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HPLC Determination of dexamethasone in human plasma and its application to an *in vitro* release study from endovascular stents

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Received January 26, 2006, accepted February 28, 2006

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Pharmazie 61: 908–911 (2006)

A simple, accurate and precise HPLC assay was developed and validated for determination of dexamethasone in human plasma. Triamcinolone acetonide was used as internal standard (I.S.) and plasma samples were extracted using liquid-liquid extraction with ethyl acetate. Chromatography was performed on a C-18 column with acetonitrile-triple distilled water (28 : 72% v/v, pH adjusted to 2.3 with phosphoric acid) at a flow rate of 1.2 mL/min and detection wavelength 254 nm. The assay was linear at a concentration range of 0.25–6 μg/mL with recoveries >77%. Precision and accuracy were within the acceptable limits. The method was used to determine dexamethasone release from different material coated endoluminal vascular stents in *in vitro* human plasma experiments. The results were useful in identifying a good coating material for the stents.

1. Introduction

Prednisolone, methylprednisolone, triamcinolone, and dexamethasone are among the most commonly prescribed glucocorticoids for systemic use (Schimmer and Parker 1996). Dexamethasone shows the highest anti-inflammatory activity among these steroids and is used systemically to treat acute and severe inflammatory, immunological, and allergic disorders (Hochhaus et al. 2001). With conventional systemic drug delivery, achieving therapeutic concentrations at a specific site is often difficult to achieve with problems associated with systemic toxicity and drug residence complications. Localized therapies such as stents have been evaluated for implantable devices and dexamethasone has shown good results (Giuseppe et al. 2005a, 2005b; Shin et al. 2005). Dexamethasone release from endoluminal vascular stent surfaces has been investigated for reduction of in-stent restenosis due to inflammation (Liu et al. 2004). The present work was aimed at evaluating different material coated endovascular stents for dexamethasone release. Since accurate estimation of the compound is vital for such investigations, a reliable, accurate and economical analytical technique such as HPLC is required. There are HPLC bioanalytical methods available for dexamethasone determination but they involve either normal phase chromatography, chemical derivatization, solid phase extraction and/or multiple steps sample clean up (Haughey et al. 1988; Katayama et al. 1993; Plezia et al. 1985; Santos-Montes et al. 1994). Therefore, it was deemed necessary to validate a simple, precise and reproducible reverse phase HPLC method for quantitation of dexamethasone in human plasma for routine analysis. The present communication describes HPLC method validation and a successful application of the method to determine dexamethasone release from different material coated endoluminal vascular stents in *in vitro* human plasma experiments in over 200 samples.

2. Investigations, results and discussion

Good chromatographic separation for dexamethasone and triamcinolone acetonide (I.S.) was achieved on a C-18 column with isocratic condition in mobile phase standards. The HPLC system reproducibility was confirmed with five replicate injections of analytical standards at 0.5, 2 and 6 μg/mL and % coefficient of variation (CV) was <7.0% indicating that the system yields reproducible data. The analysis run time was 16 min and retention time of dexamethasone and I.S. was 9.9 and 12.6 min, respectively.

Specificity of the assay method was defined as non-interference of endogenous substances in the regions of interest for the accurate determination of concentration. For plasma samples, extraction using ethyl acetate yielded clean chromatograms with no endogenous interference. Representative chromatograms of extracted plasma blank and test sample are shown in Fig. 1. The calibration curve was linear in the concentration range of 0.25–6 μg/mL ($r = 0.999$). A linear regression scheme ($y = m \cdot x + c$) was used to draw the calibration curve. A typical calibration curve was $y = 0.1591 \cdot x - 0.0114$, where y represents the peak height ratio of dexamethasone to I.S., x represents the concentration of dexamethasone and the slope of the line is expressed as m (0.1591).

The values obtained for accuracy and precision for dexamethasone in plasma are listed in Table 1. The results show that the method was precise and accurate with intra- and inter-batch variations within acceptable limits of

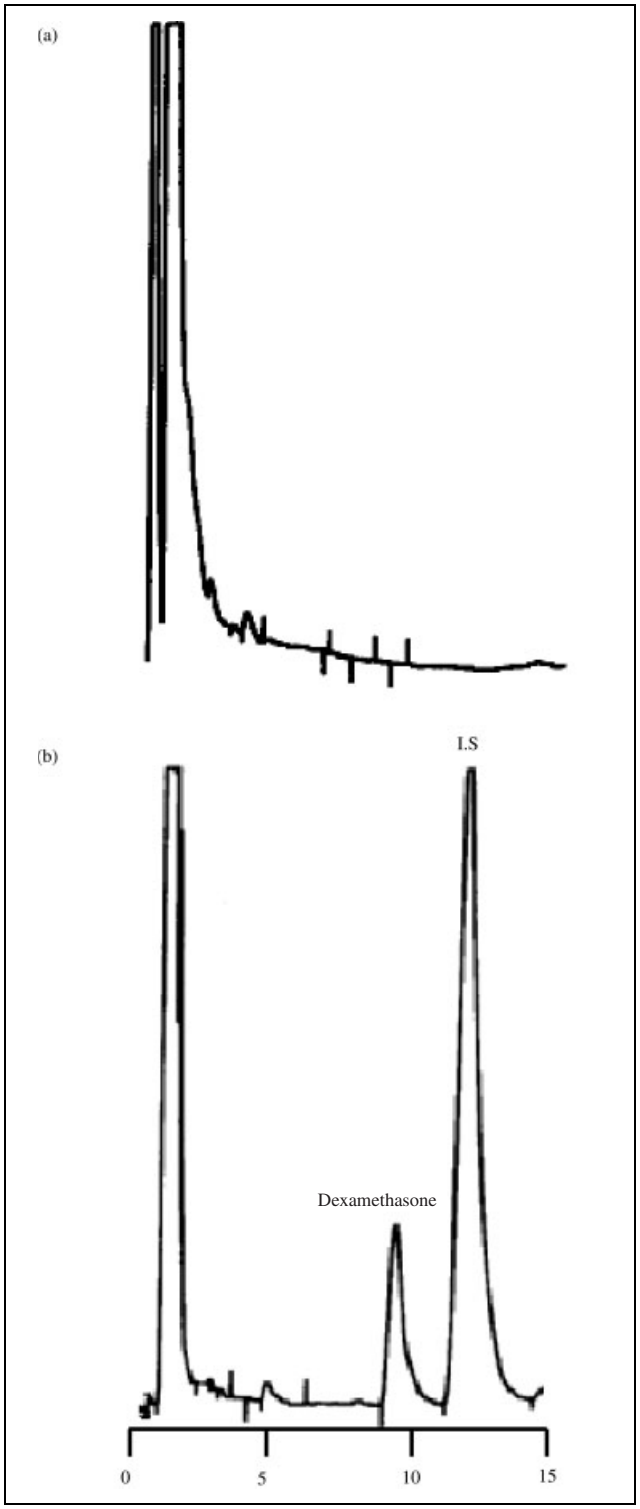

Fig. 1: Representative chromatograms of (a) Blank plasma and (b) Test samples of dexamethasone and I.S.

Table 1: Accuracy and precision of dexamethasone determination in human plasma

Concentration (μg/mL)	Accuracy (% bias)		Precision (% RSD)	
	Intra day	Inter day	Intra day	Inter day
0.5	3.9	1.9	11.1	2.3
2	1.9	1.1	3.9	5.1
6	−2.1	−2.3	7.8	4.2

±20% at low concentration and ±15% at other levels (Shah et al. 2000). The limit of quantitation was defined as the concentration within an accuracy and precision limit of 20% and was found to be 0.25 μg/mL for this method. The mean recoveries of dexamethasone in low, me-

Table 2: Recoveries of dexamethasone from spiked human plasma

Sample	Concentration (μg/mL)	Recovery % (mean ± SD)
Low	0.5	77.2 ± 4.9
Medium	2	82.1 ± 4.7
High	6	81.5 ± 3.6

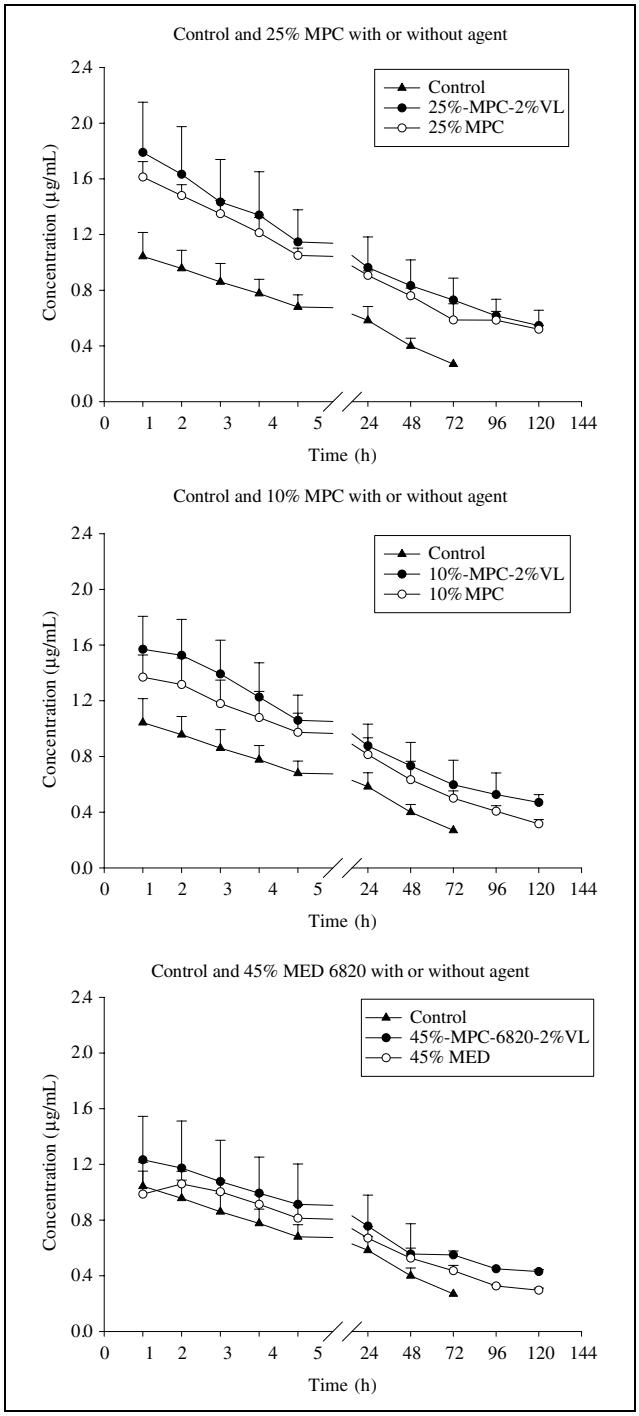

Fig. 2: Dexamethasone release profile in human plasma samples over 120 h with different coating materials

dium and high quality control samples after five days of validation were in the range of 77–82% with a % CV <5% at all concentration levels (Table 2).
Stock and working stock solutions of dexamethasone and I.S. were stable in methanol for at least 2 months at 4 °C. The analytes after extraction and reconstitution in mobile phase were stable at ambient temperature for at least 24 h in an auto sampler.
The validated method was successfully applied for analysis of dexamethasone release with different surface modified stainless steel stents in *in vitro* human plasma samples. The dexamethasone release profile in human plasma samples over 120 h with different coating materials is shown in Fig. 2. Among various studied materials, 25% MPC with coupling agent (2% VL) provided a superior release of dexamethasone in comparison to other materials up to 120 h.
In conclusion, a HPLC-UV method for estimation of dexamethasone in human plasma was developed, validated and applied for sample analysis. The method was found to be specific, accurate and precise. There were no stability problems for dexamethasone during sample processing and storage. The method was used to determine dexamethasone release from different material coated endoluminal vascular stents in *in vitro* human plasma experiments. The results were helpful in deciding a suitable coating material for endoluminal vascular stents.

3. Experimental

3.1. Chemicals and reagents

Dexamethasone and triamcinolone acetonide, used as I.S., were obtained from Sigma, USA. Acetonitrile, methanol and ethyl acetate were of HPLC grade and procured from Fisher Scientific, USA. Triple distilled water (TDW), and Ultrapure water, was obtained from an in house distillation unit. All other chemicals were of analytical grade and procured from Fisher Scientific, USA unless specified. Human blank plasma was obtained from Civitan Regional Blood Center Inc., Gainesville, Florida, USA.

3.2. Standard solutions

Stock solution of dexamethasone was prepared at a concentration of 1 mg/mL in methanol and diluted to 100 μg/mL in methanol for working stock solution. A stock solution of 1 mg/mL of I.S. was prepared in methanol and diluted to 150 μg/mL in methanol for working stock solution. All solutions were stored at 4 °C. Standard solutions of dexamethasone in mobile phase were prepared over a concentration range of 0.25–6 μg/mL and I.S. was spiked to achieve a constant concentration of 10 μg/mL. The analytical standards were used to determine HPLC system reproducibility and recovery estimation for dexamethasone from human plasma. Calibration standards of dexamethasone were prepared in human plasma by diluting blank human plasma with working stock solution to a concentration of 6 μg/mL. Blank plasma was then diluted with this solution to achieve a calibration curve in the range of 0.25–6 μg/mL. Quality control samples at low (0.5 μg/mL), medium (2.0 μg/mL) and high (6.0 μg/mL) were prepared in triplicate in the same manner and were used for method validation.

3.3. Extraction procedure

To 150 μL of plasma (spiked or test sample), 10 μL of I.S. was added. The plasma samples were mixed with 0.5 mL of ethyl acetate and vortexed for 30 s. The samples were centrifuged at 10,000 rpm for 10 min, organic layer was transferred to another Eppendorf and samples were dried in speed vac concentrator. The dry residue was reconstituted in 150 μL of mobile phase, centrifuged at 10000 rpm for 5 min and transferred to an auto injector vial for HPLC analysis.

3.4. Chromatographic conditions

The HPLC system consisted of a pump (LDC analytical constametric 3200 solvent delivery system, USA), an auto sampler (Perkin Elmer Series 200, USA), a UV detector (LDC analytical spectromonitor 3200 variable wavelength detector, USA) and an integrator (Agilent 3396 Series III, USA). Chromatographic separation was achieved on Discovery® C-18 column (150 × 4.6 mm, 5μ) attached with pellicular guard. The mobile phase composed of acetonitrile : triple distilled water (28 : 72% v/v, pH adjusted to 2.3 with phosphoric acid) at a flow rate of 1.2 mL/min. Mobile phase was degassed for 20 min in a sonicator before use. A 25 μL volume of the clear supernatant was injected onto the HPLC system and peaks were analysed at 254 nm. The chromatography was performed at room temperature.

3.5. Method validation

The method was validated in plasma for five days at low, medium and high concentrations in terms of HPLC system sensitivity, specificity, linearity, recovery, accuracy and precision. The limit of quantification (LOQ) was defined as the concentration of the sample that can be quantified with <20% deviation (Shah et al. 2000). Linearity for calibration standards ($n = 6$) was assessed by subjecting the spiked concentrations and the respective peak height ratio to least-square linear regression analysis with $y = m \cdot x + c$ equation.
For calculation of recoveries of dexamethasone, spiked quality control (QC) samples were prepared at low (0.5 μg/mL), medium (2 μg/mL) and high (6 μg/mL) concentrations. The samples were processed and the concentration of dexamethasone was determined from the analytical standard curve. The recovery was calculated by comparing the observed concentration with the spiked concentrations.
For the determination of accuracy and precision, calibration standards and QC samples at low, medium and high concentration were analyzed in triplicates for five different days ($n = 3 \times 3 \times 5 = 45$). Intra- and inter-day accuracy was determined by calculating the % bias from the theoretical concentration using the following equation:

$$\%\text{Bias} = \frac{\text{Observed concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100$$

Inter- and intra-batch precision in terms of % RSD was obtained by subjecting the data to one-way analysis of variance (ANOVA).

3.6. Dexamethasone release profile from endovascular stents in in vitro experiments with human plasma

3.6.1. Preparation of substrates and post loading of dexamethasone

316L Stainless steel substrates (1 cm × 1 cm, thickness of 0.1 mm) were coated with various polymers and methods (Table 3) using a standard laboratory protocol (Kayo 2005). Controls for this experiment included untreated 316L stainless steel. The coating materials used in the present work were 2-methacryloyloxyethyl phosphorylcholine (MPC; Dr. Ishihara, University of Tokyo) and a two part silicone resin system (Nusil, MED 6820 A, B). The coatings and coating conditions investigated in this study are summarized in Table 3. After surface modification, samples were placed in clean test tubes and washed under agitation for one week in 5 mL of Ultrapure™ water that was decanted and replaced three times. Methanol was used to prepare solutions of 0.5% (w/v) dexamethasone. The surface modified and control specimens were placed into 3 mL volumes of this loading solution and rotated gently for 24 h, after which the samples were removed and dried at room temperature for 24 h. Dexamethasone loading onto the materials was estimated by subtracting the concentration of dexamethasone in final solution (post loading) from that observed in stock solution before loading (Kayo 2005).

3.6.2. Release of dexamethasone from surface modified 316L stainless steel

Dexamethasone release *in vitro* was conducted in human plasma (donated from Shands Hospital Blood Bank, Gainesville, Florida, USA) to better emulate the blood tissue environment of endovascular stent implants. Plasma media preparation procedure has been reported previously conducted by the Biomaterials Center laboratory (Urbaniak 2004). Dexamethasone loaded specimens were placed into 10 mL of human plasma contained in 15 mL capacity centrifuge tubes at 37 °C with continuous rotation. The

Table 3: Coating and metal alkoxide treatment conditions investigated for dexamethasone release with chromium alkoxide as coupling agent

Coating material and method	25% w/v MPC Solution coated		10% w/v MPC Radiation grafted		45% v/v MED6820 Solution coated		No coat
Coupling agent	Yes	None	Yes	None	Yes	None	None

The base material used for coatings was 316L Stainless steel

samples were collected at 1, 2, 3, 4, 5, 24, 48, 72, 96, and 120 h and stored at −20 °C pending HPLC analysis. Removed aliquots were replaced with equal volume of release media at each instance.

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