Health was defined as the absence of a detectable disease as described by normal results from history, physical examination, ECG,

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blood pressure, liver- and kidney function tests, complete blood count, hepatitis virus serology and HIV test. Subjects were excluded if they were taking any prescription or OTC drugs. The study was approved by the local ethics committee. All volunteers were given a detailed description of the study and their written consent was obtained. The study was performed in accordance with the Declaration of Helsinki, the Austrian Medicines Act and the EC-GCP guidelines.

Study protocol

On the evening before the study day, approximately 12 h before drug administration, eight cantharides plasters were attached periumbilically to the abdominal skin and held in place by adhesive tape. This led to formation of blisters within 12 h [7, 8]. The experimental procedures were started at 08.00 h. Volunteers were in a supine position throughout the study period. Room temperature was kept at 25°C. A plastic cannula (Venflon®) was inserted into an antecubital vein to monitor serum concentrations of the study drug at 15 min intervals following drug administration. Subsequently, two commercially available microdialysis probes (CMA 10®, Stockholm, Sweden) with a molecular cutoff of 20 kD, an outer diameter of 500 µm and a membrane length of 16 mm were inserted, one into the musculus vastus medialis and one into the subcutaneous adipose layer of the thigh, by a previously described procedure [5, 6]. The microdialysis system was perfused with Ringer's solution at a flow rate of 1.5 µl min⁻¹ by a microinfusion-pump (Precidor®; Infors-AG, Basel, Switzerland). After a 30 min baseline sampling period, *in vivo* probe calibration was performed as described previously [1]. Following a 30 min washout period during which the microdialysis probes were flushed with Ringer's solution, paracetamol was administered as a single oral dose. Sampling of microdialysates was continued at 30 min intervals for 240 min. Cantharides blisters were punctured, one per time point, at 30 min intervals with sterile insulin needle syringes. Saliva was sampled at 30 min intervals.

Cantharides skin blister fluid method

Cantharides induced skin blisters are caused by a toxic reaction leading to the formation of a subepidermally located blister. To induce skin blisters, cantharides impregnated plasters were employed. Cantharides plasters were prepared as described in the literature [7, 8]. An ointment containing 0.25% cantharidin was prepared by mixing pure cantharidin with ointment base. On the evening before puncturing the blisters (–12 h), eight 1 × 1 cm 0.25% cantharides-impregnated plasters were applied to the abdominal skin of each subject. The patches of polyethylene sheeting were held in place by adhesive tape over 12 h. This led to formation of blisters within 12 h. On the study day, approximately 1 ml of the blister fluid was aspirated into a syringe by puncturing the blister with a fine needle at the defined time points. Blister fluid was filled into Eppendorf cups and immediately frozen in upright position at –80°C.

Microdialysis

Microdialysis is based on sampling of analytes from the extracellular space by means of a semipermeable membrane at the tip of a microdialysis probe [5, 6, 13]. The probe is constantly perfused with a physiological solution at a low flow-rate (0.5–10 µl min⁻¹). Once the probe is implanted into the tissue, substances present in the extracellular fluid (concentration C_{tissue}) are filtered by diffusion out of the extracellular fluid into the probe, resulting in a concentration ($C_{\text{dialysate}}$) in the perfusion medium. Samples are collected and analyzed. For most analytes, equilibrium between extracellular tissue fluid and the perfusion medium is incomplete; therefore: $C_{\text{tissue}} > C_{\text{dialysate}}$. The factor by which the concentrations are interrelated is termed recovery.

Assessment of microdialysis probe recovery

In vitro experiments In order to ascertain that the diffusion process for paracetamol through the microdialysis membrane is concentration-independent over a wide concentration range and to characterize the transfer rate of the probes, *in vitro* recovery of paracetamol was assessed: there was a linear correlation between paracetamol concentrations in the dialysate and paracetamol concentrations in the surrounding medium. Regression analysis of *in vitro* experiments revealed a mean *in vitro* recovery of 79% [5].

In vivo experiments *In vitro* recovery may be substantially different from *in vivo* recovery [5, 13]. Therefore, mean *in vivo* recovery rates were assessed according to the retrodialysis method [1, 14]. The principle of this method relies on the assumption that the diffusion process is quantitatively equal in both directions through the semipermeable membrane. Therefore, the compound of interest can be included in the perfusion medium and the disappearance rate (delivery) through the membrane can be calculated subsequently. The *in vivo* recovery value was calculated as:

$$\text{recovery (\%)} = 100 - (100 \cdot \text{analyte concentration}_{\text{out}} \cdot \text{analyte concentration}_{\text{in}}^{-1}).$$

In vivo recovery was assessed for each experiment by dialysing the tissue (muscle or skin) with a perfusion medium containing 10 µg ml⁻¹ paracetamol for 30 min. The *in vivo* recovery data ranged from 24% to 90% (mean recovery for muscle tissue was 52 ± 10% and 55 ± 12% for subcutaneous adipose tissue). Each individual's recovery was used for calculation of absolute interstitial values.

Measurement of drug concentrations in saliva

At the defined time points, the volunteers were requested to donate as much saliva as possible into polyethylene cups after having rinsed the mouth 5 min before sampling with plain water for 1 min. Subsequently, saliva fluid was aspirated into 2 ml syringes and immediately stored frozen at –80°C. In order not to affect drug concentrations in saliva, salivary flow was not stimulated in any way. A probe has not been used in the salivary duct.

Study drug Paracetamol was administered as a single oral dose of 2000 mg (Mexalen Tabletten[®], Merckle Ges.m.b.H., Vienna, Austria).

Drug assay Total paracetamol concentrations in blister fluid, microdialysates, saliva and in serum were analyzed by using an Fluorescence Polarisation Immunoassay (FPIA assay; Abbott, Abbott Park, IL, USA) [15]. To be able to measure paracetamol in low concentrations in the small volumes obtained by microdialysis the assay had been adapted for use on a COBAS Fara II[®] analyzer (Roche Diagnostics, Basel, Switzerland). The CV of the assay was <5% in the concentration range between 0.1 and 10 µg ml⁻¹. No extraction was required prior to analysis.

Data analysis

All data are presented as means ± s.e.mean. For microdialysis experiments interstitial concentrations were calculated by the following equation [5]:

$$\text{tissue concentration} =$$

$$100 \cdot \text{sample concentration} \cdot \text{mean in vivo recovery value}^{-1},$$

$t_{\max}$ and $C_{\max}$ values were estimated according to a two compartment model for serum and to a one compartment model for interstitial fluids by means of a commercially available computer program (Topfit 2.0, Gustav Fischer, Stuttgart, Jena, New York, 1993). Areas under the curve from 0 to 240 min (AUC (0, 240 min) [µg ml⁻¹ min]) for individual drugs were determined for serum and tissues according to the trapezoidal rule. The ratios $\text{AUC}_{\text{blister}}/\text{AUC}_{\text{serum}}$, $\text{AUC}_{\text{muscle}}/\text{AUC}_{\text{serum}}$, $\text{AUC}_{\text{subcutaneous}}/\text{AUC}_{\text{serum}}$ and $\text{AUC}_{\text{saliva}}/\text{AUC}_{\text{serum}}$ were calculated as a measure of drug penetration into the peripheral compartments. The time course of, the $\text{concentration}_{\text{blister}}/\text{concentration}_{\text{serum}}$ -ratios, the $\text{concentration}_{\text{muscle}}/\text{concentration}_{\text{serum}}$ -ratios, the $\text{concentration}_{\text{subcutaneous}}/\text{concentration}_{\text{serum}}$ -ratios and the $\text{concentration}_{\text{saliva}}/\text{concentration}_{\text{serum}}$ -ratios was calculated for each individual data set and subsequently presented as means ± s.e. mean. The Student's *t*-test for paired data was employed to test whether $\text{AUC}_{\text{interstitial}}$ -values were significantly different from $\text{AUC}_{\text{serum}}$ -values. A *P* value < 0.05 was considered the level of significance. Due to technical reasons one complete dataset of microdialysates was not available for data analysis. One volunteer moved the leg with the probes in muscle and adipose tissue rather vehemently during the initial phase of the experiment. This movement led to a shift in the position of the probes, which could not be corrected adequately. The experiment was continued though, but the function of the probes was evidently impeded, which could be seen by the smaller volumes of dialysates collected. Therefore, the microdialysis experiments were stopped but saliva and blister sampling were continued. Skin blisters could be induced in all volunteers. In two of seven volunteers, however, only one blister could be induced. Therefore, only five complete datasets were available for pharmacokinetic calculation. One dataset of saliva had to be dismissed due to extremely high initial paracetamol concentrations (130 mg l⁻¹ after 30 min),

which probably resulted from the incomplete removal of the study drug from the volunteer's buccal mucosa.

Results

The time *vs* concentration curves for paracetamol obtained by *in vivo* sampling in serum, saliva, cantharide blister fluid, muscle and subcutaneous tissue, following single p.o. dose administration are depicted in Figure 1. Pharmacokinetic data and the mean ratios $\text{AUC}_{\text{interstitial}}/\text{AUC}_{\text{serum}}$ are summarized in Table 1. The time courses of the $\text{concentration}_{\text{interstitial}}/\text{concentration}_{\text{serum}}$ -ratios are shown in Figure 2. Data from representative experiments are depicted in Figure 3.

The mean $\text{AUC}_{\text{serum}}$ (2923 ± 363 µg ml⁻¹ min) was significantly different (*P* = 0.001) from the mean $\text{AUC}_{\text{saliva}}$ (3677 ± 374 µg ml⁻¹ min). The mean $\text{AUC}_{\text{saliva}}/\text{AUC}_{\text{serum}}$ ratio was 1.83 (95% CI, 1.39–2.27). The mean $\text{AUC}_{\text{serum}}$ was not significantly different (*P* = 0.35) from the mean $\text{AUC}_{\text{blister}}$ (1829 ± 397 µg ml⁻¹ min). The mean $\text{AUC}_{\text{blister}}/\text{AUC}_{\text{serum}}$ ratio was 0.88 (0.50–1.26). The mean $\text{AUC}_{\text{serum}}$ was not significantly different (*P* = 0.89) from the mean $\text{AUC}_{\text{muscle}}$ (2742 ± 435 µg ml⁻¹ min). The mean $\text{AUC}_{\text{muscle}}/\text{AUC}_{\text{serum}}$ ratio was 1.08 (0.67–1.49). The mean $\text{AUC}_{\text{serum}}$ was not significantly different (*P* = 0.49) from the mean $\text{AUC}_{\text{subcutaneous}}$ (2303 ± 417 µg ml⁻¹ min). The mean $\text{AUC}_{\text{subcutaneous}}/\text{AUC}_{\text{serum}}$ ratio was 0.96 (0.41–1.51).

Discussion

To validate the assumption that skin blister fluid sampling, microdialysis and saliva sampling yield comparable results for the measurement of the unbound, free drug fraction in the interstitial fluid [7, 10], time *vs* peripheral compartment concentration curves of paracetamol obtained simultaneously by these techniques were compared in the present study. Paracetamol was chosen as a model drug, because it has been convincingly shown that paracetamol exhibits a protein binding of less than 5% [12]. Since paracetamol was also shown to be freely diffusible into the interstitial space *in vivo* [5], serum concentrations were assumed to equal interstitial concentrations.

In our experiments the concentration profiles after single oral administration differed among the three methods. The time course of the $\text{concentration}_{\text{peripheral}}/\text{concentration}_{\text{serum}}$ -ratios showed that cantharides blister and microdialysis data closely paralleled serum levels (Figures 1 and 2). In contrast, saliva data overestimated the corresponding serum concentrations.

In blister fluid, paracetamol levels reached serum concentrations after an equilibration period of almost 2 h, identifying cantharides-induced blisters as a slowly equilibrating compartment. Cantharides blister fluid has been suggested to be representative of the peripheral compartment [7] and monitoring drug concentrations—particularly antibiotics—is considered a useful tool for assessing tissue penetration of drugs [8, 16]. However, cantharides blisters are formed due to chemical irritation of the skin, and blister fluid mimics inflamed tissue fluid rather than a physiological protein free fluid. In blister fluid, high protein concentrations were observed [7], possibly being the reason for the cumulation

Figure 1 Time *vs* paracetamol concentration curves in cantharides blister fluid (○) (a; *n*=5), saliva (□) (b; *n*=6), muscle microdialysate (△) (c; *n*=6) and subcutaneous microdialysate (▽) (d; *n*=6). For direct comparison, serum (■) concentration curves obtained from the same volunteers are shown in each panel. Note that serum concentration curves may vary between panels since, for technical reasons, groups of volunteers were not identical. Results are presented as means $\pm$ s.e. mean. 0=time of administration.

	C_{max} ($\mu\text{g ml}^{-1}$)	t_{max} (min)	AUC ($\mu\text{g ml}^{-1} \text{ min}$)	$AUC_{interstitial}/$ AUC_{serum}
Serum (<i>n</i> =7)	18.2 ± 3.0	90 (30–135)	2923 ± 363	—
Saliva (<i>n</i> =6)	19.9 ± 2.9	135 (60–180)	3677 ± 374	1.83 (1.39–2.27)
Blister (<i>n</i> =5)	10.9 ± 1.7	120 (90–240)	1829 ± 397	0.88 (0.50–1.26)
Muscle (<i>n</i> =6)	15.8 ± 2.5	135 (75–165)	2742 ± 435	1.08 (0.67–1.49)
Subcutaneous (<i>n</i> =6)	14.4 ± 2.5	120 (45–165)	2303 ± 417	0.96 (0.41–1.51)

C_{max} , peak concentration.

t_{max} , time to reach peak concentration.

AUC, area under the time *vs* concentration curve.

$AUC_{interstitial}/AUC_{serum}$, AUC for interstitial fluid obtained by serum-, saliva-, blister fluid-, or microdialysate sampling versus corresponding AUC_{serum} -ratio. (Mean with 95% CI.)

Table 1 Pharmacokinetic parameters for serum, saliva, skin blister fluid, muscle and subcutaneous adipose tissue microdialysates after administration of a single oral dose of 2000 mg paracetamol.

of protein bound drugs in blister fluid [17]. This cumulation led some authors to the conclusion that cantharides induced skin blisters are not representative of the peripheral compartment in the measurement of highly protein bound

drugs [4]. Paracetamol, however, a drug that minimally binds to plasma proteins at therapeutic plasma concentrations, reached a blister fluid/serum concentration ratio of about 1 after a period of slow equilibration. Thus, it may be

Figure 2 Time course of the individual cantharides blister fluid/serum—(a), saliva/serum—(b), muscle microdialysate/serum—(c) and subcutaneous microdialysate/serum—(d) concentration ratios for paracetamol for the experiments shown in Figure 1. Results are presented as means $\pm$ s.e. mean. 0 = time of administration.

suggested that skin blisters could be employed as surrogate for peripheral compartments, at least for non- or low-protein bound drugs. Drug kinetics in skin blisters might be influenced by differences in blister size [18]. However, cantharides induced skin blisters in our study had an average size of 1 cm² (CV < 50%), which we believe only minimally influenced blister data.

In microdialysis experiments, the time course of subcutaneous adipose tissue concentrations closely resembled the time course in muscle (Figure 1). Mean ratio $AUC_{\text{subcutis}}/AUC_{\text{serum}}$ was 0.96 (0.41–1.51), mean ratio $AUC_{\text{muscle}}/AUC_{\text{serum}}$ was 1.08 (0.67–1.49). The present results are in agreement with the notion that paracetamol is relatively uniformly distributed throughout most body fluids [19]. Furthermore, our present data corroborate previous results on paracetamol obtained by *in vivo* microdialysis [5].

In contrast, saliva concentrations clearly overestimated corresponding serum concentrations throughout the observation period. Looking for simple, non-invasive ways to monitor therapeutic drug concentrations, saliva sampling was claimed to provide access to the unbound fraction of corresponding serum and tissue concentrations [20]. The salivary excretion of paracetamol has previously been examined [9–11]. The saliva/plasma concentration ratio following oral administration was high during the first 60 min [9], however during the elimination phase saliva

concentrations closely paralleled the plasma concentrations [9]. In the present study we had to dismiss one saliva dataset due to extremely high initial paracetamol concentrations. We speculate that the volunteer had not properly rinsed the buccal mucosa, subsequently leading to the contamination of the first saliva sample. In the other volunteers, paracetamol concentrations in saliva were also significantly higher than serum concentrations throughout the observation period. The saliva/serum ratio showed higher levels in saliva (mean ratio $AUC_{\text{saliva}}/AUC_{\text{serum}}$ was 1.83 with 95% CIs of 1.39–2.27) and a variability in saliva/serum ratios throughout the observation period. Possible reasons for the differences in the salivary data could be explained as pH/pKa effects. It has been suggested that for lipid soluble acidic compounds such as paracetamol (pKa = 9.5) the saliva/serum concentration ratio could be predicted on the basis of the degree of ionisation in the two compartments [21]. Another possible explanation for the higher saliva/serum concentration ratios is ongoing absorption of paracetamol into the arterial system [9].

In conclusion, skin blister sampling and microdialysis closely mirrored corresponding serum concentrations and, thus, proved to be suitable techniques for the assessment of peripheral compartment pharmacokinetics. In contrast, saliva data overestimated the corresponding serum concentrations. The results derived from the present study may only be

Figure 3 Time courses of representative concentration curves for paracetamol: Time *vs* concentration curves for individual microdialysis experiments are depicted in the left panel. Individual time courses of paracetamol concentrations in saliva (□) and skin blister fluid (○) are shown in the right panel. (■) Serum. Results are presented as absolute values. 0 = time of administration.

extrapolated to low and non protein bound drugs. For other compounds with different pharmacokinetic properties a reevaluation of the three sampling techniques would be unavoidable to detect similarities or differences between the techniques.

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