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Determination of the glucocorticoid fluticasone propionate in plasma by automated solid-phase extraction and liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive, robust and high throughput mass spectrometry based method is described for the determination of the glucocorticoid fluticasone propionate in plasma. The method employs solid-phase extraction in 96 well microtitre plate format which has been automated by means of a custom built Zymark robotic system. The extracts are analysed by liquid chromatography–tandem mass spectrometry using thermally and pneumatically assisted electrospray ionisation and selected reaction monitoring. The method is both accurate and precise with both intra- and inter-assay precision (C.V.) of less than $<6\%$. The method provides a lower limit of quantification of 20 pg/ml from 0.5 ml of human plasma, sufficient to monitor systemic concentrations of inhaled fluticasone propionate at therapeutic doses.

Keywords: Fluticasone propionate; Glucocorticoid

1. Introduction

Asthma is a respiratory condition characterised by airway inflammation, airway obstruction (at least partially reversible), and airway hyper-responsiveness to such stimuli as environmental allergens, viral respiratory-tract infections, irritants, drugs, food additives, exercise, and cold air. Inflammatory cells, such as the eosinophils, $CD4^+$ lymphocytes, macrophages, and mast cells, release a broad range of mediators including interleukins, leukotrienes, histamine etc., which are responsible for the bronchial hyperreactivity, bronchoconstriction, mucus secretion, and sloughing of endothelial cells. Because asthma is an inflammatory disease, early treatment with inhaled glucocorticoids is recommended for optimum long-term control, with the addition of beta-agonists as needed [1–3].

Fluticasone propionate (FP) is a novel glucocorticoid having potent topical anti-inflammatory properties. Unlike most other corticosteriods, the structure of FP is based on the androstane, rather than the pregnane, corticosteroid nucleus (see Fig. 1). The molecule is designed to maximise topical anti-inflammatory activity and minimise the unwanted systemic effects associated with other glucocor-

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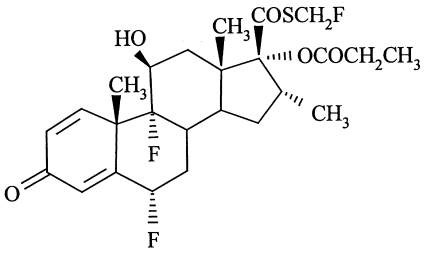

Fig. 1. Structure of the glucocorticoid fluticasone propionate (FP).

ticoids (e.g. suppression of the hypothalamopituitary-adrenal axis). This separation of activities is possible because FP is rapidly metabolized to the inactive 17β-carboxylic acid metabolite [4–6]. The combination of potent topical anti-inflammatory and low side effect profile make FP an ideal compound to treat asthma and seasonal and perennial rhinitis [3,7]. The human pharmacology of FP has been reviewed [8] and more recently the preclinical and clinical experience with FP has been updated [3].

At clinical doses of inhaled glucocorticoids it is estimated that only 20% of the drug reaches the lung with the majority of drug being swallowed and entering the gastrointestinal (GI) tract. FP itself is poorly absorbed from the GI epithelium and undergoes extensive first pass metabolism to the 17β-carboxylic acid. The oral bioavailability is less than 1%. While advantageous in inhalation therapy, this results in low plasma concentrations as any systemic levels are due almost entirely to adsorption from the lung [5]. This presents a significant challenge to the bioanalyst to provide the sensitive methods required to generate pharmacokinetic data following therapeutic doses.

Due to the lack of a suitable chromophore or fluorophore in the molecule and the suitability of steroid molecules for raising high affinity, selective antibodies, the first method developed for FP was a radioimmunoassay (RIA). Using a FP-carboxymethoximethyroglobulin conjugate for antibody production, the resulting method provided a lower limit of quantitation (LLOQ) of 500 pg/ml [5]. In order to be able to provide pharmacokinetic information after inhaled doses, it was necessary to further enhance the method by the inclusion of a solid-phase extraction (SPE) step prior to the RIA [6]. This extended the LLOQ to 50 pg/ml although reduced the sample throughput. As improved delivery devices and new formulations of FP are developed, there is a continued need for an even more sensitive, specific bioanalytical method for FP with improved sample throughput capability.

More recently, Li et al. have reported the development and validation of a method for FP in human plasma using a combination of protein precipitation and solid-phase extraction followed by liquid chromatography mass spectrometry (LC–MS) with atmospheric pressure chemical ionisation [9]. The authors employed selected ion monitoring and used budesonide as an internal standard. Despite having good selectivity and adequate accuracy and precision in spiked control matrix, the method provided a lower limit of quantification of only 200 pg/ml from a sample size of 1 ml of plasma.

Solid-phase extraction (SPE) in the 96 well format has rapidly become the sample preparation technique of choice for LC–MS–MS in our laboratory and we have previously reported on the use of a robotic sample processor (RSP) to semi-automate the extraction of 96 well SPE blocks [10]. More recently, we have developed an improved, fully automated approach using a Zymark robotic system [11], and in the present work we describe the application of this sample preparation technology in the development of a sensitive, high-throughput LC–MS–MS method for the determination of FP in plasma.

2. Experimental

2.1. Chemicals and reagents

AnaLar grade ammonium formate, sodium hydroxide and formic acid were obtained from BDH (Poole, U.K.), and used to prepare 0.1 *M* (pH=7.3) and 25 m*M* (pH=5) ammonium formate buffers for the SPE extraction and HPLC mobile phase, respectively. HPLC grade methanol was obtained from Romil Chemicals (Cambridge, UK). FP and its isotopically labelled internal standard ($^{13}C_3$-FP) were both synthesised at GlaxoWellcome Research Laboratories, and primary stock solutions (1 mg/ml) were prepared in methanol and stored at +4°C. Secondary solutions of FP were prepared at 10.0, 1.0 and 0.1 μg/ml and these were then used to spike control human plasma to produce calibration stan-

dards at 20, 25, 50, 100, 350, 600, 800 and 1520 pg/ml. Quality control (QC) samples were similarly prepared from independent stock solutions (i.e. separate weighing) by spiking control plasma at 50, 200 and 800 pg/ml, and validation controls (VC) at 20, 50, 200, 800 and 1500 pg/ml were also prepared for the initial validation of the method.

2.2. Solid-phase extraction

All plasma samples, calibration standards and QC or VC samples were centrifuged (approximately 1000 *g* for 10 min) prior to solid-phase extraction. Samples were extracted on 96 well MicroLute II™ SPE blocks (Porvair Sciences Ltd., Shepperton, Middlesex, UK) packed with 50 mg of Varian C18. The extraction was automated by means of a custom built Zymark robotic system (Runcorn, Cheshire, UK). This system comprises of a Zymate XP robot, a refrigerated storage carousel for SPE consumables and final extracts, a custom built SPE station consisting of a combined vacuum manifold and reagent addition station (RAS), and a MultiPROBE 104DT robotic sample processor (Canberra Packard Ltd., Pangboume, Berks., UK). Following conditioning on the SPE station with 400 μl methanol and 400 μl water, the robot transfers the block to the RSP which sequentially aspirates 500 μl of 100 m*M* ammonium formate buffer (pH=7.4) containing the internal standard (1 ng/ml), and 500 μl of the plasma sample. These aliquots were transferred to individual channels of the MicroLute II™ block, and the dispensing speed is such that they are effectively mixed. After returning to the SPE station for washing with 400 μl of water and 400 μl of 40:60 (v/v) aqueous methanol, the samples were eluted into a deep well 96 well collection plate (Porvair Sciences) with 200 μl of methanol. The SPE procedure is detailed in Table 1.

After the extraction procedure the collection plate is transferred to a 96 well sample concentrator and the elution solvent taken to dryness under a stream of heated nitrogen. The extracts were then reconstituted in 100 μl of 50:50 (v/v) methanol and 25 m*M* ammonium formate (pH=5), and the collection plate sealed with aluminium foil prior to analysis by LC–MS–MS.

2.3. LC–MS–MS method

Extracts were analysed by LC–MS–MS using a Jasco liquid chromatograph (comprising a PU-980 pump, a LG-980-02 ternary gradient unit and a DG 980-50 3 line degasser), a modified Gilson 233 XL autosampler, a Jasco CO 960 column oven, and a PE-Sciex API-III$^+$ triple quadrupole mass spectrometer (Ontario, Canada). An 80 μl aliquot of the SPE extract was injected onto a 150×4.6 mm I.D. column packed with Varian, ResElut C8 BD 5μm (Walton on Thames, Surrey, UK) operated at 40°C using an isocratic mobile phase of 80:20 (v/v) methanol and 25 m*M* ammonium formate (pH=5) at 1 ml/min. The flow from the column was split 1:30 with 336 μl/min directed to a TurboIonspray interface at 500°C using nitrogen as both the nebuliser (approximately 400 kPa) and auxiliary gas (2.0 l/min). Analytes were ionised by positive ion electrospray (5000 V) and detected by tandem mass spectrometry (MS–MS) using selected reaction monitoring (SRM) and a collision energy of 25 eV (laboratory frame). The transitions m/z=501 to 313 and m/z=504 to 313 were monitored for FP and

Table 1
Detailed SPE procedure on Zymark SPE system

Step	SPE Process	Solvent	Volume (ml)	Bleed factor	Vacuum (s)
1	Condition	MeOH	0.4	400	4
2	Condition	Buffer	0.4	150	5
3	Load	Sample	0.5	10	180
		Buffer/ISTD	0.5		
4	Wash	Water	0.4	10	100
5	Wash	40% (v/v) aq. MeOH	0.4	10	120
6	Elute	MeOH	0.2	10	120

$^{13}C_3$-FP, respectively, with dwell times of 250 ms per transition. Argon was used as the collision gas at an indicated target thickness of nominally 250.

LC–MS–MS SRM peaks were integrated by the PE-Sciex MacQuan processing software. Calibration curves were constructed by plotting peak area ratios of FP to internal standard against concentration, using a weighted (1/X) linear regression model. Concentrations of the analytes in unknown, QC and VC samples were subsequently interpolated from these curves. A one-way analysis of variance (ANOVA) of the validation data was performed by an Excel macro (Microsoft Corp.)

3. Results and discussion

3.1. Sample preparation

The SPE procedure for FP employed in the earlier enhanced RIA method [5,6] was used as the starting point in the development for the automated procedure used in the present work. The original, individual C18 200 mg Bond-Elut cartridges were replaced by MicroLute II™ 96 well format blocks packed with 50 mg of sorbent. The tricine buffered saline was replaced by the more mass spectometry compatible ammonium formate and the procedure was then transferred to the 96 well format by scaling down and re-optimising the volumes and composition of the buffers and solvents used in the conditioning, washing and elution steps.

The 96 well SPE blocks are extracted by means of a custom built Zymark robotic system. This system is based around a Zymate XP robot and consists of a refrigerated (+4°C) storage carousel, which acts as both a warehouse for all the SPE labware and as a repository for the final extracts, a custom built SPE station and a RSP. A schematic of the system is shown in Fig. 2. The SPE station incorporates a vacuum manifold, a reagent addition strip (RAS) for rapid dispensing of SPE solvents a row at a time (eight wells), and a solvent switching valve which allows up to nine different reagents to be used on the system. The vacuum manifold and RAS allow all the conditioning, washing and elution steps to be performed much faster then the RSP in the original semi-automated procedure [10]. This allows the RSP to do what it does best, i.e. the transfer samples from sample tubes to the 96 well SPE blocks, diluting samples and/or adding internal standard as required.

The system has a user-friendly Visual Basic™ interface to the Zymark Easylab™ software, which also controls the RSP. To run an existing method the operator has simply to select the method and enter the number of samples. The operator then loads the

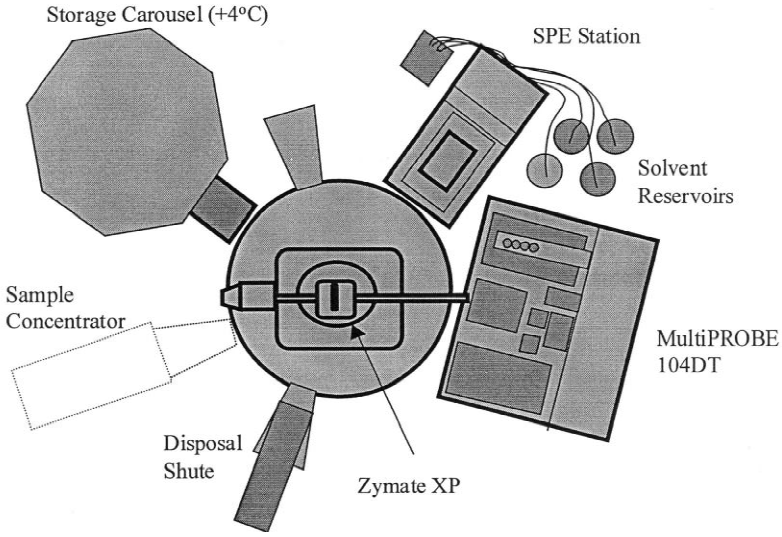

Fig. 2. Schematic of the Zymark 96 well SPE robotic system.

required number of blocks and plates into the pre-assigned towers of the storage carousel. The buffer reservoir together with the centrifuged samples, calibration standards and QC's are then placed onto the deck of the RSP.

For the FP method the sequence begins by the Zymate robot collecting the appropriate SPE block from the storage carousel and placing it onto the SPE station. The SPE station has been designed to work with all current available SPE block formats, e.g. MicroLute™ blocks or Empore™ blocks. The RAS arm is designed to push down on top of the blocks on the vacuum manifold and ensure that the vacuum is applied consistently. In setting up the SPE procedure the operator may vary both the time the vacuum is applied and the strength of the vacuum. The latter is controlled by bleeding air into the vacuum line by means of a control value. Once the block is conditioned the robot transfers it to the RSP for sample loading, including any dilutions, and the addition of internal standard. The four probes of the RSP with independent liquid detection and Varispan™ provide the flexibility to handle different mitrices of varying volumes and viscosity's, in a variety of tube sizes. The loaded block is returned to the SPE workstation for vacuuming followed by the wash steps. Prior to the final elution step, the appropriate collection plate, e.g. standard microtitre plate or a deep well collection plate, is collected from the carousel and placed in the SPE workstation underneath the SPE block. Once the samples have been eluted, the used SPE block is placed in a waste receptacle and the collection plate containing the eluants is returned to the cooled storage carousel.

After extraction the operator transfers the collection plates from the storage carousel to an off-line 96 well sample concentrator and the elution solvent taken to dryness under a stream of heated nitrogen. The concentration step takes approximately 45 min. More recently, an automated sample concentration unit from Zymark has been incorporated into the system (see Fig. 2). After re-constitution the collection plates are sealed with aluminium foil and transferred to an autosampler modified to accept the 96 well format with a capacity for up to six collection plates. The automated SPE procedure for FP results in recoveries of approximately 80%, which compares favourably with the recoveries achieved with the original RIA method using manual extraction on a conventional vacuum manifold [6].

3.2. LC–MS–MS

The full scan, positive ion TurboIonSpray spectrum of FP is presented in Fig. 3. The base peak of the spectrum is the protonated molecule (MH^+) at $m/z=501$, with only minor adduct ions observed, e.g. sodiated species at $m/z=523$ but very little fragmentation. A similar spectrum (not shown) was obtained for the isotopically labelled internal standard ($^{13}C_3$-FP), with an MH^+ ion 3 a.m.u. higher at $m/z=504$. The absence of an ion at $m/z=501$ resulting from unlabelled FP confirmed the isotopic purity of the internal standard. The respective MS–MS product ion spectra of these MH^+ ions are presented in Fig. 3 and it can be seen that both yield almost identical spectra with several abundant prod-

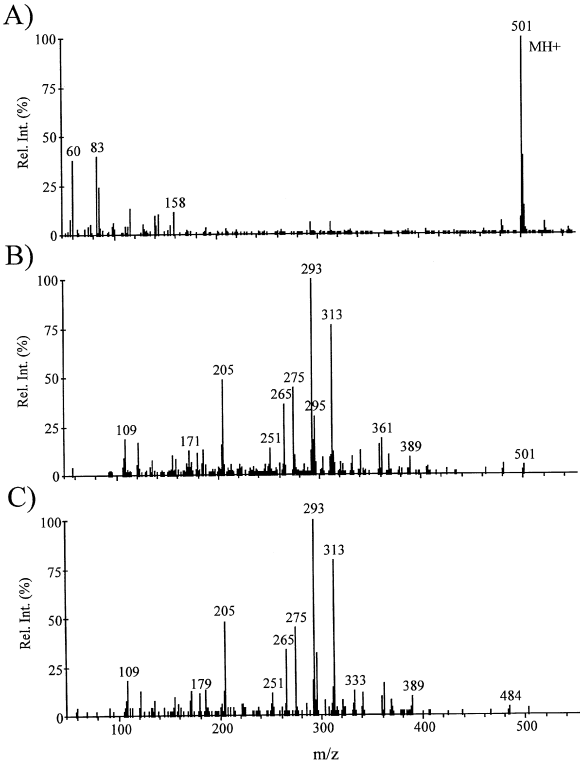

Fig. 3. (A) Positive ion TurboIonspray spectra of FP, and MS–MS product ion spectra of (B) FP and (C) the internal standard $^{13}C_3$-FP.

uct ions suitable for use as selected reaction monitoring (SRM) transitions. Initially, the product ion at $m/z=293$ was selected as the Q3 transition for both FP and the internal standard. However, an endogenous peak in the plasma extracts gave rise to interference's in both SRM channels and the slightly less abundant product ion at $m/z=313$ was therefore selected. As a common product ion was used for both analyte and internal standard, it was necessary to have Q2 settling on in order to prevent cross-talk between the two SRM channels.

The raw LC–MS–MS SRM chromatograms, resulting from the analysis of an extract of calibration standard at the lower limit of quantification (20 pg/ml), are presented in Fig. 4. The signal-to-noise ratios and lack of interferences in the chromatograms illustrate the sensitivity and specificity of LC–MS–MS, respectively. With a mobile phase of 80% (v/v) methanol in 25 m*M* ammonium formate at pH=5, both FP and the internal standard elute in under 4 min with good peak shapes. Acetonitrile was also evaluated as the organic component of the mobile phase and although similar retention characteristics could be obtained, methanol provided better resolution from the endogenous components mentioned above.

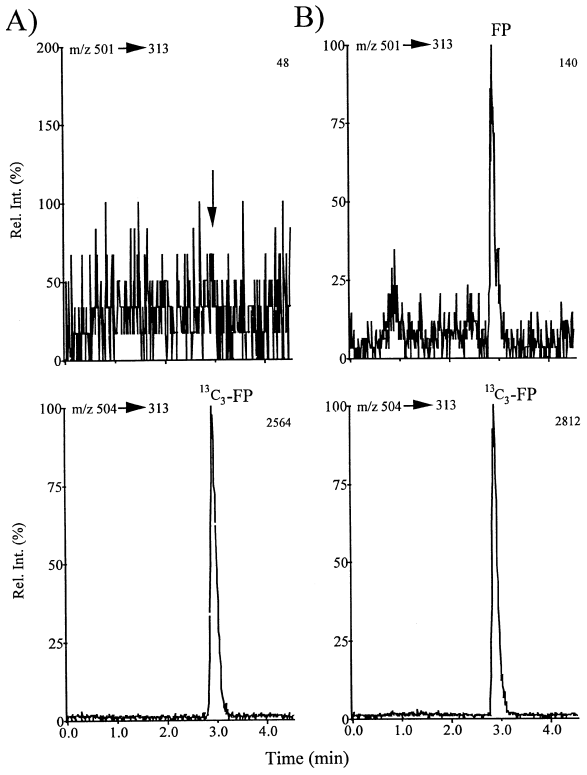

Fig. 4. LC–MS–MS SRM chromatograms of FP and the internal standard $^{13}C_3$-FP (1 ng/ml) resulting from 80 μl injections of (A) an extracted blank human plasma sample and (B) an extracted calibration standard at the LLOQ (20 pg/ml).

3.3. Method validation

The LC–MS–MS method was formally validated prior to the analysis of clinical study samples and the precision and accuracy of the method were determined and its specificity assessed. The stability of FP in frozen human plasma has been assessed in earlier work [6].

The precision of the method (%C.V.) was determined by assessing the agreement between replicate measurements of validation control samples prepared independently from the calibration standards. Validation control samples at five concentrations were analysed in replicates of six, on four separate occasions. The data for FP were examined by analysis of variance (ANOVA) to give estimates of the inter- and intra-assay precision of the method. The results are presented in Table 2 and show that at all validation control concentrations the precision (%C.V.) is better than 6% over the limits of quantification (20–1520 pg/ml). The accuracy of the method (% bias) was determined by assessing the agreement between the measured and actual (weighed) concentrations of the validation control samples, the measured concentration being the mean of the concentrations obtained during the precision assessment. The results are presented in Table 2 and show that at all validation control concentrations the bias do not exceed ±8%.

The specificity of the method was assessed by the examination of SRM chromatograms of extracts of both pooled blank plasma and blank plasma from several individual volunteers, together with pre- and post-dose plasma samples from clinical trials. Control plasma was also spiked with the 17β-carboxylic acid metabolite and, since FP is used in combination therapy, the beta-agonist salmeterol. Total ion current chromatograms of plasma extracts were also examined to ensure that FP was well resolved from

Table 2
Accuracy and precision data for FP from the pre-Study validation

	VC1	VC2	VC3	VC4	VC5
Nominal concentration (pg/ml)	20	50	200	800	1500
n	23	24	24	24	24
Mean	20.15	50.94	196.20	738.36	1439.12
S.D.	1.06	2.40	9.89	14.86	66.43
Accuracy (%Bias)	0.8	1.9	−1.9	−7.7	−5.3
Precision (%C.V.)					
Intra-assay	5.3	4.7	4.1	2.1	4.3
Inter-assay	1.0	1.3	3.3	negligible	1.3

the solvent front and any potential endogenous interference's. Due to the combination of chromatographic retention time and two stages of mass selectivity, LC–MS–MS provides extremely high specificity and this was illustrated by the lack of interference's in any of the SRM traces (see Fig. 4). The absence of a response in the blank extract again confirms the isotopic purity of the internal standard. The specificity is such that without any modifications it has been possible to validate the method at the same LLOQ in plasma from several pre-clinical species, including dog and mini-pig.

3.4. Method application

The LC–MS–MS method has been used to support several clinical studies. Fig. 5 shows a representative FP plasma profile obtained by LC–MS–MS after a single inhaled dose of 500 μg FP via a Diskus™ Inhaler. The profile demonstrates the ability of LC–MS–MS to provide pharmacokinetic data at therapeutic doses.

The extraction procedure on the robot takes approximately 45 min per 96 well block, and up to 4 blocks can be processed on one occasion (384 samples). This is significantly faster then the time required to prepare a similar number of samples by manual pipetting, using individual SPE cartridges and conventional vacuum manifolds. Even with the concentration step, sample preparation by 96 well SPE on the robotic system now makes the LC–MS–MS analysis the rate limiting step, which, with an injection every 4 min, still takes approximately 7 h to analyse a complete 96 well plate (study samples

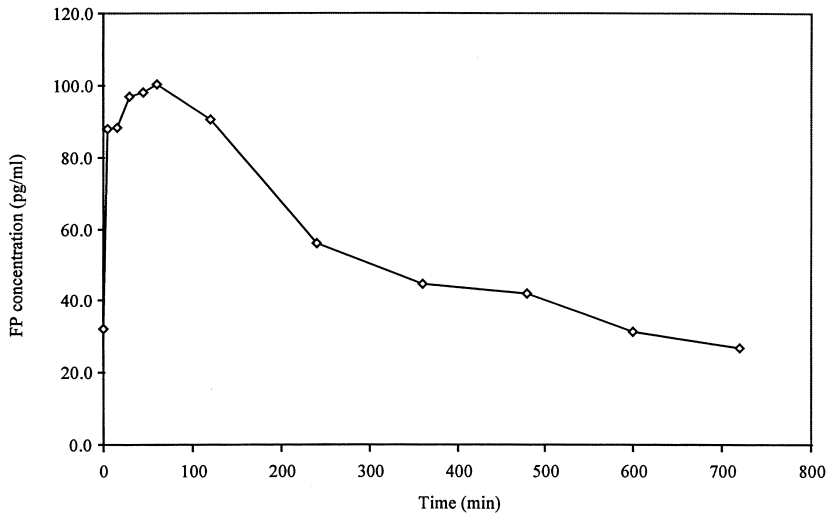

Fig. 5. Representative FP profile in human plasma after an inhaled dose of 500 μg.

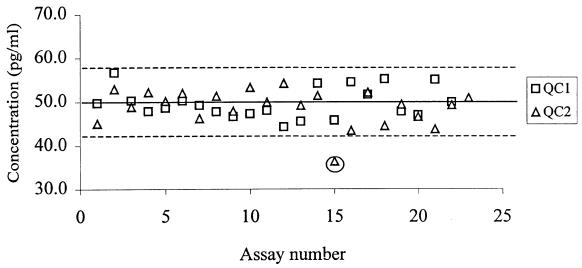

Fig. 6. Control chart for FP low QC (50 pg/ml) generated during support to a clinical study. - - - upper and lower control limits (±15%).

plus standards, quality controls and blanks). It is therefore possible to extract 2 blocks per day, which can be analysed by LC–MS–MS overnight, and still accommodate the recycle of the cryogenic vacuum system of the API-III$^+$ triple quadrupole. The performance of the method is illustrated by the QC control chart in Fig. 6 generated from 23 individual assays in support of a clinical study representing some 1600 clinical samples. Duplicate QC samples, at three concentrations representing the low, medium and high regions of the calibration range, are included in every assay. The upper and lower control limits at 15% are indicated in Fig. 6, and it can be seen that only one of low QC samples (50 pg/ml) was rejected during the entire study (circled).

4. Conclusions

A sensitive, robust and high throughput LC–MS–MS method has been developed for the glucocorticoid FP in human plasma. The LC–MS–MS method offers increased specificity and sensitivity over the previous RIA method, with an LLOQ of 20 pg/ml from only 0.5 ml of plasma. This approach to automated SPE has proved to be a very successful replacement to manual extraction offering reduced sample preparation times, which are more compatible with the fast analysis times possible with LC–MS–MS. The fact that the analyst has minimal manual pippetting, and no longer has to handle, cap, label, etc. individual vials and/or cartridges is a significant advantage of this system. It has been found that the off-line batch processing approach suits a high throughput laboratory dealing with multiple methods/projects, where dedicating one type of sample preparation system on-line with a mass spectrometer can often cause scheduling problems.

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