Journal of Chromatography B, 878 (2010) 2967–2973

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous HPLC analysis of triamcinolone acetonide and budesonide in microdialysate and rat plasma: Application to a pharmacokinetic study

Manuela de L.T. Vieira [a,b], Rajandra P. Singh [a], Hartmut Derendorf [a,*]

[a] Department of Pharmaceutics, College of Pharmacy, University of Florida, Gainesville, FL 32610, USA
[b] Capes Foundation, Ministry of Education of Brazil, Brasilia, DF 70359-970, Brazil

ARTICLE INFO

Article history:
Received 4 June 2010
Accepted 30 August 2010
Available online 9 September 2010

Keywords:
Triamcinolone acetonide
Budesonide
HPLC
Microdialysis
Solid-phase extraction

ABSTRACT

A specific and reliable HPLC-PDA method for the quantitative determination of triamcinolone acetonide, budesonide and fluticasone propionate (as internal standards) in small volumes of microdialysate and rat plasma was developed. An efficient solid-phase extraction (SPE) procedure for plasma samples yielded extremely clean extracts with overall recovery of 104.3% and 95.7% for triamcinolone acetonide (TA) and fluticasone propionate, respectively. Plasma extracts obtained after SPE and microdialysis samples were directly injected on a C18 columm to separation. The method has been validated with good linearity, sensitivity, specificity and high accuracy (RE −5.28% to 9.14%) and precision (CV 0.50% to 6.62%) on both matrices. In stability studies, TA and budesonide were stable during storage and assay procedures. The method was applied to a pharmacokinetic study in rodents using microdialysis to determine protein unbound TA concentrations in blood and muscle.

1. Introduction

Microdialysis (MD) is a sampling technique that enables the monitoring of free drug concentration–time course in extracellular fluids, i.e. tissue interstitial fluid and plasma. The technique is based on passive diffusion of small substances across the semipermeable probe membrane inserted into the target site. Some of the advantages of MD are the low invasiveness of the technique, and the possibility of direct measurement of free drug levels at the biophase and continuous sampling without net fluid (blood) loss. This latter feature of the technique usually provides higher temporal resolution compared to conventional blood sampling, and is especially useful for pharmacokinetic investigations in populations, like small animals and pediatrics, who have limitations of total blood volume that can be withdrawn [1]. Additionally, simultaneous MD measurement in blood and selected tissues is feasible and aids in the understanding of the drug distribution characteristics. Interstitial MD has been widely performed in preclinical and clinical pharmacokinetics (PK) studies; however, continuous monitoring of drug PK by intravenous MD has been applied in only a few studies so far [2,3].

PK studies involving MD rely on an efficient analytical method to determine free drug concentrations in microdialysate and at the same time, total concentration in plasma to assess the relationship between the unbound and bound levels. In addition, the assay must be sensitive to measure considerably low concentration of the analyte in small sample volumes since only a few microliters are obtained from microdialysis sampling (depending on the flow rate and sampling period used in the investigation, usually 20–30 μL). Another prerequisite of the bioanalytical assay is the simultaneous quantification of the analyte and the calibration standard added to the microdialysis perfusion solution. Nevertheless, MD technique has the advantage of generation of protein-free samples. Thus, complex and time-consuming sample preparation becomes redundant and potential for enzymatic degradation is reduced [2].

Triamcinolone acetonide (TA) is a routinely prescribed corticosteroid due to its potent anti-inflammatory and immunosuppressant activity. TA has been used for the treatment of a wide range of diseases in humans and animals, and proved to be the most efficacious corticosteroid for the treatment of psoriasis and inflammatory diseases of the eye [4]. Although TA has vast clinical importance, it exerts considerable side effects [5]. Thus, investigation of TA free fraction in blood and target-sites is needed to manage a favorable benefit/risk ratio. Since protein unbound drug is directly correlated to pharmacological effects, the assessment of its concentration is more relevant for pharmacokinetic/pharmacodynamic investigations.

The relative recovery of TA by the microdialysis probe should be determined a priori and monitored by a continuous internal

* Corresponding author at: Department of Pharmaceutics, College of Pharmacy, University of Florida, 1600 SW Archer Road P3-27, Gainesville, FL 32610, USA. Tel.: +1 352 2737856; fax: +1 352 3923249.
E-mail address: hartmut@ufl.edu (H. Derendorf).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.08.048

recovery control. Budesonide was chosen as microdialysis calibration standard to account for changes in the recovery of the TA since it has physicochemical properties similar to the analyte [6].

Few LC methods for determination of triamcinolone acetonide have been reported [7–13]. The HPLC methods determined TA concentrations in human plasma after intravenous, intramuscular, oral or inhaled administration and were characterized by a laborious plasma extraction procedure and limited concentration ranges [7–11]. Also, these methods are not suitable for the purpose of the proposed PK study, since none of them simultaneously determines TA and budesonide. An ultra-sensitive reversed-phase capillary LC coupled to tandem mass spectrometry (μLC/MS/MS) was able to quantify TA in porcine plasma following suprachoroidal administration; however, this approach requires more sophisticated instrumentation [12]. Nevertheless, the main disadvantage of previously reported methods rests on the large plasma volume required for sample preparation, minimum of 750 μL, and/or the sample volume, minimum of 20 μL, subjected to the HPLC analysis.

Therefore, the purpose of this study was to develop an efficient method for reliable quantification of TA in small volumes of rat plasma and microdialysate using common laboratory equipment. A simple plasma solid-phase extraction followed by HPLC-PDA detection was developed. The method was validated according to international guidelines [14,15] in terms of specificity, linearity, precision and accuracy for both matrices. The assay was applied to the measurement of total TA levels in plasma and unbound TA and budesonide in blood and muscle microdialysis samples.

2. Experimental

2.1. Chemicals and reagents

The synthetic corticosteroids triamcinolone acetonide, budesonide and fluticasone propionate were obtained from Sigma (St. Louis, MO, USA) (Fig. 1). HPLC grade methanol and phosphoric acid were purchased from Fischer Scientific (Fair Lawn, NJ, USA). Blank male rat plasma was obtained from Lampire Biological Laboratories (Pipersville, PA, USA). Lactated Ringer's Injection USP were purchased from Baxter (Deerfield, IL, USA) and used as artificial microdialysate. Solid phase-extraction (SPE) cartridges with C18 phase (1/cc capacity and 100 mg sorbent) were purchased from Bakerbond SPE™ (JT Baker, Deventer, Netherlands).

2.2. Apparatus and chromatographic conditions

The HPLC system consisted of an Agilent 1100 series autosampler (G1329A), column oven (G1316A), degasser (G1379A), quaternary pump (G1311A) and DAD detector (G1315B). Instrument control, data acquisition and processing were performed using ChemStation software.

Chromatographic separations were obtained using a Kromasil C18 analytical column (5 μm particle size, 250 mm × 4.6 mm i.d., Hichrom, Reading, UK) which was protected by a Kromasil C18 guard column (5 μm, 10 mm × 3.2 mm, Hichrom, Reading, UK). The column temperature was maintained at 25 °C and the detection wavelength was set at 254 nm. The isocratic mobile phase consisted of methanol:water in the ratio of 72:28 (v/v). A flow rate of 0.8 mL/min was used to achieve desired chromatographic separation. The mobile phase was filtered through 0.45 μm cellulose membrane filter (Millipore, Bedford, MA, USA) and degassed in an ultrasonic bath (FS110H, Fisher Scientific, USA) prior to use. The injection volume was 10 μL for microdialysates and 20 μL for extracted plasma samples. Samples were maintained at 4 °C in the autosampler prior to injection.

2.3. Preparation of stock and working solutions

Primary stock solutions of TA, fluticasone propionate (plasma internal standard, IS) and budesonide (microdialysis calibration standard) were prepared in methanol to yield for each solution concentrations of 1 mg/mL. These stock solutions were further diluted in methanol to get intermediate concentrations of 100 μg/mL for TA, 75 μg/mL for IS and 75 μg/mL for budesonide.

Working solutions of TA (1.5–750 μg/mL) and budesonide (7.5–150 μg/mL) required for spiking plasma and microdialysate calibration and quality control samples were subsequently diluted from primary and intermediate stock solutions. All methanolic solutions were stored at −20 °C, protected from the light, until used.

2.4. Preparation of calibration standards and quality control (QC) samples

A 7% spiking with TA working solutions of appropriate concentrations were done in either blank rat plasma or Ringer's solution to obtain the desired concentration of TA for calibration and quality control (QC) samples. Four levels of quality control samples at the lowest limit of quantification (LLOQ), low (LQC), medium (MQC) and high (HQC) end of the calibration curve were prepared for both matrices. Microdialysate calibration standards (0.1, 0.25, 0.5, 1, 2.5, 5 and 10 μg/mL) and QC samples (LLOQ = 0.1 μg/mL, LQC = 0.2 μg/mL, MQC = 2 μg/mL and HQC = 7 μg/mL) were prepared prior to each analytical run, whereas plasma calibration standards (0.5, 1, 2.5, 5, 10, 25 and 50 μg/mL) and QC samples (LLOQ = 0.5 μg/mL, LQC = 1 μg/mL, MQC = 20 μg/mL and HQC = 40 μg/mL) were stored at −70 °C until analysis.

Microdialysis samples were also spiked with budesonide working solutions of appropriate concentrations to obtain the desired level of budesonide for calibration (0.5, 1, 2.5, 5, and 10 μg/mL) and QC samples (LLOQ = 0.5 μg/mL, MQC = 2 μg/mL and HQC = 7 μg/mL).

2.5. Plasma sample pre-treatment: SPE procedure

Spiked plasma samples were completely thawed in a water bath at room temperature and vortexed adequately. To 140 μL of the plasma sample, 10 μL of internal standards solution (fluticasone propionate 75 μg/mL containing budesonide 75 μg/mL) were added to yield a concentration of 5 μg/mL. Samples were mixed 1:1 with 4% phosphoric acid solution to release protein-bound drug. After thorough mixing, samples were extracted using solid phase-extraction (SPE) cartridges with C18 phase. The extraction was carried out on a Vac Elut SPS 24 solid-phase extraction manifold (Varian, Palo Alto, CA, USA). Each cartridge was conditioned by 1 column volume of methanol followed by 1 column volume of water. The diluted plasma samples were loaded onto the conditioned SPE cartridges at a flow rate of 1 mL/min. Washing was done with 600 μL of 2% phosphoric acid. Then, a low vacuum (2–5 mmHg) was applied for 2–5 min to remove the aqueous part. The analytes were eluted using 300 μL of methanol and a 20 μL aliquot of each sample was subjected to HPLC analysis.

2.6. Method validation

2.6.1. Specificity

Six different sources of blank rat microdialysate and rat plasma were screened for potential endogenous interferences in the retention times of TA, budesonide (calibration standard) and fluticasone propionate (IS).

2.6.2. Linearity

The linearity range of the method for TA was evaluated by seven-point standard curves in the concentration range of 0.1–10 μg/mL

for microdialysate and 0.5–50 μg/mL for plasma on three validation days. Budesonide calibration curves in microdialysate were prepared in the range of 0.5–10 μg/mL. Microdialysate calibration curves were constructed by plotting the analyte peak area *vs.* concentration using $1/x^2$ linear regression whereas for plasma calibration curves, the TA/IS (fluticasone propionate) peak area ratios *vs.* TA concentrations were plotted using $1/x^2$ linear regression.

2.6.3. Accuracy and precision

The intra-day precision and accuracy of the method for quantifying TA were determined by analysis of four sets of plasma and six sets of microdialysate QC samples at the LLOQ, LQC, MQC and HQC levels in a single day. The inter-day precision and accuracy were estimated by analysis of all QC samples over the three validation days. The accuracy and precision of the method for budesonide were determined by analysis of six sets of microdialysis QC samples at the LLOQ, MQC and HQC. Accuracy was calculated as the mean percent deviation (RE) of the observed concentration (C_{obs}) from the nominal concentration at each QC level, $\%RE = [(C_{obs} - C_{nom})/C_{nom}] \times 100$. Precision was expressed as percent of coefficient of variation, $\%CV = (SD/\text{mean } C_{obs}) \times 100$.

2.6.4. Extraction recovery

The extraction recovery from plasma were carried out in plasma QC samples at low, medium and high TA concentrations (1, 20 and 40 μg/mL) and at one concentration (5 μg/mL) of the IS (fluticasone propionate). The absolute percentage recovery was determined by comparing the mean peak area of four replicates of extracted samples with mean peak areas of unextracted standards of equivalent concentration, %AR = (peak area sample/peak area standard) × 100.

2.6.5. Stability

Stability tests were performed under settings likely to be encountered during sample collection, storage, preparation and analysis: microdialysate kept at room temperature (25 ± 2 °C) for 12 h, process stability (autosampler at 4 °C for 24 h), long-term stability of plasma at −70 °C for 2 months and plasma samples freeze–thaw stability (three cycles). Experiments were performed using three replicates of LQC, MQC and HQC samples of the corresponding matrix.

2.7. Microdialysis study

The proposed method was applied in a pharmacokinetic study in rats involving the simultaneous collection of microdialysate from blood and muscle to monitor free drug concentrations at steady-state in the central and peripheral (tissue) compartments. The relative recovery of TA was monitored by the calibrator budesonide. The study was approved by the Institutional Animal Care and Use Committee of University of Florida and all procedures involving animals were conducted in accordance with the Principles of Laboratory Animal Care. Adult male Sprague–Dawley rats weighting 250–300 g were used in the study (Harlan Lab, Tampa, FL, USA). Animals were anesthetized by isoflurane inhalation and placed in a heating pad in the dorsal position. Microdialysis probes (CMA/20 Elite probe, 14/10 PAES, membrane length 20 mm, cut-off 20 kDa; CMA Microdialysis, Stockholm, Sweden) were placed into the right jugular vein and right hind leg muscle of rats with the aid of a needle and guide cannula.

2.7.1. Microdialysis in vivo calibration

As a result of the continuous perfusion of the microdialysis probe, the drug concentration measured in the microdialysate is only a fraction of the true concentration in the sampling site (blood or muscle extracellular fluid). This fraction is referred to as the probe's relative recovery and its value is commonly estimated *in vivo* by the retrodialysis method prior to drug administration.

Using this approach of probe calibration, the relative recoveries by loss (diffusion out of the membrane) of the drug and calibrator are determined and related to each other by the calculation of their ratio ($\text{Recovery Ratio}_{TA:budesonide} = \text{Recovery}_{TA}/\text{Recovery}_{budesonide}$). It is assumed that the recovery by loss of the calibrator (budesonide) into the sampling site is representative for the recovery by gain of the drug (TA) from the biological fluid during the experiment [16].

For *in vivo* relative recovery ($n = 3$), probes were perfused with Ringer's solution containing TA (5 μg/mL) and the calibration standard budesonide (10 μg/mL) at a flow-rate of 1.5 μL/min with 20 min sampling interval. TA and budesonide concentrations in the microdialysate samples (C_{dial}) and in the perfusate (C_{perf}) were determined by HPLC. The relative recovery of each analyte was calculated by the following equation: $\text{Recovery}(\%) = [(C_{perf} - C_{dial})/C_{perf}] \times 100$.

2.7.2. Drug administration and sampling

After vascular and muscular probe implantations, probes were perfused with Ringer's solution containing budesonide (10 μg/mL) at 1.5 μL/min and allowed to equilibrate for 1 h. Basal microdialysates were collected at 20 min intervals. Total plasma and free blood and muscle concentrations of TA were determined at steady-state after a loading dose (50 mg/kg) of prodrug TA phosphate (triamcinolone acetonide dihydrogenphosphate dipotassium salt, Volon A soluble, Dermapharm, Germany) followed by 23 mg/kg/h infusion (0.5 mL/h) via a catheter in the tail artery. Blood and muscle microdialysates were continuously collected every 20 min, stored at 4 °C and analyzed within 24 h. Blood samples (300 μL) were collected every 30 min, centrifuged and stored at −70 °C until analysis. The free TA levels in blood and muscle ($C_{TA\,free}$) were converted from the microdialysate concentrations ($C_{TA\,dial}$) as follows: $C_{TA\,free} = (100 \times C_{TA\,dial})/(\%\text{Recovery}_{budesonide} \times \text{Recovery Ratio}_{TA:budesonide})$, using the calibrator recovery obtained at each sampling interval ($\%\text{Recovery}_{budesonide}$).

3. Results and discussion

3.1. Development of the sample pre-treatment procedure

Microdialysate samples lack proteins due to the semipermeable membrane of the probe (molecular cut-off 20 kDa), thus samples were directly injected into the HPLC system. However, the pre-analytical treatment of plasma samples was essential to obtain cleaner extracts. Different liquid–liquid and solid-phase extraction (SPE) procedures were tested. Liquid–liquid extraction using ethyl-acetate, dichloromethane and *tert*-butyl methyl ether as previously used by other investigators [8,11,12] were tested. However, our results showed relatively low recovery for TA, around 60%, poor reproducibility and specificity. Subsequently, SPE procedures were tested using octadecyl phase sorbent because of its extreme retentive nature for hydrophobic compounds. Initially, the pretreatment of the plasma samples with 4% phosphate acid (pH = 1) aimed on the release of TA from plasma proteins which is relatively high in rats (90% plasma protein binding). The optimization of the SPE procedure was done by varying the proportion of methanol in water used as washing solvent to minimize polar matrix interferences. The method proved to be unsuccessful as it did not improve the specificity of the method. However, an acidic wash (2% phosphoric acid) removed interfering endogenous substances without causing elution of the analytes. The volume of methanol as elution solvent was also optimized to improve the recovery of the analytes. A higher volume of eluate (300 μL instead of 150 μL) resulted in excellent

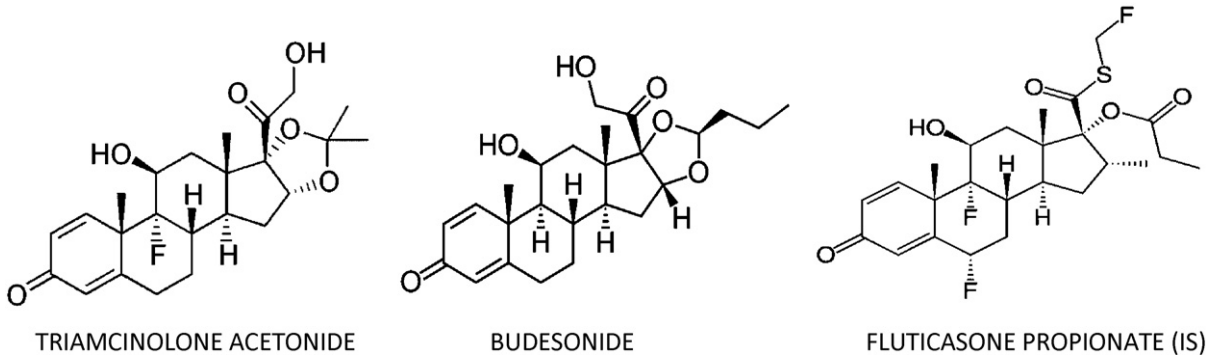

Fig. 1. Chemical structures of triamcinolone acetonide, the calibrator budesonide and the internal standard (IS) fluticasone proprionate.

recovery and minimal residual matrix. All these efforts helped us achieve an efficient SPE procedure with one wash and one elution step with no drying and reconstitution. Thus, this is a simple and economical plasma extraction procedure with increased sensitivity, specificity and throughput for determination of corticosteroids in small volume of plasma samples.

3.2. Method validation

3.2.1. Specificity

The specificity is the ability of an analytical method to differentiate and quantify the analyte in the presence of potential interfering compounds.

Chromatograms obtained from blank microdialysate, blank plasma, the peak response of TA at the medium end of the calibration curve in plasma and microdialysate calibration standards, and blood microdialysate and plasma samples obtained after constant-rate infusion of TA phosphate to a rat are shown in Figs. 2 and 3. TA, budesonide and IS (fluticasone propionate) were eluted at 6.8, 12.3 and 14.0 min, respectively. The results demonstrated there is no interference in the determination of the analytes, granting good method selectivity.

3.2.2. Linearity

The weighted linear regression (weighting factor: $1/\text{concentration}^2$) analysis was used since this option provided an improvement in the residuals with a similar coefficient of determination (r^2) to the linear model. Calibration curves for TA in both matrices exhibited consistently good coefficients of determination, $r^2 \geq 0.992$ for all microdialysate curves and $r^2 \geq 0.996$ for all plasma curves. Detailed results for linearity parameters for TA in microdialysate and plasma are listed in Table 1. Slope and intercept among microdialysate calibration curves ($n = 6$) were not statistically different ($P = 0.7407$) allowing the construction of one common curve with slope of 15.49 (±0.12) and intercept of −0.1046 (±0.0297), $r^2 = 0.9959$. Since differences between slopes and intercepts of plasma calibration curves ($n = 6$) were not significant ($P = 0.3727$), the pooled slope equals 0.2138 (±0.0027) and intercept −0.0041 (±0.0031) with $r^2 = 0.9962$. Good linearity values were also found for budesonide in microdialysate curves (Table 1). The common curve ($P = 0.8998$) has a slope of 17.07 (±0.22) and intercept of −4.053 (±0.218) with $r^2 = 0.9932$.

3.2.3. Accuracy and precision

The mean back-calculated concentrations of TA in microdialysate and plasma calibration standards with resulted accuracy

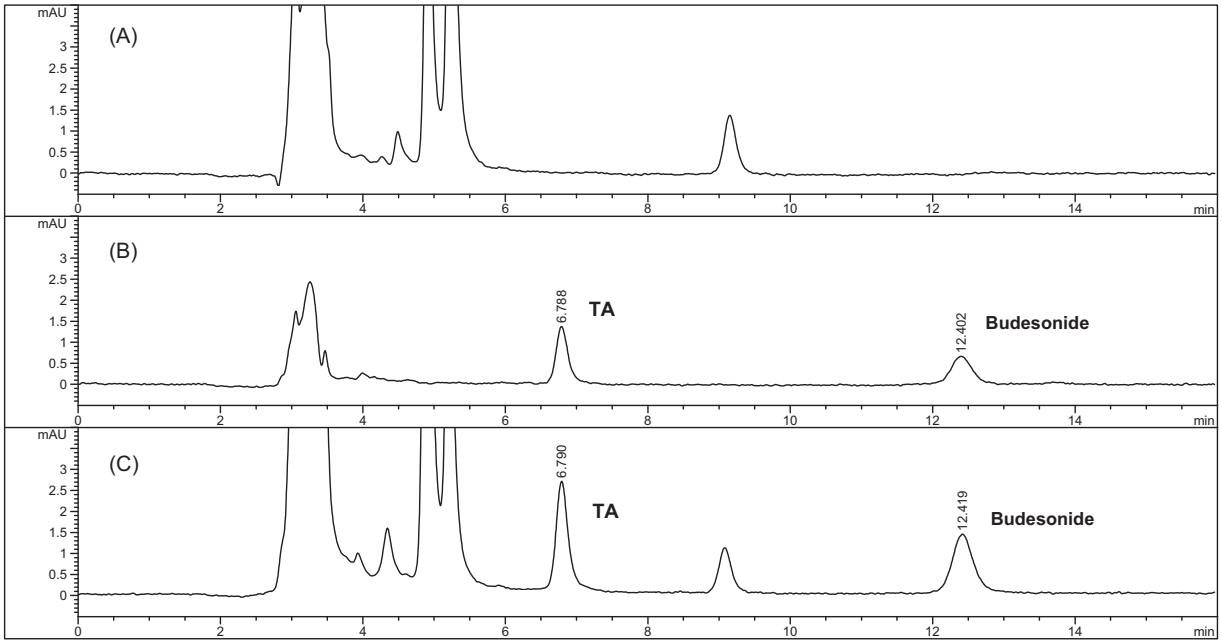

Fig. 2. Representative chromatograms of (A) blank microdialysate, (B) microdialysate calibration standard spiked with TA (1 μg/mL) and budesonide (1 μg/mL) and (C) microdialysate sample containing TA (2.86 μg/mL) and budesonide (1.92 μg/mL).

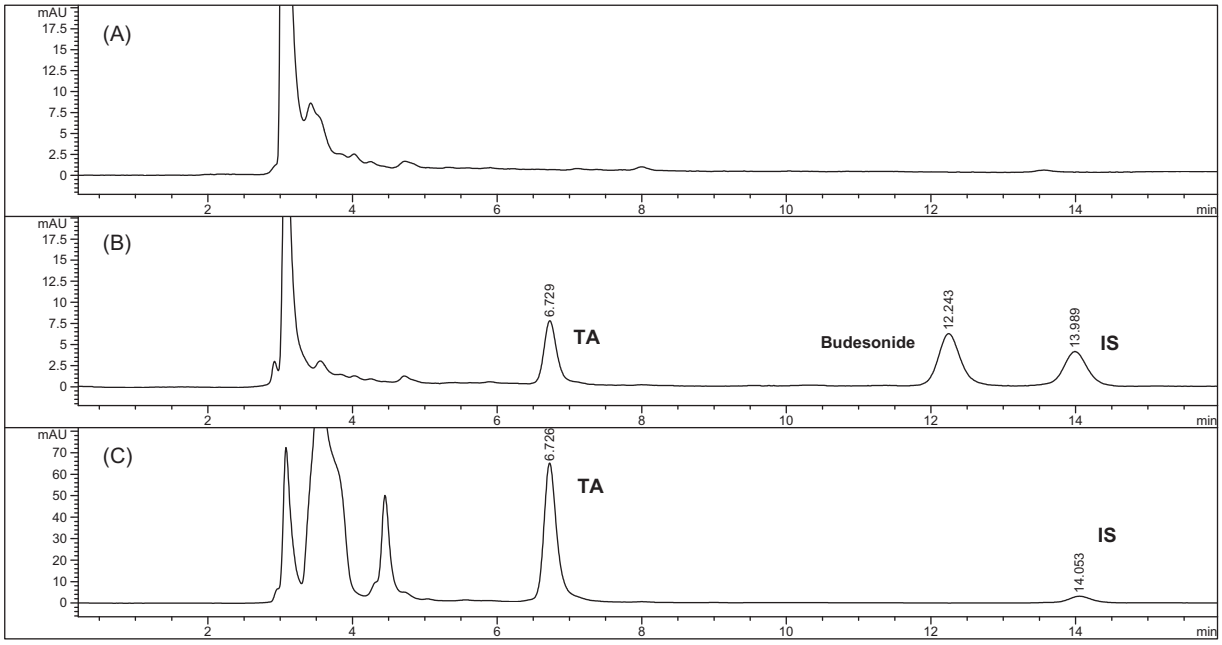

Fig. 3. Representative chromatograms of (A) blank rat plasma, (B) plasma calibration standard spiked with TA (5 μg/mL), budesonide (5 μg/mL) and IS (5 μg/mL) and (C) plasma sample containing TA (42.6 μg/mL).

Table 1
Linear regression parameters for plasma and microdialysate calibration standards.

Linearity	TA plasma			TA microdialysate			Budesonide microdialysate		
	Intercept	Slope	r^2	Intercept	Slope	r^2	Intercept	Slope	r^2
1	−0.0019	0.2156	0.9996	−0.0386	15.27	0.9916	−4.388	17.22	0.9917
2	−0.0047	0.2234	0.9997	−0.1201	15.15	0.9935	−3.807	16.91	0.9887
3	−0.0064	0.2163	0.9973	−0.1697	15.43	0.9952	−4.458	17.89	0.9992
4	−0.0083	0.2205	0.9961	−0.0262	15.34	0.9975	−3.821	17.03	0.9941
5	0.0003	0.2023	0.9979	−0.2037	16.10	0.9987	−4.210	17.08	0.9934
6	−0.0038	0.2053	0.9996	−0.0692	15.63	0.9992	−3.634	16.33	0.9995
Mean		0.2138			15.49			17.07	
SD		0.0083			0.36			0.50	
CV (%)		3.9			2.3			3.0	

(%RE) and precision (%CV) are listed in Table 2. The accuracy values of budesonide for various concentrations in microdialysate calibration standards ranged from −4.81% to 5.21% with precision between 1.74% and 5.51%.

The intra-day precision of TA QC samples for both matrices ranged between 0.50% and 6.62%, and accuracy was between −5.28% and 9.14%. The Inter-day precision was between 3.35% and 6.46%, and the accuracy values ranged between −3.19% and 6.30%. The mean observed value, coefficient of variation and relative errors of the plasma and microdialysate QC samples used on the three validation days are presented in Table 3.

The intra-day accuracy of budesonide in microdialysate QC samples (n = 6 at each concentration) ranged from −1.42% to 9.14% and precision values were between 1.80% and 4.50%. The inter-day accuracy values were 0.73% for LLOQ and 3.18% for MQC with CV of 3.39% and 4.89%, respectively.

The accuracy and precision values were well within acceptable limits stated for bioanalytical method validation: ±15% at low, medium and high range of concentrations and ±20% at the LLOQ.

3.2.4. Extraction recovery

The extent of recovery of the TA and IS from plasma was reproducible and equivalent. The mean absolute recovery (n = 4 at each concentration) at low, medium and high QC samples were 109%, 103% and 99.6% with precision of 2.02%, 4.57% and 3.94%, respectively. The mean recovery of the IS was 95.7% with CV of 1.81%.

Table 2
Summary of observed TA concentration in microdialysate and plasma calibration standards (n = 6 at each concentration).

Microdialysate					Plasma				
C_{nom} (μg/mL)	Mean C_{obs} (μg/mL)	SD	%RE	%CV	C_{nom} (μg/mL)	Mean C_{obs} (μg/mL)	SD	%RE	%CV
0.1	0.100	0.004	0.36	3.52	0.5	0.485	0.015	−3.06	3.12
0.25	0.253	0.010	1.26	3.75	1	1.05	0.04	5.16	3.69
0.5	0.481	0.007	−3.75	1.41	2.5	2.65	0.20	6.15	7.51
1	0.987	0.031	−1.30	3.11	5	4.71	0.12	−5.76	2.59
2.5	2.45	0.11	−2.03	4.59	10	9.52	0.41	−4.84	4.35
5	5.01	0.18	0.14	3.49	25	25.1	1.1	0.34	4.19
10	10.5	0.2	5.35	1.59	50	50.9	2.6	1.86	5.12

Table 3
Intra-day and inter-day accuracy (%RE) and precision (%CV) of observed TA concentrations (μg/mL) in plasma and microdialysate quality controls.

Nominal concentration	Intra-day			Inter-day		
	Mean C_{obs}	%RE	%CV	Mean C_{obs}	%RE	%CV
Microdialysate ($n = 6$)						
LLOQ (0.1 μg/mL)	0.103	3.12	5.47	0.104	4.31	4.69
LQC (0.2 μg/mL)	0.207	3.54	4.19	0.206	3.44	5.24
MQC (2 μg/mL)	1.96	−2.11	3.10	1.97	−1.44	5.36
HQC (7 μg/mL)	6.72	−3.96	1.83	7.03	0.42	3.35
Plasma ($n = 4$)						
LLOQ (0.5 μg/mL)	0.495	−0.91	4.99	0.506	0.25	6.46
LQC (1 μg/mL)	1.05	4.64	2.20	1.06	6.30	5.29
MQC (20 μg/mL)	19.4	−3.01	2.19	19.4	−3.19	5.19
HQC (40 μg/mL)	39.6	−1.08	0.89	39.2	−1.94	3.68

Table 4
Stability results of TA in rat plasma and microdialysate under various conditions ($n = 3$ at each concentration).

Storage condition	LQC			MQC			HQC		
	C_{nom}	%RE	%CV	C_{nom}	%RE	%CV	C_{nom}	%RE	%CV
Plasma									
3 freeze–thaw cycles	1	97.9	2.68	20	103	3.58	40	99.1	6.17
2 months at −70 °C	1	100	2.79	20	95.1	2.62	40	97.4	0.90
Process 4 °C, 24 h	1	102	2.78	20	96.0	0.75	40	102	3.03
Microdialysate									
Room temperature 12 h	0.2	111	1.68	2	108	6.55	7	100	2.62
Process 4 °C, 24 h	0.2	101	1.84	2	97.9	4.07	7	100	1.72

3.2.5. Stability

The results of stability test of TA in plasma and microdialysate QC samples are summarized in Table 4. TA and budesonide in spiked microdialysate proved to be stable after sample preparation and storage in the sample tray of the autosampler at 4 °C for 24 h and at room temperature for at least 12 h. Average stability for budesonide at MQC was 99.2% with precision of 3.95% and 109% with precision of 6.57% under these respective conditions. The results of process stability of plasma QC samples demonstrated that the postextraction solution is stable at 4 °C for at least 24 h. TA was also stable after three freeze–thaw cycles and after storage at −70 °C for 2 months.

Overall, the conducted stability tests indicated reliable stability for TA and budesonide under the experimental conditions.

3.3. Application to a microdialysis study

3.3.1. Microdialysis probe recoveries

The validated bioanalytical method has been applied in a microdialysis study of TA using budesonide as calibration standard. The relative recovery of TA in blood and muscle was 61.3 ± 4.8% and 58.3 ± 3.9%, respectively; whereas budesonide recovery in blood and muscle was 87.0 ± 2.0% and 86.1 ± 4.9%, respectively. The calculated ratio of relative recoveries TA:budesonide, 0.70 ± 0.06, and budesonide recovery determined at each sampling interval were used to back calculate the free TA levels in blood and muscle tissue after drug administration as follows: $C_{TA\,free} = 100 \times C_{TA\,dial}/\%Recovery_{budesonide} \times 0.7$.

3.3.2. TA concentrations

The corrected unbound TA concentrations determined at steady state in blood and muscle microdialysates were 4.66 ± 0.37 μg/mL and 5.60 ± 0.18 μg/mL, respectively. The free TA levels in rat plasma is in accordance with the simultaneously measured total TA concentration, 43.1 ± 0.7 μg/mL, when binding to plasma proteins (90%) is taken into account. There was a good correlation between free drug levels in the muscle and the free plasma concentrations.

4. Conclusion

The pharmacokinetic analysis of TA via microdialysis relies on an efficient assay capable of quantifying TA in both plasma and microdialysate matrices at a wide concentration range after systemic administration. Additionally, the relatively low concentration of TA and budesonide in minimal sample volume of microdialysates requires a sensitive assay. Both of these challenges were met by the analytical method reported here. The present HPLC-PDA analysis was found to be specific and highly reproducible for both matrices. TA and budesonide were stable during storage and sample processing. Plasma sample clean-up involves a simple solid-phase extraction procedure with no reconstitution step, allowing sufficient sample-throughput to be applied to PK studies. Usefulness of the developed method was confirmed in preclinical microdialysis studies of TA using budesonide as calibrator. In conclusion, the method developed may be applied for bioanalysis of TA in future (pre)clinical studies using free drug monitoring via microdialysis with precision and accuracy.

Acknowledgement

The authors acknowledge the CAPES/Fulbright program for providing the doctorate scholarship.

References

[1] C.S. Chaurasia, M. Müller, E.D. Bashaw, E. Benfeldt, J. Bolinder, R. Bullock, P.M. Bungay, E.C. DeLange, H. Derendorf, W.F. Elmquist, M. Hammarlund-Udenaes, C. Joukhadar, D.L. Kellogg Jr., C.E. Lunte, C.H. Nordstrom, H. Rollem, R.J. Sawchuk, B.W. Cheung, V.P. Shah, L. Stahle, U. Ungerstedt, D.F. Welty, H. Yeo, Pharm. Res. 24 (2007) 1014.
[2] N. Plock, C. Kloft, Eur. J. Pharm. Sci. 25 (2005) 1.
[3] J.P. Elshoff, S. Läer, J. Pharm. Tox. 52 (2005) 251.
[4] T. Yilmaz, C.D. Weaver, M.J. Gallagher, M. Cordero-Coma, R.A. Cervantes-Castaneda, D. Klisovic, A.J. Lavaque, R.J. Larson, Ophthalmology 116 (2009) 902.
[5] S.M. Gawchik, C.L. Saccar, Drug Saf. 23 (2000) 309.
[6] SIS Specialized Information Service. Division of the National Library of Medicine (NLM) Available from http://chem.sis.nlm.nih.gov/chemidplus/ (last accessed on 16 February 2010).
[7] J. Qu, Y. Qu, R.M. Straubinger, Anal. Chem. 79 (2007) 3786.

[8] H. Derendorf, P. Rohdewald, G. Hochhaus, H. Möollmann, J. Pharm. Biomed. Anal. 4 (1986) 197.
[9] S. Rohatagi, G. Hochhaus, H. Möllmann, J. Barth, E. Galia, M. Erdmann, H. Sourgens, H. Derendorf, J. Clin. Pharmacol. 35 (1995) 1187.
[10] H. Möllmann, P. Rohdewald, E.W. Schmidt, V. Salomon, H. Derendorf, Eur. J. Clin. Pharmacol. 29 (1985) 85.
[11] S.A. Döppenschmitt, B. Scheidel, F. Harrison, J.P. Surmann, J. Chromatogr. B 682 (1996) 79.
[12] S.-Q. Zhang, H.R. Thorsheim, S. Penugonda, V.C. Pillai, Q.R. Smith, R.J. Mehvar, J. Chromatogr. B 887 (2009) 927.
[13] C. Rojas, N.V. Nagaraja, A.I. Webb, H. Derendorf, J. Pharm. Sci. 92 (2003) 394.
[14] Food and Drug Administration of the United States, Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), Washington, DC, 2001.
[15] International Conference on Harmonization. ICH Q2(R1): Validation of Analytical Procedures: Text and Methodology, ICH, 1996.
[16] E.C. de Lange, A.G. de Boer, D.D. Breimer, Adv. Drug Deliv. Rev. 45 (2000) 125.