

Development and validation of RP HPLC method for determination of betamethasone dipropionate in gingival crevicular fluid

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A simple RP HPLC method for quantification of betamethasone dipropionate (BDP) in gingival crevicular fluid (GCF) has been developed and validated. GCF represents a valuable matrix for therapeutic monitoring of drugs used in the treatment of periodontal disease. The proposed method involves single step extraction for sample preparation. The calibration curve for BDP was linear over the concentration range of 0.10–2.00 $\mu\text{g mL}^{-1}$ ($R^2 = 0.9971$). RSD values of intra- and inter-day precision ranged 2.2–4.5 and 1.6–5.7 %, while accuracy values were higher than 96.6 and 97.0 %, respectively. The described method can be successfully applied for determination of betamethasone concentrations in GCF obtained from patients with chronic periodontitis after local treatment with BDP cream 0.5 mg g^{-1} .

Keywords: betamethasone dipropionate, gingival crevicular fluid, HPLC, periodontal disease

Accepted June 10, 2013

Periodontal disease is a chronic inflammatory disease of the oral cavity affecting the soft- and hard-supporting structures of the teeth (1, 2). Although bacterial in etiology, current findings imply that the pathogenesis of periodontitis is mediated by the host's response (3). Corticosteroids can act as host's modulation agents and represent a therapeutic approach in periodontal therapy (4–6). One of the mostly used corticosteroids in clinical practice is betamethasone dipropionate (BDP). Therapeutically, it is used in the treatment of periodontal disease by local application in the periodontal pocket, after scaling and root planing. Compared to other corticosteroids intended for local application, such as hydrocortisone or triamcinolone acetonide, BDP shows greater anti-inflammatory activity probably due to stabilization of lysosomal membranes and blocking the actions of the invasive mediators of inflammation (6, 7).

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According to our knowledge, no HPLC method for determination of BDP concentration in gingival crevicular fluid (GCF) could be found in literature, while some RP HPLC and HPLC-MS methods have been developed for determination of BDP in biological samples (8, 9).

Thus, the aim of our study was to develop, optimize and validate a new bioanalytical HPLC method for determination of BDP in GCF.

EXPERIMENTAL

Chemicals and materials

Betamethasone dipropionate and alclomethasone dipropionate (internal standard, IS) were provided by Farmabios (Italy) and Crystallpharma (Spain), respectively.

Methanol (HPLC grade) was supplied by Merck, Germany and potassium dihydrogen phosphate (analytical grade) was purchased from Fluka, Switzerland. HPLC grade water was used for chromatographic analysis. Whatman 3MM chromatography paper strips 2 x 5 mm (Whatman Lab Sales Ltd., UK) were used for GCF collection. Human serum was obtained from healthy volunteers from the Department of Periodontology, Faculty of Dentistry, University of »Ss. Cyril and Methodius«, Skopje, Macedonia.

Collection of GCF samples

GCF samples were obtained from fifty patients suffering from localized periodontitis at the end of the periodontal treatment (one week after the beginning of the scaling and root planing procedure). Quadrants consisting of 5 teeth were treated with 0.1 mL 0.5 mg g⁻¹ BDP cream containing 27 µg betamethasone dipropionate (Beloderm® cream 0.5 mg g⁻¹, Belupo, Croatia), using blunt needle. Samples for analysis were taken 15 minutes after treatment. Patients were in good general health and did not take any antibiotic or anti-inflammatory drug in the previous three months. The procedure was approved by the Ethical committee of Faculty of Dentistry, University of »Ss. Cyril and Methodius«, Skopje, Macedonia. GCF was collected applying the method of Koss *et al.* (10) and taken by placing 2 x 5-mm Whatman paper strips into the pocket (depth 4–6 mm) until mild resistance was felt and left there for 30 s. Strips contaminated with blood were excluded from analysis.

Apparatus and chromatographic conditions

The HPLC analysis was conducted on Agilent 1100 series (USA) equipped with UV diode array detector. The chromatographic separation was performed on Purospher STAR RP 18-e 120 Å, 150 x 4.6 mm, 5-µm, using LiChroCART® 4-4 guard column (Merck, Germany). The HPLC system operated at 25 °C with MeOH/0.04 mol L⁻¹ KH₂PO₄ (70:30, V/V) as mobile phase. Flow rate was 1.3 mL min⁻¹. The injection volume was 100 µL. UV detection was performed at 245 nm. The total run time for the HPLC analysis was 14 min.

Preparation of standard solutions and quality control (QC) samples

Stock standard solutions of betamethasone dipropionate (1.00 mg mL^{-1}) and alclomethasone dipropionate (1.00 mg mL^{-1}) were prepared in methanol and refrigerated at 4°C . Working standard solutions of BDP were made daily by diluting the stock standard solution of BDP with mobile phase to concentrations of 50.00, 125.00, 250.00, 375.00 and $500.00 \text{ }\mu\text{g mL}^{-1}$, respectively. Working standard solution of IS was made daily by diluting the stock standard solution of IS with mobile phase to concentrations of $100.00 \text{ }\mu\text{g mL}^{-1}$. Calculations were performed assuming a volume of one μL GCF. It is based on the volume that could be collected in the periodontal pocket and which is consistent with dental practitioner experience. Calibration curve standard solutions were prepared using working standard solutions of BDP and IS on six paper strips previously spiked with $1 \text{ }\mu\text{L}$ of serum, in the following manner: One μL of separate BDP working standard solution was added to separate paper strip, $10 \text{ }\mu\text{L}$ of IS working standard solution was added to each paper strip. Paper strips were extracted with $500 \text{ }\mu\text{L}$ MeOH/ H_2O mixture (70:30, V/V). Final concentrations of calibration curve standard solutions were 0.10, 0.25, 0.50, 0.75, 1.00 and $2.00 \text{ }\mu\text{g mL}^{-1}$. The starting calibration curve standard solution containing $2.00 \text{ }\mu\text{g mL}^{-1}$ BDP was prepared from the stock standard solution of BDP in methanol. QC samples were prepared in the same manner as calibration curve standard solutions in concentrations of 0.10, 0.25, 1.00 and $1.75 \text{ }\mu\text{g mL}^{-1}$ and stored at -20°C . GCF exists as a serum transudate, therefore QC samples and calibration curve standard solutions were prepared in serum because GCF is not commercially available nor easily collectable in large volumes (11, 12).

GCF sample preparation

After collection of GCF, paper strips were removed and placed in preweighed Eppendorf tubes and kept at -20°C until analysis. Before the HPLC analysis, GCF samples were thawed at room temperature. $10 \text{ }\mu\text{L}$ from $100 \text{ }\mu\text{g mL}^{-1}$ IS solution was added to the GCF sample. After adding a mixture of methanol/water (70:30, V/V) as an extracting solvent up to volume of $500 \text{ }\mu\text{L}$, the GCF sample solutions were Vortex mixed for 3 minutes. The liquid content of the tubes was transferred to glass autosampler vials. A $100\text{-}\mu\text{L}$ aliquot was injected into chromatographic system. The mass of the fluid was calculated from the differences between masses of the strips with GCF and dry strips. The obtained value, expressed as μg , was converted to volume in μL assuming the density of GCF was 1 mg mL^{-1} (13).

Validation procedure for the bioanalytical HPLC method

The validation of the developed bioanalytical RP HPLC method for determination of BDP in GCF was performed according to EMEA Guideline for validation of bioanalytical methods (14).

The calibration line was constructed with six calibration standards in the range from 0.10 to $2.00 \text{ }\mu\text{g mL}^{-1}$ BDP, including LLOQ and the upper limit of quantification (ULOQ). The calibration curve was obtained using linear regression analysis of BDP peak area to internal standard peak area ratio *vs.* concentration. Limit of detection (LOD) was calculated as the concentration level resulting in peak area three times the baseline noise

while lower limit of quantification (LLOQ) was calculated as analyte response area at least five times the blank response. Analyte calculated concentration should be at reproducible with a precision of $\leq 20\%$ and accuracy of 80.0–120.0 %.

Selectivity. – Selectivity of the method was investigated by comparing blank GCF sample solutions and QC sample solutions containing $0.10\ \mu\text{g mL}^{-1}$ BDP.

Accuracy and precision. – Intra-day accuracy and precision were determined using five replicates of each of the following QC sample solutions: 0.10, 0.25, 1.00 and $1.75\ \mu\text{g mL}^{-1}$ which represent LLOQ, low QC sample (LQC), medium QC sample (MQC) and high QC sample (HQC), respectively. The QC sample solutions were analyzed on the same day to establish intra-day accuracy and precision and on three different days to investigate inter-day accuracy and precision.

Recovery. – The extraction recovery for betamethasone dipropionate and aclofate dipropionate were calculated by comparing the peak areas measured after extraction of five replicates of QC sample solutions in the following concentrations: 0.10, 0.25, 1.00 and $1.75\ \mu\text{g mL}^{-1}$ with peak areas of solutions of the same concentration prepared in mobile phase.

Stability. – BDP sample solution stability was tested by chromatographic analysis of QC samples at LQC and HQC levels. The freeze-thaw stability (36 h at $-20\ ^\circ\text{C}$, three cycles), short-term stability (2 h, room temperature), long-term stability (14 days, $-20\ ^\circ\text{C}$) and autosampler stability (immediately after extraction and 16 hours after preparation) were investigated.

RESULTS AND DISCUSSION

HPLC method optimization

During preliminary investigations, mobile phase composition and flow rate of the mobile phase were optimized. Several mobile phases containing buffer $0.04\ \text{mol L}^{-1}\ \text{KH}_2\text{PO}_4$ and methanol were investigated where the composition of the organic phase varied from 60–80 % (organic phase/buffer, V/V). The best result was obtained using mobile phase containing 30 % $0.04\ \text{mol L}^{-1}\ \text{KH}_2\text{PO}_4$ and 70 % methanol (V/V). The flow rate was investigated in the range from $0.8\text{--}2.0\ \text{mL min}^{-1}$ and the final flow rate was set at $1.3\ \text{mL min}^{-1}$.

HPLC method validation

Linearity. – Linearity was observed within the range $0.10\text{--}2.00\ \mu\text{g mL}^{-1}$. Coefficient of determination R^2 was 0.9971, the LOD was $0.05\ \mu\text{g mL}^{-1}$ and the LLOQ was $0.10\ \mu\text{g mL}^{-1}$.

Selectivity. – The chromatograms of blank GCF and blank GCF spiked with BDP and IS are presented in Fig. 1.

As shown in Fig. 1b, BDP and the IS were well separated under the applied HPLC conditions and resolved with good symmetry. The retention times for BDP and IS were 11.01 min and 6.5 min, respectively. No endogenous interfering peaks at the retention times of BDP and IS were observed in blank GCF samples, confirming the selectivity of the method (Fig. 1a).

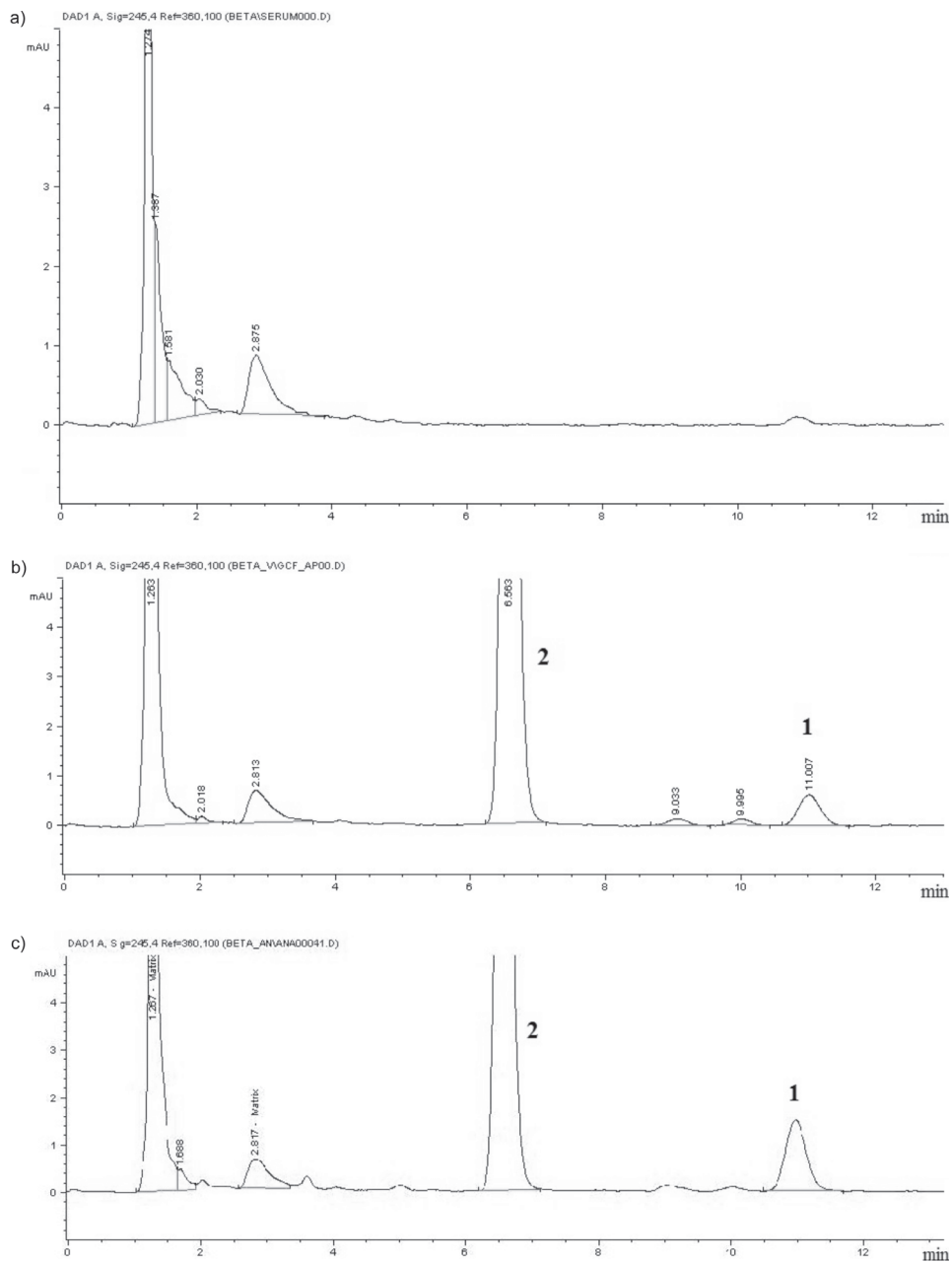


Fig. 1. Representative chromatogram of: a) blank GCF sample, b) QC sample containing 0.10 $\mu\text{g mL}^{-1}$ BDP (1) and 2.00 $\mu\text{g mL}^{-1}$ IS (2), c) patient GCF sample containing 2.00 $\mu\text{g mL}^{-1}$ IS (2) and 0.25 $\mu\text{g mL}^{-1}$ BDP (1).

Table I. Intra-day and inter-day accuracy and precision

	BDP in QC sample ($\mu\text{g mL}^{-1}$) ^a	Found concentra- tion ($\mu\text{g mL}^{-1}$) ^a	Accuracy (%) ^b	Precision (RSD, %) ^b
Intra-day assay	0.100	0.109 \pm 0.004	109.6	3.5
	0.250	0.246 \pm 0.009	99.7	4.0
	1.000	0.967 \pm 0.025	96.6	2.2
	1.750	1.728 \pm 0.078	98.7	4.5
Inter-day assay	0.100	0.106 \pm 0.152	106.0	4.4
	0.250	0.247 \pm 4.651	99.1	3.3
	1.000	0.969 \pm 2.231	97.0	1.6
	1.750	1.767 \pm 0.101	101.0	5.7

^a Each sample solution contains 2.00 $\mu\text{g mL}^{-1}$ IS.

^b Mean \pm SD, $n = 5$.

Accuracy and precision. – The intra-day and inter-day accuracy and precision are shown in Table I.

Intra-day precision ranged from 2.2–4.5 % and intra-day accuracy ranged from 98.7–109.6 %. Inter-day precision and accuracy ranged from 1.6–5.7 % and 97.0–106.0 %, respectively.

Recovery. – The mean extraction recovery of BDP from the matrix at four concentration levels (0.10, 0.25, 1.00 and 1.75 $\mu\text{g mL}^{-1}$) were 87.9, 99.6, 101.0 and 99.5 %, respectively. The recovery for the internal standard, alclomethasone dipropionate, was 98.2 %. The recovery of BDP from the matrix, using the described procedure, was consistent and efficient.

Stability. – The stability experiments were aimed at testing all possible conditions that samples may encounter between collection and analysis. Stock solution of BDP and IS were stable at room temperature for 24 h (recovery: 99.5 and 99.8 %, respectively) and at 2–8 °C for one month (recovery: 99.1 and 98.5 %, respectively).

The results showed that GCF samples spiked with BDP are stable after three freeze-thaw cycles, after 16 hours in the autosampler and at room temperature for 2 hours. The study also indicated that samples could be kept frozen at –20 °C for two weeks. The results are presented in Table II.

Analysis of patient GCF samples

The validated HPLC method was applied for determination of BDP concentrations in GCF samples obtained from patients after the local periodontal treatment with BDP 0.5 mg g^{–1} cream. A representative chromatogram of a patient GCF sample with BDP is shown in Fig. 1c.

Table II. Stability data for betamethasone in GCF samples

Storage condition/ BDP in QC sample ($\mu\text{g mL}^{-1}$)	Determined BDP ^a	
	Fresh sample ($\mu\text{g mL}^{-1}$)	Relative to fresh sample (%)
Freeze-thaw stability		
0.100	0.099 ± 0.005	99.0 ± 3.5
1.750	1.726 ± 0.004	99.0 ± 0.4
Short-term stability		
0.100	0.100 ± 0.001	100.3 ± 2.0
1.750	1.722 ± 0.001	98.6 ± 0.4
Autosampler stability		
0.100	0.102 ± 0.002	101.2 ± 0.7
1.750	1.732 ± 0.003	98.0 ± 0.3
Long-term stability		
0.100	0.101 ± 0.004	101.0 ± 3.9
1.750	1.737 ± 0.010	99.7 ± 0.7

^a Mean \pm SD, $n = 5$.

The concentrations of BDP in analyzed GCF varied in the range 0.10–1.80 $\mu\text{g mL}^{-1}$ per collected sample. The variability in concentrations of BDP in GCF among patients is probably a result of different pocket depths, as assumed by Tsai *et al.* (15), since deeper periodontal pockets contain larger volumes of excreted GCF.

CONCLUSIONS

An isocratic RP HPLC method with UV detection has been developed and validated for determination of BDP in GCF. Alclomethasone was used as internal standard. The simple extraction of BDP with water-methanol mixture without sample concentration, evaporation or reconstitution yielded high value of recoveries. Further, the validation results show that the proposed bioanalytical RP HPLC method is suitable for analysis of BDP in GCF and therefore can be applied for therapeutic drug monitoring in patients undergoing therapy.

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