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Short communication

Liquid chromatography–tandem mass spectrometric assay for clobetasol propionate in human serum from patients with atopic dermatitis

Rolf W. Sparidans[a,*], Sara G.A. van Velsen[b], Marlise P. de Roos[a], Jan H.M. Schellens[a,c], Carla A.F.M. Bruijnzeel-Koomen[b], Jos H. Beijnen[a,c,d]

[a] Universiteit Utrecht, Faculty of Science, Department of Pharmaceutical Sciences, Section of Biomedical Analysis, Division of Drug Toxicology, Sorbonnelaan 16, 3584 CA Utrecht, The Netherlands
[b] University Medical Centre Utrecht, Department of Dermatology and Allergology, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands
[c] The Netherlands Cancer Institute, Department of Clinical Pharmacology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[d] Slotervaart Hospital, Department of Pharmacy & Pharmacology, Louwesweg 6, 1066 EC Amsterdam, The Netherlands

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ABSTRACT

A bioanalytical assay for the topical corticosteroid clobetasol propionate was developed and validated. For the quantitative assay 0.5 ml human serum samples, supplemented with clobetasone butyrate as internal standard, were extracted with hexane–ether. Evaporated and reconstituted extracts were injected on a polar embedded octadecyl silica column with isocratic elution using formic acid in water–methanol as mobile phase. The eluate was led into the electrospray interface with positive ionization and the analyte was detected and quantified using the selective reaction monitoring mode of a triple quadrupole mass spectrometer. The assay was validated in the range 0.04–10 ng/ml, the lowest level of this range being the lower limit of quantification. Precisions were 5–10% and accuracies were between 102 and 109%. The drug was stable under all relevant conditions. Finally, the assay was successfully applied on patients suffering from severe atopic dermatitis treated topically with clobetasol propionate.

1. Introduction

Topical corticosteroids represent the mainstay account for the most part of treatment of atopic dermatitis. Clobetasol propionate (Fig. 1) is a potent topical corticosteroid often used for the treatment of severe, active atopic dermatitis. Although clobetasol propionate is highly effective in controlling this disease, percutaneous absorption of the drug during daily application of 20–30 g Dermovate® ointment (0.05%, w/w clobetasol propionate) can lead to suppression of the adrenal gland function with a decrease in cortisol production [1–3]. The amount of percutaneous absorption of clobetasol propionate, and the effect on the adrenal gland, probably depends on many factors such as the extent of body surface affected by atopic dermatitis, the mode of application and the amount used per day [4].

In order to quantify the (low) systemic levels of clobetasol propionate in a pharmacokinetic study, a radioimmunoassay was used by Hehir et al. [3]. As far as we know, other assays for systemic levels of clobetasol propionate have not been published hitherto.

The development and validation of a sensitive bioanalytical assay for clobetasol propionate in human serum using LC-triple quadrupole MS is reported hereafter. The topical use of clobetasol propionate leading to low systemic serum levels (approximately 1 ng/ml, 24 h after treatment [3]) particularly demands the high sensitivity of the presented method.

2. Material and methods

2.1. Chemicals

Clobetasol propionate (≥98%) and clobetasone butyrate (≥98%; IS) were both obtained from Sigma (St. Louis, MO, USA). LC–MS grade water, gradient grade acetonitrile, methanol of HPLC quality and *n*-hexane (p.a.) were from Biosolve (Valkenswaard, The Netherlands). Water not used as eluent was home-purified by reversed osmosis on a multi-laboratory scale. Formic acid and diethyl ether were of analytical grade and originated from Merck (Darmstadt, Germany). Human serum was supplied by Innovative Research (Southfield, MI, USA).

Abbreviations: CID, collision induced dissociation; LLOQ, lower limit of quantification; SRM, selected reaction monitoring; QC, quality control.

* Corresponding author. Tel.: +31 30 2537391; fax: +31 30 2535180.
E-mail address: R.W.Sparidans@uu.nl (R.W. Sparidans).

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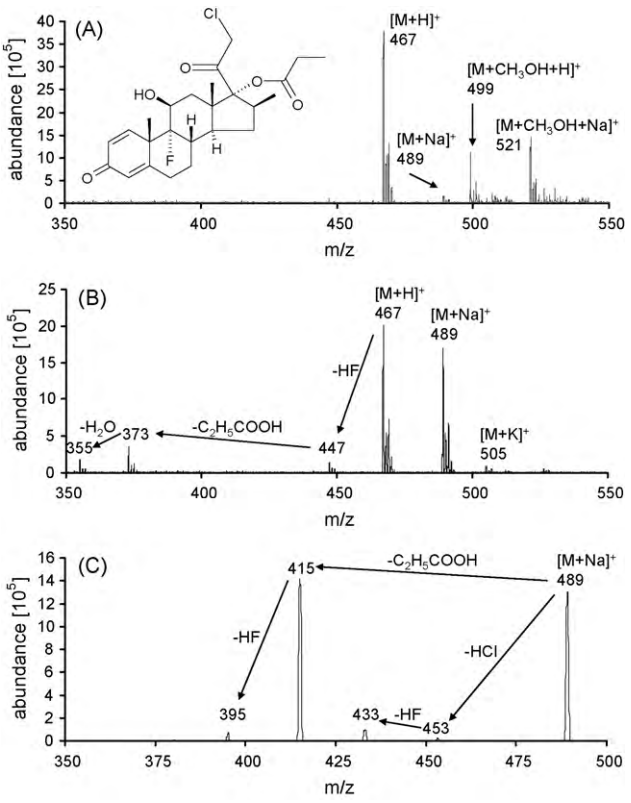

Fig. 1. Chemical structure and MS spectra of clobetasol propionate, recorded during chromatographic elution after injection of 20 μl of 1 μg/ml clobetasol propionate. (A) ESI spectrum without up-front CID (mass resolution *m/z* 0.2); (B) ESI spectrum with up-front CID at −22 V (mass resolution *m/z* 0.2); (C) product spectrum (CID = −10 V) of the sodium adduct at *m/z* 489.0 (mass resolution *m/z* 0.7).

2.2. Equipment

The LC–(UV)–MS/MS equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler, two LC10-ADvp-μ pumps, a SPD10-Avp spectrophotometric UV–vis detector (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Electron, Waltham, MA, USA). For data recording and system controlling the Finnigan Xcalibur software (version 1.4, Thermo Electron) was used.

2.3. LC–MS/MS conditions

Partial-loop injections (20 μl) were made on a Polaris® 3 C18-A column (50 mm × 2 mm, d_p = 3 μm, average pore diameter = 10 nm, Varian, Middelburg, The Netherlands) with a corresponding 10 mm pre-column. The column temperature was maintained at 40 °C and the auto-injector sample racks were maintained at 4 °C. The mobile phase (flow rate 0.5 ml/min) was composed of a mixture of water containing 1% of formic acid and methanol (30:70, v/v). The positive electrospray detection started 0.6 min after injection, ionization voltage was 4600 V, capillary temperature 268 °C and nitrogen sheath, ion sweep and auxiliary gasses were 45, 16 and 17 arbitrary units, respectively. The up-front collision induced dissociation (CID) voltage was −22 V. Using unit mass resolutions, selected reaction monitoring (SRM) transitions (with collision energy, tube lens off set and dwell time) were 489.0 → 415.0 (−10 V; 110 V; 0.4 s) and 501.1 → 413.0 (−13 V; 120 V; 0.1 s) for clobetasol propionate and IS respectively.

2.4. Sample pre-treatment

To a volume of 0.5 ml human serum, pipetted into a 10-ml borate glass tube with a Teflon lined screw cap, 50 μl of 20 ng/ml IS in 50% (v/v) methanol and 4 ml diethyl ether/*n*-hexane (1/3; v/v) were added. The tube was closed and shaken by a rotary mixer at 50 rpm for 10 min. After centrifugation of the sample at 2643 × *g* at ambient temperature for 5 min, the tube was placed in the freezer at −30 °C for ca. 1 h. Afterwards, the organic extract was poured off in a conical 10 ml glass tube and evaporated at 35 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of 50% (v/v) methanol using vortex mixing. After centrifugation at 2643 × *g* for 5 min the sample was pipetted into a 250 μl glass insert placed in an injection vial.

2.5. Validation

A laboratory scheme based on international guidelines [5,6] was used for the validation procedures.

2.5.1. Calibration

Stock solutions of clobetasol propionate at 1 and 2 mg/ml and IS at 1 mg/ml were prepared in methanol. The stock solutions were stored at −30 °C.

The 1 mg/ml stock solution of clobetasol propionate was diluted to a 10 ng/ml calibration sample (stored at −30 °C) in pooled human serum. Additional calibration samples were prepared daily at 4, 1, 0.4, 0.1 and 0.04 ng/ml by dilution with the blank matrix. All calibration samples were processed in duplicate for each daily calibration. Least-squares linear regression with the reversed square of the concentration of the analyte ($1/x^2$) was employed to define the calibration curves using the ratios of the peak area of clobetasol propionate and IS.

2.5.2. Precision and accuracy

The 2 mg/ml stock solution of clobetasol propionate was used to obtain validation (quality control (QC)) samples in pooled human serum at 8 ng/ml (QC-high), 0.5 ng/ml (QC-med) and, 0.05 ng/ml (QC-low). The QC samples were stored −30 °C. Precisions and accuracies, including between day variations of the accuracy, were determined by sextuple analysis of each QC in three analytical runs on three separate days for all QCs (total: n = 18). Relative standard deviations were calculated for both the within day precision and the between day precision. The average concentrations found (n = 18) were reported relatively to the spiked levels to represent the accuracy.

2.5.3. Lower limit of quantification (LLOQ)

A serum sample at the LLOQ level (0.04 ng/ml), was analyzed in six-fold. Precision and accuracy were then calculated.

2.5.4. Selectivity

Six individual serum samples were processed to test the selectivity of the assay. These samples were processed as double blanks (no clobetasol propionate, no IS) and after spiking with 0.05 ng/ml clobetasol propionate (QC-low) and addition of the IS. The spiked samples were also used to assess the inter-batch variation of the matrix effect.

2.5.5. Recovery

The extraction efficiency (recovery) was determined in quadruplicate by comparing responses of processed samples (QC-high, -med and -low) with extracts of drug-free human serum reconstituted in reconstitution solvent spiked with the analytes at the same levels. Ionization efficiency (ion suppression or enhancement) was

Table 1
Stability data (recovery [%]; ±SD) of clobetasol propionate in human serum, reporting the percentage of the initial concentration.

Condition	QC-high	QC-low	*n*
8 h at ambient temperature	100.3 ± 4.4	80.4 ± 6.0	3
3 freeze–thaw cycles	100.8 ± 5.0	107.9 ± 11.5	3
8 weeks at −30 °C	94.9 ± 3.9	96.4 ± 14.3	4
8 weeks at −80 °C	93.6 ± 10.2	93.9 ± 9.2	4

assessed by comparing responses of the spiked reconstitution solvent at the three QC levels with and without the presence of reconstituted blank extraction residue. The extraction and ionization efficiencies (recoveries) of the IS were assessed using identical procedures at the IS concentration used in the assay.

2.5.6. Stability

The stability of clobetasol propionate was investigated in QC-high and -low serum samples. Triplicate or quadruplicate analysis of these samples was performed after storage under relevant conditions (see Table 1).

The stability of clobetasol propionate and clobetasone butyrate was also investigated in the methanolic stock solutions using LC–UV at 240 nm.

2.6. Patient samples

Two patients suffering from severe atopic dermatitis were treated with 30 g ointment containing 0.05% (w/w) clobetasol propionate (Dermovate®) twice daily over the whole body during hospitalization for 7 days and once daily for 5 days (patient A: female, 19 years) and twice daily for 10 days (patient B: female, 66 years). Blood samples were collected in 10 ml Vacutainer® tubes (BD, Plymouth, UK) from the antecubital vein before and after the first day of treatment, on the last day of hospitalization and on 2 days in between. After coagulation for approximately 30 min at ambient temperature, serum was separated by centrifugation at 1811 × *g* for 10 min. Serum samples were stored at −30 °C for not longer than 1 month and stored further at −80 °C until analysis. Sample analysis of non-blank samples (6 out of 8 were still available) was duplicated 3 months after the initial analysis.

3. Results and discussion

3.1. Method development

Because of the high sensitivity that had to be achieved, a simple protein precipitation as pre-treatment procedure was expected useless due to insufficient selectivity. Liquid–liquid extraction is a more selective procedure with the possibility to concentrate the analyte by evaporation of the extract. It has been successfully applied for several LC–MS/MS assays for corticosteroids in plasma [7–9]. The hexane–diethyl ether mixture was chosen as the extraction solvent mixture because of the possibility to optimize the ratio of both solvents. The extraction procedure reported herein was based on the method of dos Santos Pereira et al. [8]; 25% (v/v) diethyl ether resulted in optimal recovery of clobetasol propionate.

Steroids can be ionized by different atmospheric ionization techniques, ESI, APCI and APPI, using both, positive and negative ionization. Positive ESI has been used most frequently for endogenous steroids [10] and this combination was also shown to be suited for several synthetic glucocorticoids [11]. On the other hand, Antignac et al. [12] reported negative ionization to be the first choice for corticosteroids, due to the selective loss of the base (formate or acetate, depending on the eluent additive) and formaldehyde during CID of the negative base adduct.

Table 2
Assay performance data of clobetasol propionate resulting from 18 validation (QC) samples in 3 analytical runs, including between day variations of the accuracy.

Nominal concentration [ng/ml]	Within day precision [%]	Between day precision [%]	Accuracy [%]
8	5.4	8.5	101.9 ± 8.3
0.5	9.3	10.0	109.7 ± 3.8
0.05	9.7	9.9	108.7 ± 2.4

For clobetasol propionate this transition of $[M + HCOO]^-$ showed a low response using ESI. Using APCI, however, a specific propanoic acid loss from the deprotonated parent molecule during CID (in the second quadrupole) was observed that could lead to a sensitive LC detection. No prominent base adduct was observed with negative APCI. In the positive mode, both, ESI and APCI, showed a prominent protonated molecule with comparable abundances. CID of the protonated clobetasol propionate showed sequential losses of hydrogen fluoride, propanoic acid and water with almost equal maximal responses for $[M\text{-}HF\text{-}HOCOC_2H_5 + H]^+$ and $[M\text{-}HF\text{-}HOOCC_2H_5\text{-}H_2O + H]^+$. In addition to the protonated molecule, ESI in the positive ion mode produced sodium adducts and solvent clusters (Fig. 1A). Using methanol as the organic modifier, methanol loss of the sodiated methanol cluster was the most abundant SRM transition. The use of this transition, however, did not result in sensitive detection in positive ESI because of its poor specificity. The abundance of the sodiated molecule in positive ESI could be increased by up-front CID (shown in Fig. 1A and B) and the CID in the second quadrupole of this stable sodium ion resulted in a specific loss of propanoic acid at a low CID voltage (Fig. 1C). During the MS/MS optimization experiments the two very promising SRM transitions with specific propanoic acid loss (from $[M-H]^-$ with APCI and from $[M + Na]^+$ with ESI) were investigated further. Their use with LC–MS/MS in the SRM mode showed the highest sensitivity for positive ESI which was then chosen for developing and validating the presented assay. As expected and based on the structural resemblance, this transition was also observed for the IS clobetasone butyrate.

3.2. Validation

SRM chromatograms of clobetasol propionate and the IS are depicted in Fig. 2, showing chromatograms of a double blank (no analyte, no IS), an LLOQ spiked and a patient sample.

3.2.1. Calibration

The response function was evaluated in six analytical runs (72 calibration samples) and proved clearly to be sufficiently linear and monotone (data not shown) with average precisions for each concentration in the range 94–108%.

3.2.2. Precision and accuracy

Assay performance data from the validation samples at three concentrations are reported in Table 2. Between day variations and deviations of the accuracy ≤10% were observed for all levels. Therefore, the upper limit of the calibration range could be assigned to the upper limit of quantification and precisions, and deviations of the accuracy met the required ±15% [5,6].

3.2.3. Lower limit of quantification

The six-fold analysis of the 0.04 ng/ml serum sample resulted in a precision of 9.7% and an accuracy of 104.2%. This level, also being the lowest level of the calibration range, could be attributed to the LLOQ because precision and accuracy met the required ±20% [5,6].

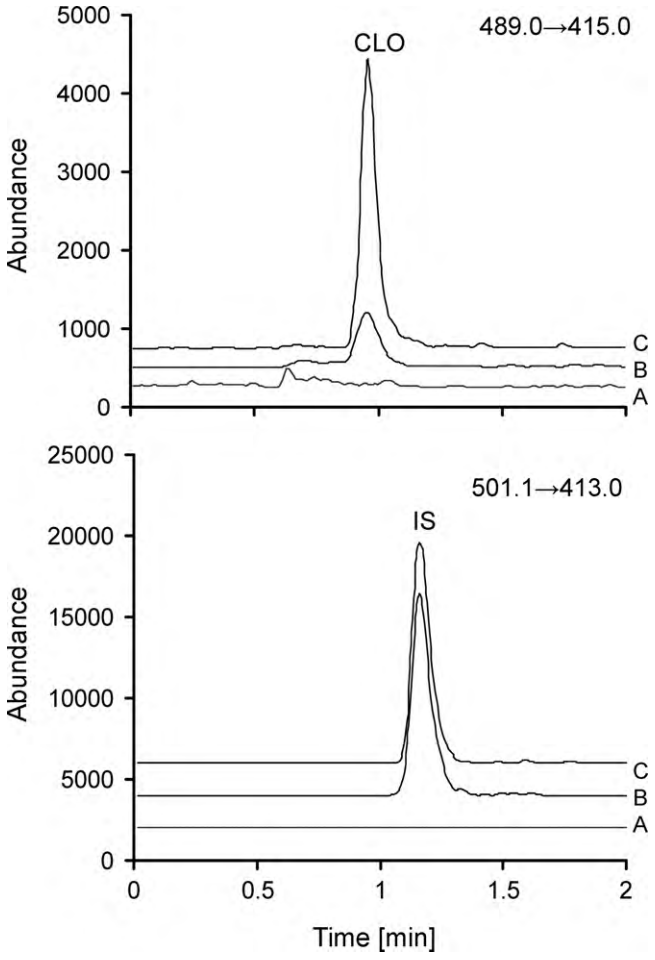

Fig. 2. SRM chromatograms of (A) blank serum without IS, (B) serum spiked with clobetasol propionate at the LLOQ level (0.04 ng/ml), and (C) patient serum sample (last sample of patient B in Fig. 3) containing 0.149 ng/ml clobetasol propionate.

3.2.4. Selectivity

The analysis of six batches of blank samples showed no interfering peaks in the SRM traces for clobetasol propionate and IS in human serum. Blank responses could not be distinguished from the detector noise (signal-to-noise ratio <3) for both, clobetasol propionate and IS and were all each below 15% of the LLOQ response of clobetasol propionate (20% is required [6]) and below 0.2% of the regular signal of the IS. The absence of any interference in these experiments is a proof of the high selectivity of the assay [5,6]. The average response of the QC-low-spiked blank samples (0.05 ng/ml; $n = 6$, ±SD) was 0.047 ± 0.004 ng/ml clobetasol propionate. In addition to the absence of interference this result shows no increased variation due to an inter-batch variability of the matrix effect.

3.2.5. Recovery

The recovery experiments showed only small extraction losses (<15%) for clobetasol propionate at the three QC levels and the recovery of the extraction of the IS was 76 ± 14% ($n = 4$). Ion suppression was below 17% for all QC levels of clobetasol propionate and was 20 ± 7% ($n = 4$) for the IS. These low losses of analyte and IS, during both, extraction and ionization, and the low inter-batch variability of the matrix effect all contributed to the successful validation of this assay [5,6].

3.2.6. Stability

Recoveries of clobetasol propionate in serum after different storage procedures are shown in Table 1. From the recoveries in the range of 80–108% only the 80% of the QC-low sample at ambient temperature for 8 h needed some attention. Therefore, additional experiments at a 0.2 ng/ml level (4 h at ambient temperature and 2 h at 37 °C) were performed. Recoveries at these conditions were 105 ± 5% ($n = 4$) and 106 ± 10% ($n = 4$), respectively. Finally, sufficient stability of the serum samples all under relevant conditions was demonstrated.

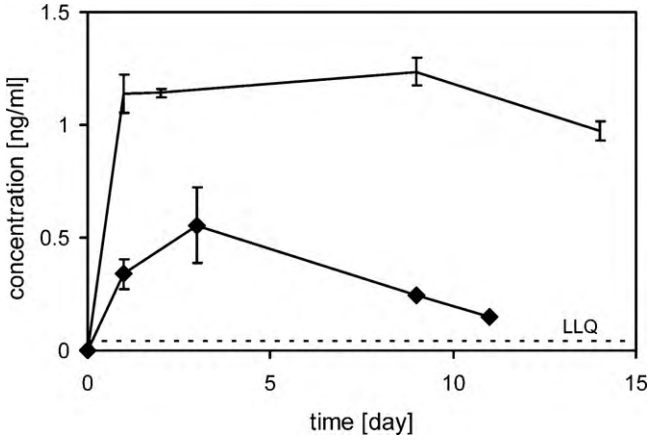

Fig. 3. Pharmacokinetic profile of two patients treated with 30 g ointment containing 0.05% (w/w) clobetasol propionate (Dermovate®) twice-daily during hospitalization for 14 (patient A, treatment reduced to once daily after 7 days; 4 duplicate sample analysis, only error bars are shown) and 10 days (patient B; 2 duplicate sample analyses; ♦), respectively.

Recoveries of the analytes in methanolic stock solutions were 100.4% for clobetasol propionate and 97.1% for clobetasone butyrate (both with $n = 2$ at 2 mg/ml) after being subjected for 6 h to ambient temperature. After storage at −30 °C for 1 year the recovery of clobetasol propionate was 101.2% ($n = 2$ at 1 and 2 mg/ml respectively) and 101.3% for clobetasone butyrate ($n = 1$ at 1 mg/ml). These results facilitate the potential use of stock solutions of both corticosteroids during a whole year.

3.3. Patient samples

Pharmacokinetic plots of clobetasol propionate levels in serum *vs.* time are shown in Fig. 3 for two patients. The assay clearly shows its ability to quantify the clobetasol propionate serum levels of these patients during treatment with low variations of duplicate analyses. In addition, levels after termination of the clobetasol propionate treatment showed to be quantifiable for about 3 remaining days (data not shown).

4. Conclusions

The first validated assay for clobetasol propionate in human serum has now been reported. The LC–MS/MS assay meets commonly accepted criteria for precision, accuracy, recovery and stability [5,6]. The sensitivity of the method is suitable for monitoring the drug in serum in patients treated topically with the drug until 3 days after the last whole body treatment and meets the sensitivity of analogous assays for other corticosteroids [7–9]. The new assay showed to be a valuable tool for clinical studies with hospitalized patients suffering from atopic dermatitis during topical treatment with clobetasol propionate.

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