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Short Communication

Determination of nifedipine in gingival crevicular fluid: a capillary gas chromatographic method for nifedipine in microlitre volumes of biological fluid

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ABSTRACT

This paper describes a sensitive capillary gas chromatographic (GC) method for the determination of nifedipine in sub-microliter samples of gingival crevicular fluid (GCF) in order to assess if nifedipine is present in the GCF and if so, whether the local tissue concentrations of this drug are an important determinant in the development of gingival overgrowth. Liquid–liquid and solid-phase extraction were combined to give adequate sample clean-up and concentration for measurement by automated capillary GC with electron capture detection. Nifedipine and its principal metabolite, M-I, were analysed in both plasma and GCF in 9 adult male patients who had been taking nifedipine for over six months. M-I could not be measured in GCF. Plasma nifedipine and M-I levels were nomal, but the nifedipine levels found in the GCF of 7 patients (including all those with overgrowth) were remarkably elevated, 15 to 316-fold greater. This massive concentration of nifedipine into the GCF is therefore linked with gingival overgrowth. This is the first time that a GC method has been developed which permits determination of GCF pharmacokinetics of a drug which causes gingival overgrowth, and further investigation will lead to a better understanding of the tissue mechanisms involved.

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INTRODUCTION

The calcium channel blocker, nifedipine, is used in the management of angina and hypertension [1]. A well reported unwanted effect is the development of gingival overgrowth in approximately 10% of dentate adults taking the drug [2]. The mechanism of gingival overgrowth is uncertain, although a reaction between nifedipine and/or its metabolite with components of the gingival tissues is fundamental. The significance of plasma levels of nifedipine, and perhaps more importantly, local tissue levels have not yet been determined.

The gingival tissues form a protective, loosely adherent cuff around each tooth. The gingival connective tissues exude a biological fluid into the crevice between this cuff and the tooth known as the gingival crevicular fluid (GCF) (Fig. 1). GCF is a transudate derived from serum, although local tissue activity may alter the constituents and the amount of fluid produced [3–6]. Minute quantities of fluid are produced by the healthy gingiva but this increases in the presence of inflammation, under which conditions 0.1–1.0 μl can be sampled [7]. We are the first to determine whether nifedipine is present in the crevicular fluid [8], or if concentrations of the drug in this transudate are important determinants for the incidence and severity of nifedipine-induced gingival overgrowth. Previous investigators have detected the presence of other drugs, notably tetracycline and minocycline in GCF [9–11], however their methods of sample analysis were qualitative only.

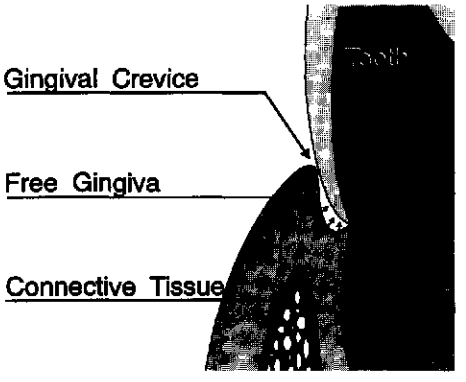

Fig. 1. Structure of gingival crevice showing direction of fluid movement.

The difficulties in analysing nifedipine and its metabolites are reflected in the multitude of methods in print, in excess of 50 [12]. The best reported sensitivity, using state-of-the-art detection such as negative-ion chemical ionisation mass spectrometry, [13] can result in a limit of detection of 0.03 μg/l from a sample of 1.0 ml (or detection of 30 pg nifedipine). However, if we assume that GCF levels of nifedipine are equivalent to those present in plasma we would need to detect 10 to 70 μg/l from a 1 μl sample, or 10 to 70 pg nifedipine. The method for the analysis of nifedipine in plasma in use in our laboratory is based on the method of Schmid *et al.*, using electron capture detection [14], and requires a relatively large sample (0.5 ml) and has a detection limit of 1.0 μg/l (500 pg). Clearly, our plasma method is not of direct applicability to the submicroliter sample volume of GCF available. A method under development in our laboratory for the analysis of nisoldipine in plasma samples (0.01 to 10.0 μg/l) [15] gave us insight into the potential of sample concentration and clean-up using liquid–liquid extraction followed by solid-phase extraction.

Accordingly, a sensitive capillary GC method for the analysis of nifedipine in small GCF samples was developed in order to assess if nifedipine is present in the GCF and if so, whether the local tissue concentrations of this drug are an important determinant in the development of gingival overgrowth.

EXPERIMENTAL

Chemicals

The following compounds were used and were a gift of Bayer plc (Newbury, UK): 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester (nifedipine), 2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester (M-I), and 2-methylpropyl-methyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-pyridine-3,5-carboxylate (nisoldipine).

Glassware

Amber screw-top 4-ml vials with PTFE lined caps, were supplied by Zinsser Analytic, Maidenhead, UK. Tapered 600-μl amber vials with grey PTFE lined crimpcaps, and PTFE sleeves to fit the Hewlett-Packard 7673A autosampler were supplied by Chromacol, London, UK. Glass pasteur pipettes for sample transfer were supplied by Bilbate, Daventry, UK.

Preparation of solutions

Unless otherwise stated all solid dihydropyridines, stock solutions, standards in plasma and patient samples were handled under yellow light to avoid photodegradation of nifedipine [16].

For calibrating the GCF assay nifedipine (154 mg/l) was dissolved in methanol in amber glass volumetric flasks and subsequently diluted (1:10) in blank centrifuged human plasma to give a stock solution of 15.4 mg/l in plasma. Serial dilutions of this standard gave solutions of 10 mg/l, 7.7 mg/l, and 3.85 mg/l in plasma.

Reagents

Methanol, propan-2-ol and acetone, of Distol® grade were obtained from FSA, Loughborough, UK. Propan-2-ol was acidified immediately prior to use to a concentration of 0.17 *M* acetic acid (BDH, Poole, UK). Pesticide analysis grade toluene was used (BDH).

Apparatus

Solid-phase extraction (SPE) cartridges (NH_2, Bond Elut 100 mg/1 ml, Varian, Harbor City, CA, USA) and a Jones Elution Manifold were supplied from Jones chromatography, Hengoed, UK. The Elution Manifold was connected to a Capex 2DC oil-free pump (Charles Austen Pumps, Weybridge, UK) via a cold solvent trap (conical side-arm flask in ice). The pump effluent was piped to a filter cabinet (Bigneat, Havant, UK) where all solvents were handled. Liquid–liquid extractions were achieved by vortex-mixing on a customised multiple-sample vortex-mixer (Vibrax-VXR, IKA Labortechnik, Janke & Kunkel, Germany). Glass gas-tight Hamilton syringes (10 μl) and the centrifugal evaporator (Centrivap) were obtained from V.A. Howe and Co., London, UK. Periotron and Periopapers were obtained from Pro Flo, New York, USA.

Sample collection and treatment

Due to the photolability of nifedipine, all clinical procedures were performed under yellow light.

Gingival crevicular fluid was collected from 9 adult male patients who had been taking a minimum of 40 mg/day of nifedipine for a minimum period of six months. Samples were collected at 0, 0.5, 1, 2, 4, and 8 h after the subjects had taken their normal dose. Concurrent plasma samples were collected and prepared using a modification of the method of Schmid *et al.* [14]. Filter paper collection strips (Periopapers) measuring 2 × 6 mm were placed within the gingival crevice of a previously cleaned, dried and isolated tooth as illustrated in Fig. 2. When a visible amount of fluid was seen (ca. 0.5 μl) the filter paper was removed and the volume of fluid present measured using a Periotron 6000 [7]. The plastic holding tip of the Periopaper was then removed prior to placing two filter paper samples into a 4-ml amber vial containing 1 ml of 20 m*M* Tris buffer pH 9.0, which contained internal standard, nisoldipine, at a concentration of 26 μg/l.

The vials were gently rocked for 48 h in a cold room at 4°C to maximise extraction from the filter paper. The filter papers were then removed from the samples to which 2.5 ml of toluene was added. Extraction into toluene was achieved by vortex-mixing for 15 min. Following centrifuga-

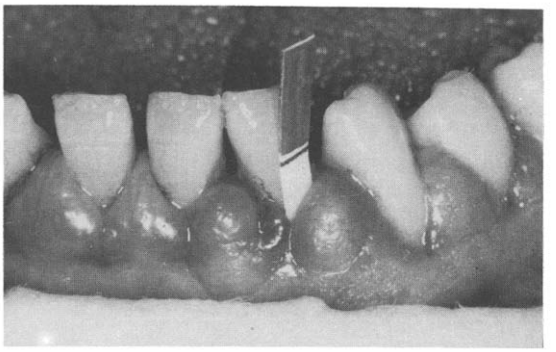

Fig. 2. Clinical photograph showing presence of overgrowth, around the lower incisors. Filter paper *in situ*.

tion (1614 *g*, 10 min) the upper toluene layer was aspirated into a solid-phase-extraction cartridge which had been previously prepared (2 column-volume rinses each of methanol, acidified propan-2-ol and toluene). The sample was subsequently eluted in 500 μl of acidified propan-2-ol into a 600-μl tapered amber vial. Collection vials were then placed into a Centrivap for 35–45 min until dry. The sample was then reconstituted in 100 μl of toluene and loaded onto the GC.

For each analysis a series of standards was produced by spiking filter papers with 1 μl of a known concentration of nifedipine. These were treated in an identical manner to the samples and the results used to produce a calibration curve. Quality control samples were produced and analysed in the same way.

Gas chromatography

A Hewlett-Packard 5890A Series II GC equipped with a ^{63}Ni electron capture detector (15 mCi) was used in combination with an HP7673A robotic autosampler. A fused silica pre-column, 7 m × 0.56 mm I.D., 0.15 μm DB1 (J & W Scientific, Folsom, CA, USA), was connected by a universal-fitting glass butt-connector (Chrompak, London, UK) to the analytical fused silica column, 40 m × 0.25 mm I.D., 0.25 μm DB1 (J & W Scientific). The on-column injection port was unheated, the detector was maintained at 300°C and chromatography was carried out isothermally at 250°C. The carrier gas was hydrogen (flow-rate 32 cm/s, 300 kPa back pressure) with nitrogen make-up gas (60 ml/min). An HP3396A computing integrator was used to process the chromatographic signals and to drive the autosampler. Under the described conditions the retention times of nifedipine, and nisoldipine were 6.9 and 9.2 min, respectively.

Standards, samples and quality controls were analysed in duplicate and the peak-area ratio of drug to internal standard was calculated by the integrator. Calibration curves were constructed and used to calculate sample concentrations.

RESULTS AND DISCUSSION

Although this sample preparation protocol is rather lengthy and requires technical expertise it has proved to be successful. The liquid–liquid extraction is known to give a recovery of at least 100% [14] and the SPE cartridges can be re-used without problems ($\geq$ 6 times) due to the relatively clean matrix passed through them. Although most workers choose a C_{18} solid phase for the extraction of nifedipine we have found that this does not give sufficient sample clean-up nor recovery for our application. Unfortunately however, using this system, the recovery of the pyridine metabolite (M-I) was low and erratic and it co-chromatographed with inteferents and therefore we cannot comment upon its presence or absence in the GCF. It is, however, believed to be pharmacologically inactive. The internal standard used by Schmid *et al.* (nitrendipine) also co-chromatographed with inteferents and was substituted by nisoldipine, another dihydropyridine drug.

Sample collection was not without problems. Prior to collection the site must be rendered completely free of plaque deposits and saliva. The sampling site was therefore thoroughly polished using a rotating rubber cup and dental polishing paste which was then washed away with a stream of water. The site was then dried and isolated from further salivary contamination with dental cotton wool rolls and a low volume aspirator. GCF can be collected by one of two methods; either the insertion of a small glass capillary within the crevicular sulcus or by the insertion of filter paper strips as described here. The capillary method of collection has disadvantages in that the insertion of the capillary is likely to induce bleeding within the sulcus and therefore cause contamination of the sample. Previous researchers measuring drugs within the GCF have opted to employ the latter method. Filter papers provide a simple, inexpensive and atraumatic method of collection. In addition this method was currently employed on the Periodontology clinic where sampling was to take place and therefore provided a convenient reliable method. Any filter

TABLE I

INTRA-ASSAY REPRODUCIBILITY FOR THE MEASUREMENT OF NIFEDIPINE IN GCF (n = 10)

Sample	Theoretical concentration (mg/l or ng/sample)	Mean measured concentration (mg/l or ng/sample)	C.V. (%)
Low QC	2.0	1.82	30
Medium QC	5.5	5.20	12
High QC	11	9.31	13.9

papers seen to be contaminated with plaque, saliva or blood were discarded.

The filter papers are supplied in a pre-packed plastic envelope so that they are sterile. Each paper has a plastic coated tip (Fig. 2) by which the papers can be more easily handled using sterile tweezers, thus reducing the risk of contamination. Initial work into the feasibility of this assay demonstrated that unless this tip was removed prior to extraction of the GCF into buffer the plasticisers within the tip leached into the buffer causing severe contamination and completely unsatisfactory chromatography. Once this was realised all plastic tips were removed with sterile scissors immediately prior to placing the filter papers within the buffer.

When producing standards for a calibration curve a further problem arose. It would be impossible to collect sufficient quantity of blank GCF in which to manufacture standards. The nearest matrix available to us for this purpose is plasma. However, as GCF represents a transudate of plasma it required modification. To this end plasma used for the manufacture of GCF standards was centrifuged to remove large protein constituents and the supernatant removed for use.

TABLE II

INTER-ASSAY REPRODUCIBILITY FOR THE MEASUREMENT OF NIFEDIPINE IN GCF (n = 8)

Sample	Theoretical concentration (mg/l or ng/sample)	Mean measured concentration (mg/l or ng/sample)	C.V. (%)
Low QC	2.0	2.32	24
Medium QC	5.5	5.55	13
High QC	11	10.78	8.7

The extent of concentration by the GCF rendered great sensitivity unnecessary. Hence the calibration curves for nifedipine in GCF were from 0.25 to 16 mg/l (or 16 ng/l μl) and were linear with intercepts not significantly different from zero and with correlation coefficients greater than 0.99. The limit of quantitation for nifedipine was 0.25 mg/l or 250 pg. The results of intra- and inter-assay reproducibility studies are shown in Tables I and II. Recovery of nifedipine in this assay was between 94 and 103%. The coefficients of variation for this assay (Tables I and II) are not ideal, but they reflect the difficulty in handling such a small sample. The many preparation steps also increase the error produced. Moreover, the patients' samples were reanalysed if there was greater than 10% difference between duplicates.

Chromatograms of extractions from blank, centrifuged plasma, a standard (3.85 mg/l nifedipine) and a typical GCF sample are shown in Fig. 3.

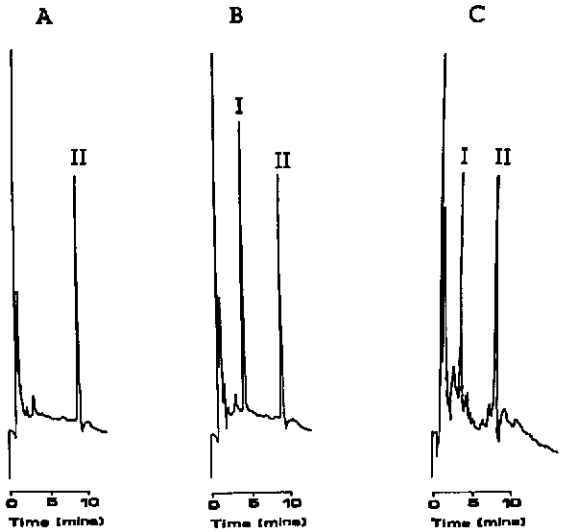

Fig. 3. Extractions from GCF. Peak I = nifedipine, peak II = nisoldipine (I.S.). (A) Blank centrifuged plasma (1 μl) added to filter paper and extracted. (B) Blank centrifuged plasma (1 μl) spiked with nifedipine to a concentration of 3.85 mg/l, added to filter paper and extracted. (C) Patient's GCF collected (0.87 μl) and extracted. The nifedipine concentration was 3.1 mg/l. Patient had taken an oral dose of 10 mg one hour previous to this sample being taken.

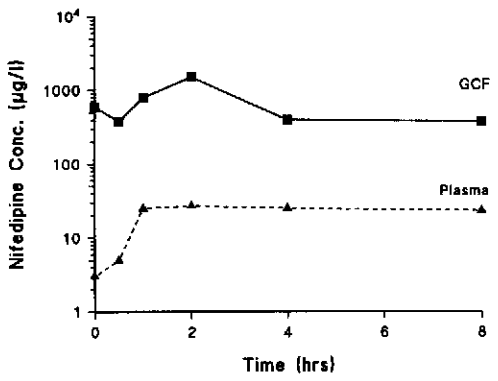

Fig. 4. Plasma and GCF nifedipine levels in a patient after 20 mg of nifedipine.

Once established the assay was used to assess the concentration of nifedipine in the GCF of nine adult patients. All patients sampled were male with an age range of 44–69 y. All the patients had been taking between 20 and 80 mg per day of nifedipine for the control of angina and/or hypertension for a minimum of 4 years. Five of the patients sampled demonstrated significant gingival changes ("responders"), and had all undergone gingival surgery in the past to remove overgrown gingival tissue. Nifedipine was detected in quantifiable amounts in the GCF of all but two of the patients sampled (both failed to exhibit clinical signs of overgrowth, "non-responders").

Plasma nifedipine and M-I levels were also analysed. These were unremarkable. Whenever nifedipine was detected in the GCF, it was at significantly higher levels than in plasma. For each patient in which nifedipine was detected within the GCF, the maximum level of nifedipine in GCF (GCF C_{max}) was compared to the maximum level seen in plasma. GCF C_{max} values varied from 920 to 9300 µg/l which represented a 15–316 fold increase from the plasma C_{max} (see Table III). The pharmacokinetic profiles of one of the patients is shown in Fig.4 in order to demonstrate this point.

In the nine patients studied the plasma levels were greatest between 0.5–2.0 h post dose, which corresponded to the peak GCF levels in all but one patient whose GCF nifedipine concentration peaked at 4 h.

Biopsy of gingival tissues is a traumatic procedure and therefore alternative means of obtaining tissue levels of the drug are sought. As GCF itself represents the medium bathing the cellular components it is logical to examine the levels of drugs seen within this fluid. Nevertheless, it is important to realise that although the GCF may give an indication of tissue levels, it may differ, as

TABLE III

PLASMA AND GINGIVAL CREVICULAR FLUID LEVELS SEEN AT T_{max}

Key: R = responder, NR = non-responder, NA = not applicable, ND = not detected.

Patient	Status	GCF C_{max} (µg/l)	Plasma C_{max} (µg/l)	GCF/plasma ratio
1	R	1540	103	15
2	R	920	37	25
3	R	7080	80	88
4	R	1440	24	60
5	R	6950	174	40
6	NR	9300	29	316
7	NR	1650	39	43
8	NR	ND	101	NA
9	NR	ND	142	NA
Mean ± S.D.		4130 ± 3500	81 ± 54	84 ± 105

its production and constituents may be affected by several factors, notably the degree of inflammation within the tissue [3]. Thus, further tissue based work is required before we can say that the levels of drug within GCF correlate with those levels seen in tissue.

CONCLUSION

The method by which the concentration of nifedipine into the GCF is achieved is uncertain although one can speculate that this may be a significant factor in the development of overgrowth. As there was nifedipine sequestration in the GCF of two non-responders there must be other factors besides this which predispose to nifedipine-induced gingival overgrowth. This assay is now being employed to measure GCF concentration of nifedipine in a larger number of patients and to relate this to their gingival overgrowth status. In addition work is now starting to relate the concentration of drug in GCF with tissue levels.

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