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Analysis of betamethasone in rat plasma using automated solid-phase extraction coupled with liquid chromatography–tandem mass spectrometry. Determination of plasma concentrations in rat following oral and intravenous administration

C.S. Tamvakopoulos*, J.M. Neugebauer, M. Donnelly, P.R. Griffin

Merck Research Laboratories, Rahway, NJ 07065, USA

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Abstract

A method is described for the determination of betamethasone in rat plasma by liquid chromatography–tandem mass spectrometry (LC–MS–MS). The analyte was recovered from plasma by solid-phase extraction and subsequently analyzed by LC–MS–MS. A Packard Multiprobe II, an automated liquid handling system, was employed for the preparation and extraction of a 96-well plate containing unknown plasma samples, standards and quality control samples in an automated fashion. Prednisolone, a structurally related steroid, was used as an internal standard. Using the described approach, a limit of quantitation of 2 ng/ml was achieved with a 50 μl aliquot of rat plasma. The described level of sensitivity allowed the determination of betamethasone concentrations and subsequent measurement of kinetic parameters of betamethasone in rat. Combination of automated plasma extraction and the sensitivity and selectivity of LC–MS–MS offers a valuable alternative to the methodologies currently used for the quantitation of steroids in biological fluids.

Keywords: Betamethasone

1. Introduction

Corticosteroids like betamethasone, dexamethasone and prednisolone are known effective drugs for the treatment of a variety of allergic diseases such as asthma, dermatitis, and allergic rhinitis [1,2]. Although chronic treatment is limited by systemic side effects, corticosteroids are regarded as the most effective treatment currently available for atopic diseases [2]. The development of animal models for inflammatory disease have relied on the use of corticosteroids such as betamethasone as positive controls. Since drug plasma concentrations often correlate to efficacy, easily applicable methods for the quantitation of steroids in biological fluids are essential in understanding inflammatory disorders.

Skrabalak et al. [3] described a method for the determination of betamethasone and its major metabolite (6β-hydroxybetamethasone) in equine urine, by micro-liquid chromatography–mass spectrometry (micro-LC–MS). It was recognized by the authors that the polar, non-volatile, heat-labile nature of steroids made analysis by gas chromatography–mass

*Corresponding author. Tel.: +1-732-594-8162; fax: +1-732-594-9545.

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spectrometry (GC–MS) difficult, while LC–MS presented a viable non-destructive alternative. Fiori et al. [4] presented studies in which several corticosteroids, including betamethasone were measured in milk replacers by LC–atmospheric pressure chemical ionization (APCI) MS. Aliquots (5 ml) of milk replacement were processed by solid-phase extraction (SPE) cartridges and subsequently evaluated by liquid chromatographic separation and detection on a single quadrupole mass spectrometer in the full scan mode. The assay's specificity relied on a combination of retention time and unique fragmentation patterns of the various analytes observed in full scan mode. The methodologies described above, for the measurement of betamethasone in biological fluids, were based on single quadrupole analysis. In more recent years, the use of LC–MS–MS coupled with robotic sample preparation, allowed the development of high throughput alternatives for analysis of steroids. Rule and Henion [5] described the quantitation of two steroids, equilenin and progesterone, in human urine by 96-well SPE and LC–MS–MS analysis in the APCI mode with limits of quantitation (LOQs) of 1 ng/ml. Similar automated sample preparation approaches have been described for the determination of analytes from different structural classes such as protease inhibitors in plasma and cartilage tissue [6]. Stolker et al. [7] demonstrated that quantitation of dexamethasone, an isomer of betamethasone, could be achieved in urine down to the 0.5 ng/ml level by SPE–LC–MS–MS. Similarly Polettini et al. [8] showed that betamethasone could be measured in urine by LC–LC–MS–MS with an LOQ of 1 ng/ml. The authors of this study reported that direct analysis of betamethasone in urine by LC–MS–MS or LC–LC–MS did not provide satisfactory results in terms of sensitivity due to matrix effects. The studies by Stolker and Polettini described improvements of earlier methods for the analysis of dexamethasone and betamethasone, respectively with increases in selectivity and sensitivity. However both approaches depend on the analysis of high volumes of biological fluids (5–7.5 ml of urine) and have not been applied towards analysis of plasma.

Our laboratory's need for selective, sensitive and automated methods for the measurement of corticosteroids in animal plasma, prompted us to develop a method for the quantitation of betamethasone of rat plasma (50 μl). A method was developed utilizing TurboIonSpray which was found to afford higher sensitivity than APCI for the measurement of betamethasone, as a result of reduced source fragmentation of the precursor ions. Since TurbolonSpray can be sensitive to interferences from the biological matrix, an effective clean up procedure (96-well solid-phase extraction) was developed in order to minimize the potential for matrix interferences and ion suppression. Ion suppression effects were described by Matuszewski et al. [9], using measurements of finasteride in human plasma. The authors cautioned against the perception that LC–MS–MS guarantees specificity and demonstrated how ion suppression of the analyte resulted in reduction of signal due to interfering compounds from the matrix that was used for analysis. In the studies with finasteride, it was shown that such effects could be minimized by effective sample clean up or by increasing high-performance liquid chromatography (HPLC) retention. The examples offered by Matuszewski et al. were based on large volumes of plasma (1 ml) in comparison to the volume used in our assay (50 μl). It should also be noted that such effects can differ from analyte to analyte. Although we did not have any evidence that ion suppression or interfering components from the biological assay (e.g., circulating metabolites in plasma), would compromise analysis for the analytes studied in this manuscript (betamethasone, prednisolone), we thought that employing the described extraction procedure would minimize such effects. We also find through our experience of employing mass spectrometry for the analysis of compounds in biological fluids that sample clean up can protect the instrumentation, thus requiring less frequent source cleaning and maintenance. The combination of automated sample extraction with LC–MS–MS analysis offered advantages over previously reported methods for the quantitation of betamethasone with increased throughput and reduced sample size that was compatible with in vivo studies in rat.

Although the pharmacokinetics of betamethasone in rabbits [10] and healthy adults [11] have been referenced in the literature, the pharmacokinetics in rat following oral and intravenous administration have not been described. The in vivo data described

in this manuscript provides a valuable application to the described methodology and a useful insight into the pharmacokinetics of betamethasone in rat.

2. Experimental

2.1. Materials

Betamethasone and prednisolone were obtained from Sigma, St. Louis, MO, USA. A different source of betamethasone was TCI-EP, Tokyo, Japan. HPLC-grade acetonitrile, methanol, ammonium acetate, ammonium formate and reagent-grade trifluoroacetic acid were purchased from Fisher Scientific, Fair Lawn, NJ, USA. Formic acid was purchased from Aldrich, Milwaukee, WI, USA.

2.2. Sample preparation

Stock solutions of both analyte and internal standard were prepared in 50% acetonitrile in water. Stock spiking solutions of betamethasone, ranging from 4 to 4000 ng/ml, were prepared and placed in a 96-well plate. A set of solutions of betamethasone at three different levels (20, 200, 2000 ng/ml) were prepared separately in order to be used for the preparation of quality control (QC) samples. Standard curves (2–2000 ng/ml) and QC samples (10, 100, and 1000 ng/ml) were prepared daily by spiking 50 μl of plasma with 25 μl of the solutions described above.

2.3. Automated 96-well preparation and solid-phase extraction procedures

A four-tip Packard Multiprobe II liquid handling system (Meriden, CT, USA) and 10 mg Waters Oasis HLB extraction plates (Milford, MA, USA) were utilized for the preparation of a 96-well plate source plate with standards, QCs and study samples. Subsequently solid-phase extraction was performed using the same system. The 96-well sample source plate was first prepared by the Packard by transferring 50 μl aliquots of blank plasma to positions of an empty 96-well plate designated for the preparation of standards and QC samples. Standards and QCs were prepared by adding 25 μl of the corresponding spiking solutions. Plasma samples from a pharmacokinetic study were provided in plate format and transferred (50 μl) onto the source plate at designated wells. Next, 50 μl aliquots of a 1 μg/ml solution of prednisolone (internal standard) were added to each sample (with the exception of the double blank sample) by the multiprobe unit. Finally 0.25 ml of water was added to all the samples of the source plate. The Multiprobe was programmed to perform solid-phase extraction as follows: the extraction plate was conditioned with 0.5 ml of methanol followed by addition of 0.5 ml of water. Sample from the source plate was transferred onto the extraction plate in two aliquots of 240 μl and aspirated to waste. The extraction plate was then washed (to waste) with 0.5 ml of water. The sample was eluted to a 96-well collection plate with 0.5 ml of 90% acetonitrile, 0.1% trifluoroacetic acid. The collection plate was then placed in a heating block at 45 °C and evaporated to dryness under constant flow of nitrogen. Prior to analysis, the samples were reconstituted with 0.2 ml of 50% acetonitrile, 10 m*M* ammonium formate, 0.1% formic acid.

2.4. Liquid chromatography

HPLC was performed using a Perkin-Elmer Series 200 Micro Pump equipped with a Perkin-Elmer Series 200 autosampler. Samples were injected from 96-well autosampler plates with an injection volume of 10 μl. Chromatography was performed using a 50×2.1 mm, 5 μm SB C_{18} Zorbax column with a 0.5 μm prefilter (Upchurch Scientific, Oak Harbor, WA, USA). The mobile phase consisted of a mixture of acetonitrile–water (50:50) containing 10 m*M* ammonium formate and 0.1% formic acid delivered at a flow-rate of 0.2 ml/min.

2.5. Tandem mass spectrometry

Tandem mass spectrometry was carried out on a Perkin-Elmer Series API 3000 mass spectrometer (Toronto, Canada) using the TurboIonSpray source in the positive ion mode. The probe temperature was set at 400 °C, ion spray voltage set at 5000 V. Orifice potential was optimized at +65 V for betamethasone and at +55 V for the internal standard (prednisolone). Collision energy was optimized for each

transition by infusing solutions of betamethasone and prednisolone with an infusion pump (Harvard Apparatus). Specific multiple reaction monitoring (MRM) transitions were optimized for betamethasone (393→372.8, 393→354.8, 393→337) and for prednisolone (361→324.8, 361→342.8). The dwell time of 200 ms was used for each transition monitored.

2.6. Animals and dosing procedure

Specific-pathogen-free, male, 350–400 g Sprague–Dawley rats (Taconic Farms, Germantown, NY, USA) were used. They arrived with cannulas placed in the femoral vein and artery. On the day of the study, the rats were dosed intravenously (i.v.) with compound (i.v.—1 mg/kg, orally—2 mg/kg) through the femoral vein or per os (orally) by gavage. Typically for discovery type of studies, two subjects were dosed for the i.v. phase and three subjects for the oral phase of the experiment. Blood samples from the femoral artery catheter were collected at various time points (i.v.—5 min, 15, 30, 1 h, 2, 4, 6, and 8; orally—15 min, 30, 1 h, 2, 4, 6, and 8) into lithium heparin Microfuge tubes. Following sample collection, the catheter was flushed with sterile, heparinized saline (20 units/ml). Plasma was frozen at −70 °C until analysis.

2.7. Kinetic parameters

The Watson–Drug Metabolism Laboratory Information Management System (Wayne, PA, USA) was used for the determination of pharmacokinetic parameters. Oral bioavailability was determined from the dose-adjusted $AUC_{(0-\infty)}$ (area under the curve) ratio following oral dose (2 mg/kg) relative to that of the intravenous dose (1 mg/kg).

3. Results and discussion

3.1. Extraction procedure using 96-well SPE

The Oasis HLB extraction 96-well plates were employed for sample clean-up prior to LC–MS–MS analysis. The use of 96-well plates allowed the development of an automated extraction procedure. The recovery of analyte was determined at three different concentrations (20, 200, and 1000 ng/ml) by comparing the area ratios of analyte to internal standard between samples spiked with betamethasone before the extraction and samples spiked post-extraction with betamethasone at the same level (internal standard was added before the extraction for both sets). The recoveries were 70, 73, and 70% for the 20, 200, and 1000 ng/ml concentrations, respectively, which was adequate for determining betamethasone concentrations in rat plasma. Similar recovery estimates were achieved by direct comparison of area counts of betamethasone before and after the extraction, suggesting that no artifacts were generated due to variation of internal standard extraction efficiency. Solid-phase extraction afforded clean extracts that are less likely to cause ion suppression when using TurboIonSpray. Liquid–liquid extraction could also be employed for the extraction of steroids from rat plasma [12]. However, we found that liquid–liquid extraction was a more difficult technique to automate in comparison to solid-phase extraction.

The intra-day precision and accuracy for the determination of betamethasone in rat plasma were assessed by preparing QC samples of betamethasone at three different concentrations (10, 100, and 1000 ng/ml) and processing those samples using the automated SPE procedure as described. Quantitation of QC samples was based on standard curves that were prepared daily. Results for intra-day precision and accuracy determined on three different days of analysis are shown in Table 1. Calculated values for QC samples at all levels tested were within 10% of nominal values. The precision of measurement, expressed as RSD, ranged from 2.3 to 6% for the 10 ng/ml level, 2.7–4.8% for the 100 ng/ml level and 0.4–3.6% for the 1000 ng/ml level. Similarly inter-assay precision and accuracy were determined for three different days of analysis at the 10, 100, and 1000 ng/ml levels. Precision ranged from 4.2% at the 10 ng/ml level to 3% for the 100 and 1000 ng/ml levels. Accuracy ranged from 98% to 103% at the 10, 100 and 1000 ng/ml concentrations. Calibration curves were constructed by linear regression of standard concentrations against the peak area ratios of analyte to internal standard. Linear cali-

Table 1
Intra-day (intra-assay) precision and accuracy for the quantitation of betamethasone in rat plasma ($n=3$)

Concentration (ng/ml)	% Accuracy			Precision (RSD, %)		
	Day 1	Day2	Day 3	Day 1	Day 2	Day 3
10	97	95	103	2.3	2.4	6.0
100	103	97	99	4.4	4.8	2.7
1000	106	100	102	3.6	1.3	0.4

bration curves were obtained in the range of 2–2000 ng/ml (10 points) with correlation coefficients of greater than 0.998 using a $1/x$ weighted linear regression model. For the 3 days of analysis described above average slopes were 0.00084 ± 0.00007 and average intercepts were 0.00085 ± 0.00013. A limit of quantitation of 2 ng/ml was routinely achieved for the assay with signal-to-noise ratio of greater than 10 for the betamethasone peak. The validation described above was satisfactory for studies at the discovery phase of pharmaceutical development. Further validation studies might be necessary depending on a laboratory's objectives or for the analysis of betamethasone in different biological fluids.

3.2. Stability of betamethasone in rat plasma

Typically sample preparation is short (15–30 min) for the described methodology. Such time frames are consistent with our practices for analysis of plasma samples in the discovery phase for which analyte plasma stability is often not known. We evaluated the stability of betamethasone in rat plasma by spiking known amounts of betamethasone (50 and 500 ng/ml) in rat plasma and by measuring concentrations of betamethasone after 1 and 2 h at room temperature. Similar experiments were performed to address freeze–thaw cycles (two cycles in total) by comparison of freshly prepared quality control samples (50 and 500 ng/ml) to samples that were frozen ($-70\,^{\circ}C$) following preparation and then were thawed at room temperature prior to extraction. At the 2 h time point we observed a slight reduction (approximately 20%) in the value of the controls at the 50 ng/ml in comparison to the reference sample (zero time point). The same magnitude of reduction was observed following the second freeze–thaw cycle. Although the reduction at the 50 ng/ml level is noteworthy, quality control values for the reference time point and for the 2 h point were within the limits of experimental variability (average reference QC: 56.5 ng/ml, average 2 h QC: 46.5 ng/ml and average cycle 2 QC: 47 ng/ml). At 500 ng/ml the 2 h and cycle 2 values were within 10% of the value of the reference point, suggesting that betamethasone is stable in rat plasma for up to 2 h and following two freeze–thaw cycles.

3.3. Liquid chromatography and mass spectrometry

Fig. 1 represents structures of betamethasone (I) and prednisolone (II). A full scan spectrum of betamethasone is shown in Fig. 2a. The base peak at m/z 393 corresponds to the protonated molecular ion $(M+H)^+$ of betamethasone. Fig. 2b represents product ion scans of precursor ion 393. Major fragment ions at 373, 355, and 337 m/z were selected for the optimization of MRM transitions. A full scan spectrum of prednisolone is shown in Fig. 3a. The peak at m/z 361 corresponded to the protonated molecular ion $(M+H)^+$ of prednisolone. Fig. 3b represents product ion scans of precursor ion 361. The most intense fragment ions were selected for the optimization of MRM transitions. Three transitions were monitored for betamethasone: 393→372.8, 393→354.8, 393→337 to ensure the assay's selectivity. Results were generated from more than one transition to ensure than determinations were not compromised by non specific interferences (e.g.,

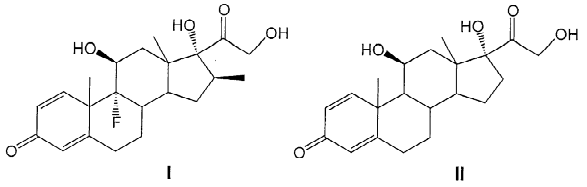

Fig. 1. Structures of (I) betamethasone, (II) prednisolone.

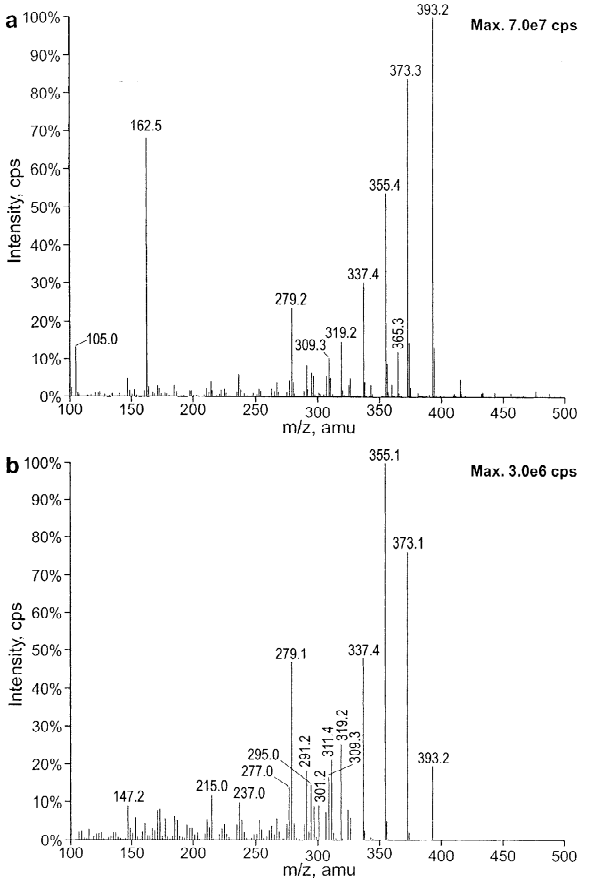

Fig. 2. (a) Positive ion full-scan spectrum of betamethasone (I), (b) product ion spectrum of precursor ion at 393.

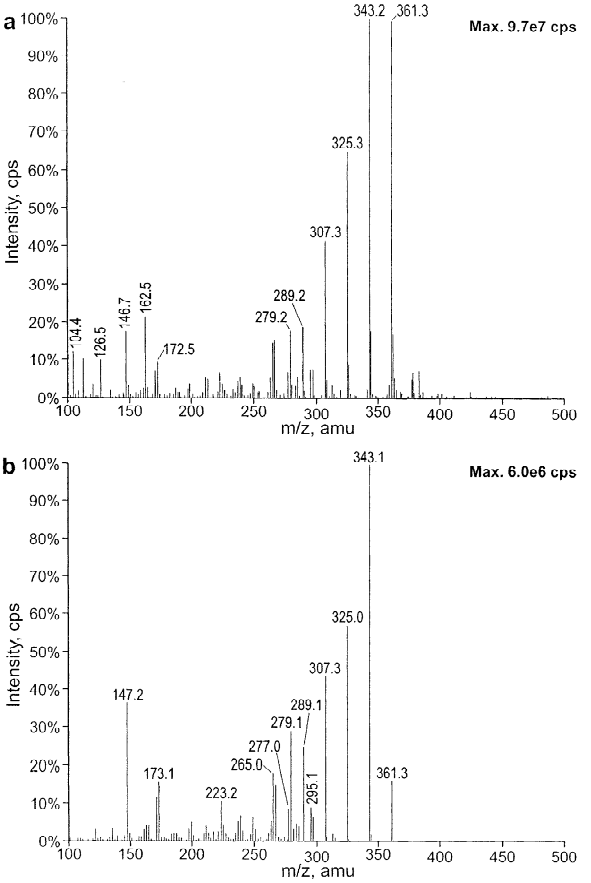

Fig. 3. (a) Positive ion full-scan spectrum of prednisolone (II), (b) product ion spectrum of precursor ion at 361.

from the biological matrix). The data presented in this manuscript was based on the transition of 393→372.8. An isocratic procedure was employed to increase sample throughput. The extraction procedure generated clean extracts from rat plasma as shown in Fig. 4a, which is a representative chromatogram of the extraction of control plasma. The absence of interfering peaks from the plasma extract in representative MRM transitions for betamethasone (393→372.8) and prednisolone (361→342.8) was established (as well as the other transitions mentioned above). Fig. 4b represents a control plasma extract spiked with prednisolone and analyzed as described. No interfering peaks were observed at the representative MRM transitions for betamethasone. Fig. 5a is a representative chromatogram of a 10 ng/ml betamethasone in plasma extract. Using the described methodology, signal-to-noise ratios that exceeded 40 ($S/N>40$) were routinely achieved at the 10 ng/ml level.

3.4. Plasma concentrations and kinetic parameters of betamethasone in rat

In the discovery phase we often aim to minimize the number of animals required per study, since studies are of exploratory nature. In this paper the determination of plasma concentrations and oral bioavailability of betamethasone in rat was based on five animals ($n=2$ for i.v. and $n=3$ for oral),

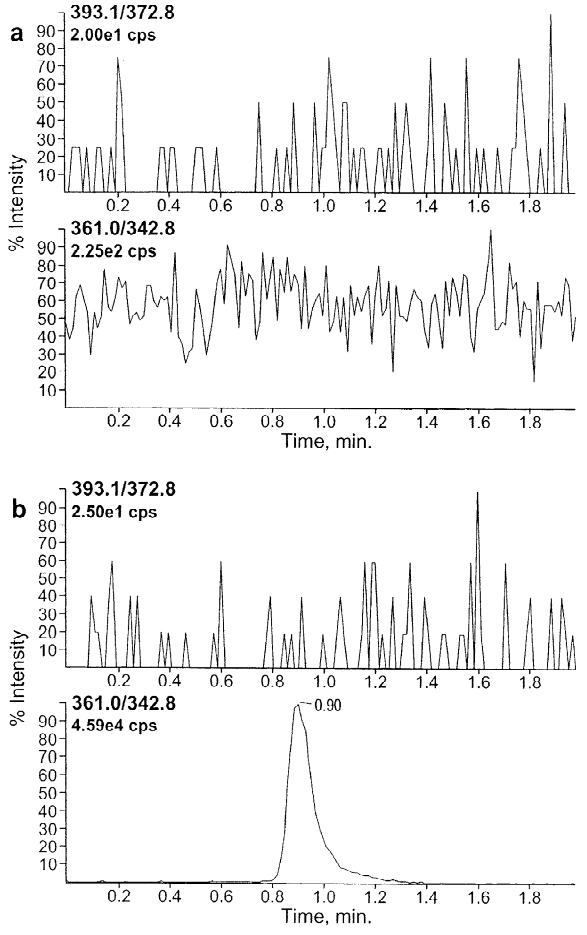

Fig. 4. (a) Chromatogram of double blank at transitions *m/z* 393→372.8 (representative of betamethasone) and 361→342.8 (representative of prednisolone). (b) Blank plasma sample, spiked with prednisolone (internal standard).

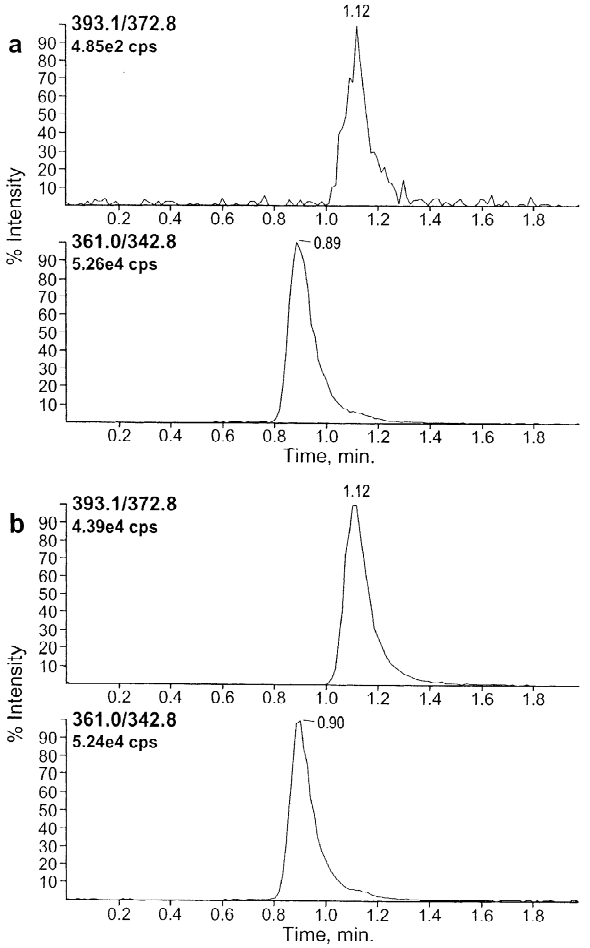

Fig. 5. (a) Chromatogram of plasma sample extract, spiked with 10 ng/ml of betamethasone and internal standard, (b) Representative chromatogram from a study sample, in which betamethasone was dosed intravenously to Sprague–Dawley rats.

potentially not capturing the magnitude of biological variability. Fig. 5b displays a representative chromatogram from the study in which plasma samples from rats that had been dosed with betamethasone were analyzed as described. Fig. 6 represents plasma profiles of betamethasone (concentration versus time) following intravenous (Fig. 6a) and oral (Fig. 6b) administration. Kinetic parameters are presented in Table 2, following intravenous administration and Table 3, following oral administration: kinetic parameters were calculated using Watson. Following intravenous administration (1 mg/kg), plasma clearance was found to be 5 ml/min/kg and $t_{1/2}$ was approximately 2 h. Following oral dosing (2 mg/kg), betamethasone was rapidly absorbed (T_{max}: 0.5 h) and average plasma concentrations ranged from approximately 100 ng/ml to a C_{max} of approximately 1500 ng/ml. The average oral bioavailability was approximately 65%. Although the administered doses of betamethasone were low, the plasma concentrations detected following oral or intravenous dose were well within the limitations of the assay. Consequently the sensitivity of the assay was

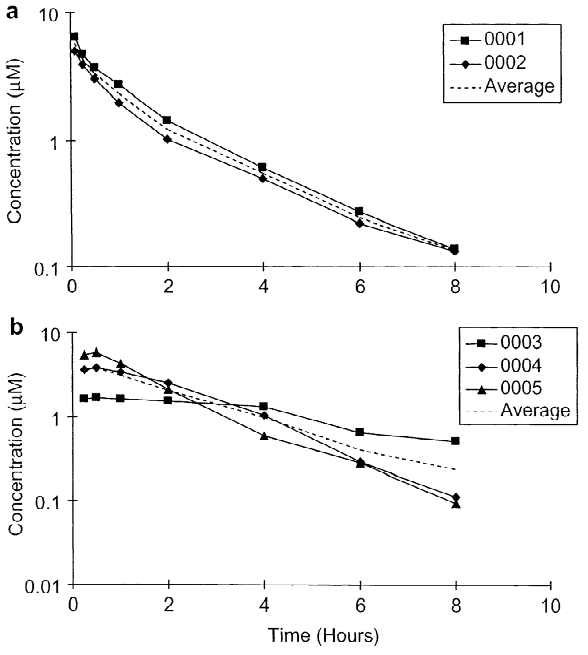

Fig. 6. Plasma concentrations of betamethasone versus time, following (a) intravenous and (b) oral administration.

Table 2
Kinetic parameters following i.v. administration (1 mg/k/g) of betamethasone in rat

		Subject 1	Subject 2	Average
Cl_p	ml/min/kg	4.43	5.63	5.03
Vd_{ss}	l/kg	0.58	0.79	0.68
$t_{1/2}$	h	1.90	2.14	2.02

adequate for the determination of kinetic parameters of the described study.

4. Conclusions

Combination of automated 96-well SPE procedure with LC–MS–MS analysis provided a rapid, sensitive and accurate methodology for the measurement of betamethasone in rat plasma. The procedure's applicability was demonstrated by the determination of plasma concentrations of betamethasone in rat following oral and intravenous administration. Investigating the applicability of this approach to the quantitation of other steroids in biological fluids would be valuable and with potential applications to various scientific disciplines in which measurements of steroids are critical.

Table 3
Kinetic parameters following oral administration (2 mg/kg) of betamethasone in rat

		Subject 3	Subject 4	Subject 5	Average	SD
C_{max}	μM	1.71	3.88	5.90	3.83	2.10
T_{max}	h	0.5	0.5	0.5	0.5	–
$F_{(0-x)}$	%	55	68	70	64	8
$F_{(0-\infty)}$	%	61	67	68	65	4

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