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Short communication

High throughput HPLC-ESI-MS method for the quantitation of dexamethasone in blood plasma

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Abstract

Dexamethasone is a synthetic glucocorticoid with potent anti-inflammatory properties. However, its administration causes significant side effects, specially in long-term therapy. A new approach for limiting adverse effects consists in the slow and constant deliver of this drug, using dexamethasone-21-phosphate-loaded erythrocytes (RBC) as circulating bioreactors converting the non-diffusible dexamethasone-21-phosphate into the diffusible dexamethasone. In order to evaluate the real possibility to use this new method of administration, a simple, cheap and rapid assay was set to manage a large number of samples originating from clinical studies. Due to the sample complexity and analite polarity, electrospray mass spectrometry (MS) is the most powerful technique to achieve qualitative and quantitative data. In order to overcome the complex, time-consuming and expensive LC–MS/MS methods reported in the literature in the present work a standard fluxes HPLC-ESI-MS method was set up for quantitative evaluation of dexamethasone. Thanks to the extraction ion chromatogram (XIC) feature of the software, it was possible to obtain sharp profiles for dexamethasone (DXM) and for the employed internal standard (IS) flumethasone (FM), in spite of the extremely complicated chromatogram obtained after HPLC separation of acetonitrile extracted plasma sample, thus avoiding the use of the expensive deuterated internal standard. This enabled us to obtain a linear response curve, allowing the quantification of DXM from blood samples at the picomoles level.

Keywords: Electrospray mass spectrometry; Dexamethasone; Cystic fibrosis

1. Introduction

High performance liquid chromatography (HPLC) is generally used to separate and detect a wide variety of compounds; however this technique, coupled with traditional detectors, is often ineffective to obtain the quantitation of molecules present in fluids of complex composition, such as the biological ones. In fact, the presence of a large number of compounds endogenous to the cells/tissues, results in a complex matrix, from which it is very difficult to separate the analyte of interest. In order to achieve reproducible and constant yields a liquid to liquid extraction was performed. In the last few years, mass spectrometer have been introduced as detector for HPLC. In fact, a large number of different ion sources has been set-up and the electrospray interface is the most frequently used technique for the ionization of polar compounds of large or low molecular weight [1–3]. In fact the ability of this source to produce ions, adding or subtracting one or more protons to or from the molecules, as such or after addition of specific modifiers, avoiding their chemical derivatization, makes this technique suitable for several qualitative and quantitative studies. Moreover, the ability of commercial softwares equipping the mass spectrometers to extract the specific analyte ions from the total ionization current, allows to overcome HPLC separation problems.

Dexamethasone (DXM) is a member of the glucocorticoid class of hormones commonly used for its anti-inflammatory properties. However, the risk of significant side effects (such as growth retardation, blood glucose abnormalities and cataract formation) associated with the need for high oral doses due to the short half-life of corticosteroids (approx. 3 h) limit its

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use for long-term therapy [4]. For this reason, a drug-delivery system able to release low and constant levels of dexamethasone in circulation is needed. Engineered erythrocytes have been proposed as drug-delivery systems [5–11], and previous experiences [12,13] have demonstrated that non diffusible pro-drug dexamethasone 21-P (DXM-21-P) can be entrapped in human erythrocytes where it is slowly dephosphorylated to the corresponding diffusible glucocorticoid analogue dexamethasone. This strategy has been recently employed in cystic fibrosis (CF) patients to reduce the progression of lung disease [13].

In this paper, a high throughput HPLC-MS method is described in details for the detection and quantitation of the DXM plasma levels in CF patients infused with DXM-21-P-loaded erythrocytes. Flumethasone (FM) was chosen as an internal standard (IS) for its structure similarity with the analyte; in fact the hydrogen present in position 6 of dexamethasone is substituted with a fluorine in flumethasone. This structural similarity resulted in a similar chromatographic retention time and allowed comparable ionizations of DXM and FM and a good reproducibility in standard curves and measures. Firstly a scan mode analysis performed on the analyte and on the IS showed the preponderance of a collision-induced dissociation (CID) fragment (*m/z* 361.5 and 379.5, respectively) for both compounds. These fragments were chosen for quantitation after extraction of the single ion currents deriving from the electrospray ionization of DXM and of FM.

2. Experimental

2.1. Sample preparation and liquid chromatography

For sample analysis 10 mL of blood collected in heparin were centrifuged at $2000 \times g$ to separate plasma from blood cells; DXM was extracted using diethyl ether, concentrated with a Speed Vac concentrator (Savant Instrument) and resuspended in 0.5 mL of acetonitrile. The procedure was carried out as previously described [12] and 0.2 μCi [^{3}H]-DXM (specific activity 80 Ci/mmol) Amersham Pharmacia Biotech (UK) was added to each plasma sample to evaluate drug recovery after the extraction procedure [13].

The extracted samples were analyzed by high performance liquid chromatography electrospray mass spectrometry (HPLC-ESI-MS), using a Hewlett-Packard (HP) 1090 liquid chromatograph, directly coupled to the mass spectrometer.

The chromatographic separation was conducted using an Hypersil Hypercarb (100 mm × 2.0 mm i.d.—5 μm particle size) column (Alltech, IL, USA) in isocratic conditions using as eluent water:acetonitrile 20:80 with thriethylamine (TEA) at the final concentration of 5 mM. The column compartment temperature was set at 50 °C. The flow was set to 0.3 mL/min and the eluent was sent directly to the MS interface without splitting. The injected sample volume was 100 μL, obtained by adding 10 μL of FM 100 μM–90 μL of standard DXM or of sample solution. The stationary phase of the column was mainly chosen for its resistance to high pH values; TEA was added to the mobile phase since the best negative ionization of both analyte and IS occurs at pH values of 8.5–9.

2.2. Mass spectrometry

All the experiments were conducted on a HP5989A single quadrupole MS-Engine equipped with a straight needle alignment, standard fluxes, and an atmospheric pressure ionization electrospray ion source HP59987A.

To optimize the ionization method, DXM and FM were subjected to direct infusion analysis (DIA), with a micro infusion pump (Harvard Apparatus, MA, USA) at 10 μL/min, by dissolving the molecules in water:acetonitrile 20:80 with 5 mM TEA. The scan analysis were carried out in the 200–500 AMU mass range. Subsequently, the flow injection analysis (FIA) using the same carrier eluent was performed at 0.3 mL/min; the source (Vcap = 4000 V, CapEx = −180 V) and mass spectrometer (entrance lens = 115 V) potentials were adjusted to get the best signal intensities of the collision-induced dissociation (CID) signals from analyte and IS, at *m/z* 361.5 and 379.5, respectively, and to achieve a constant $[M-H]^-/[M-CH_2O–H]^-$ ratio. These ions were selected to perform the DXM quantitative analysis in single ion monitoring (SIM) negative mode. The gas used was research grade air (>99,995%); the gas pressure was 120 psi to supply the drying gas at 3 L/min and to obtain a final 100 psi nebulizing pressure to generate the spray. The drying gas temperature was kept at 350 °C. The operating pressure was 3 Torr in the first stage, 0.2 Torr in the second stage and 3.5×10^{-4} Torr in the inner part of the source, the manifold front. Mass spectrometer tuning and calibration were performed manually, infusing a 10 ng/μL solution of the mono-sulfonated azo dye Acid Red 4 in isopropanol:water:ammonia 49.75:49.75:0.25 at a flow rate of 10 μL/min. Calibration parameters were further verified by DIA (0.3 mL/min) of a 20 μM DXM standard solution obtained dissolving the analyte in the LC mobile phase.

2.3. Reagents and chemicals

The solvents used for the mobile phase, HPLC grade acetonitrile (Merck, Darmstadt, Germany) and Milli-Q water (Millipore Corp., Bedford, Italy) were filtered and degassed; thriethylamine (Sigma–Aldrich, St. Louis, MO), used as an additive to modify ionization, was of analytical grade.

Air research grade (>99,995%) was purchased from SIAD (Bergamo, Italy). DXM, FM and Acid Red 4 (cas n.5858-39-9) were purchased from Sigma. Separate 5 mM stock solutions of standard DXM and FLU, prepared in acetonitrile, were stored at −20 °C and replaced monthly; working solutions for calibration curves and IS were prepared daily and diluted in acetonitrile.

3. Results and discussion

Dexamethasone (DXM) (European Pharmacopoeia 5.0 - 01/2005:0388) and flumethasone (FM) used in this study as internal standard differ only by the presence of a fluorine in position 6 thus electrospray ionization was comparable, making the use of an expensive stable isotope analogue unnecessary.

Firstly, a scan analysis of the molecules in negative ion mode (selected because of a stronger signal compare to the positive

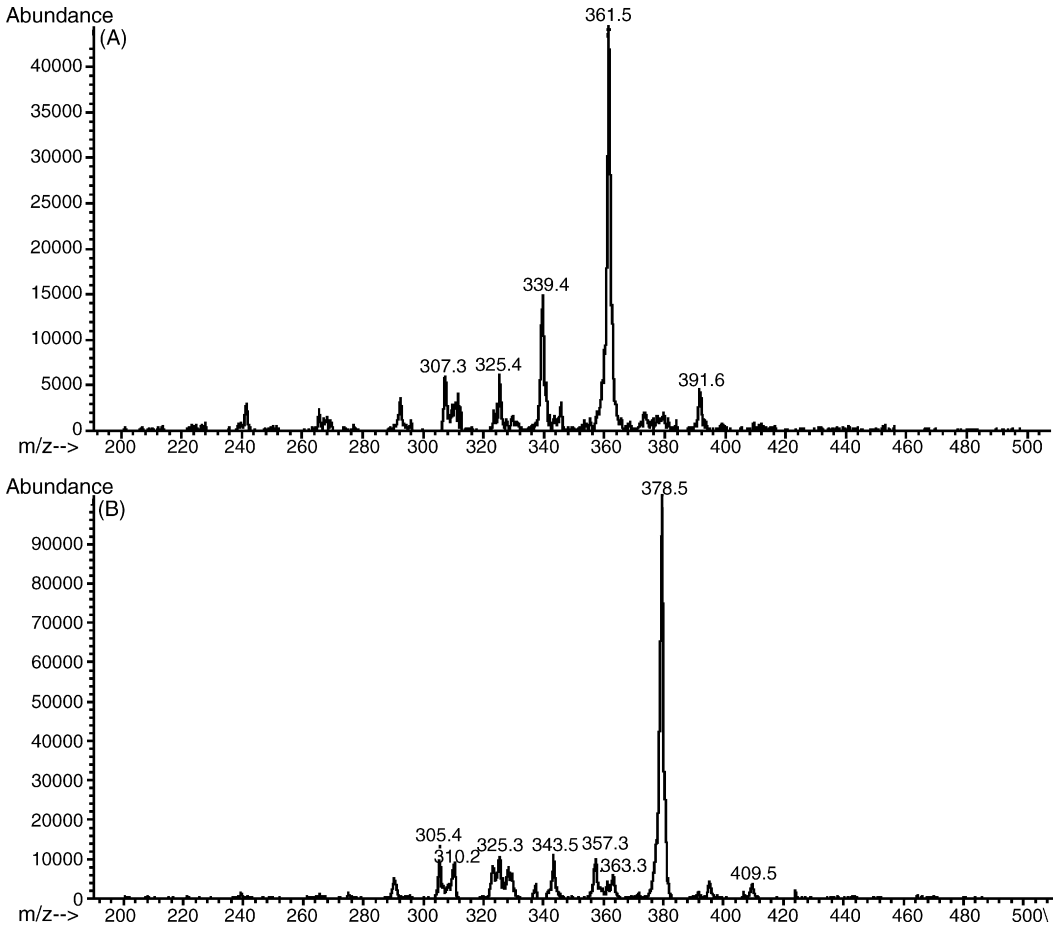

Fig. 1. Mass spectrum of standard dexamethasone (A) and flumethasone (B). Spectra were acquired in SCAN mode in a 200–500 amu mass range FIA at 0.3 mL/min. In both spectra the base peak is represented by the CID product ions at *m/z* 361.5 and 379.5, respectively.

one) was carried out to determine their behavior in the electrospray source; Fig. 1 shows the spectra collected in FIA at 0.3 mL/min using the eluent water:acetonitrile 20:80 with 5 mM TEA; for both molecules a low intesity signal at *m/z* 391.5 and 410.5, respectively, was observed corresponding to the deprotonated ($[M-H]^-$) ion together with a main peak corresponding to a collision-induced dissociation fragment, caused by the intrinsic low stability of these molecules in the CID region of the source. The DXM and FM mass spectra probably arise by a similar fragmentation mechanism: the CID product ion, at *m/z* 361.5 and 379.5, respectively, corresponds to the loss of a formaldehyde specie from the $[M-H]^-$ molecules and have been exploited to characterize DXM and FM with more specificity.

These base peak signals were selected to perform the DXM quantitative analysis in SIM mode; the dwell times were adjusted in order to obtain 4.1 cycles/s getting a high sampling for each HPLC peak. The SIM analysis was carried out at low mass resolution (0.7 amu peak width) to further increase sensitivity. Among the various modifiers used to optimize the negative ionization, TEA gave the best response. Two different approaches were conducted to separate/ionize DXM and FM in the presence of the selected modifier. On the one hand, a post-ionization was conducted on an Hypersil C18 (2.1 mm × 250 mm) column using a T union to mix TEA with the HPLC eluent, on the other hand a more robust system was set-up, employing a Hypercarb column (Fig. 2). This porous graphite carbon stationary phase was chosen for its broad operative pH-interval (pH 1–14) and because of ability of graphite to interact with planar compounds, as our analyte. The fast HPLC elution of FM and DXM (retention time was 4.3 and 4.9 min, respectively) allowed daily analysis of a high number of samples. The HPLC chromatogram obtained from patients blood samples was extremely crowded by the complexity of the biological matrix: thus the extraction ion chromatogram feature allows to obtain sharp profiles of DXM and FM out of the total ion current chromatogram (TIC) which is the sum of DXM and FM SIM signals (Fig. 2). In this way, it was possible to separately integrate the extracted chromatograms and calibration curves were calculated after normalization of DXM peak areas with FM peak areas. Validation was performed in compliance with the FDA guidance for bioanalytical method validation. In particular linearity, accuracy and precision, freeze and thaw stability, long term stability, limit of detection (LOD) and specificity of the method have been evaluated. The calibration curve was calculated from the mean of three different calibration curves, extracted from as many analyses performed over a period of one month. Linearity of the calibration curves were evaluated over a range

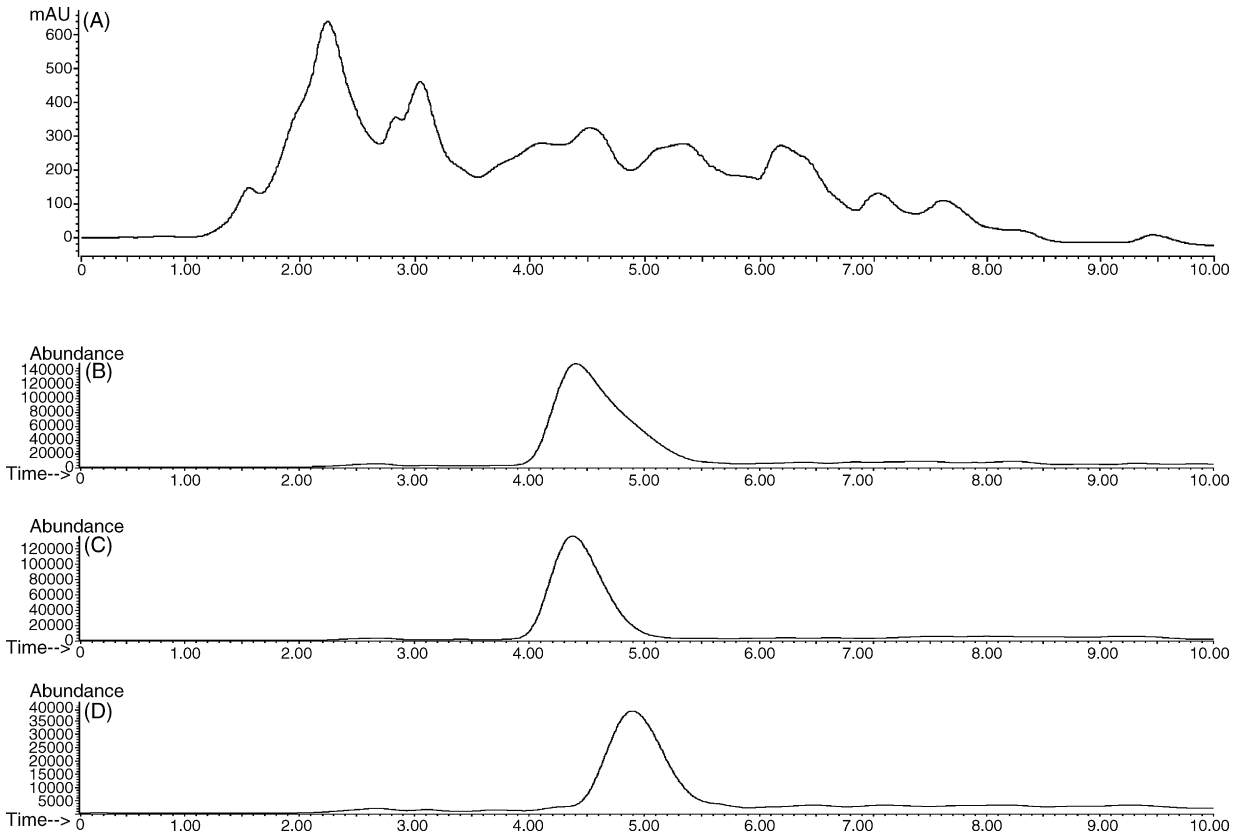

Fig. 2. UV chromatogram at 254 nm (panel A) from a patient plasma sample, extracted as reported in the experimental section; in panel B the corresponding electrospray TIC, which is the sum of DXM (361.5) and FM (379.5) SIM signals, is shown. SIM profiles of flumethasone (panel C) and dexamethasone (panel D) were obtained by extracting single ion chromatograms for ion 379.5 and 361.5, respectively, from the TIC.

between 4.5 and 450 pmol of injected standard molecule. To calculate the concentration of DXM in samples the linear regression equation $y = 990{,}53x + 45{,}895$, $R^2 = 0.998$ obtained from the calibration curve was used. Same results for the calibration curve were obtained using plasma from untreated patients spiked with appropriate volumes of the working DXM solutions. Reference DXM standard solution was prepared according to the European Pharmacopoeia 5.0. Accuracy and "in day" precision were determined using three spiked blank plasma at three different levels: 5, 15 and 30 pmol. The accuracy was in the range of −17 to +14% at the tested levels and the relative standard deviation (RSD) was in the range of 7% to 8.6%. Freeze and thaw stability was determined in triple at each of the DXM concentration reported above after two freeze and thaw cycles of 12 h and accuracy and precision was within the previously reported values; results obtained for the long term stability study, performed applying the same criteria over a period of 30 days on spiked blank plasma stored at −20 °C were in the same range of accuracy and precision of the values reported above. Limit of detection was determined at the signal to noise (S/N) ratio 5:1 giving a LOD value of 1 pmol injected. DXM was not detectable in blank plasma extracted with the method of extraction reported, this demonstrating the specificity of the method.

Before the analysis of a plasma extracted set of samples and respective standard curves all the components of the ion source working at first and second vacuum stage pressure were cleaned extensively and the instrument was tuned with Acid Red 4 and dexamethasone solution (see above in experimental section, mass spectrometry) to work at best. This method enabled detection and quantitation of DXM from patient blood samples up to 26 days after infusion with autologous erythrocytes loaded with the pro-drug dexamethasone-21-phosphate (Table 1).

Table 1
Plasma levels of dexamethasone (μM) up to 26 days post-infusion of dexamethasone 21P-loaded RBC

Patients	Time post-infusion (days)				
	0	1	7	15	26
1	0.116	0.090	0.023	0.026	0.020
2	0.200	0.035	0.031	0.028	0.021
3	0.133	0.131	0.118	N.D.	0.050

Patients received 10.7 ± 3.8 mg dexamethasone 21P by means autologous erythrocyte infusions. N.D. (sample was not detected).

4. Conclusions

In this paper, an HPLC-MS-ESI single quadrupole method was developed to reproducibly detect and quantify dexamethasone in human plasma, after infusion of autologous dexamethasone 21-P loaded erythrocytes, at the picomole level. Electrospray proved to be a reliable ion source to directly detect low molecular weight polar compounds, without the need for sample preparation or derivatization. The source flexibility to

accept standard fluxes made it suitable for the easy transfer of standard HPLC methods to the mass spectrometric detection. The whole procedure appears to be a fast, not expensive, efficient and reproducible method to quantify dexamethasone over a wide concentration range with a single quadrupole instrument, available to most mass spectrometry laboratories. The lowest dexamethasone concentration detectable whit this method was 0.015 μM (about one injected picomole).

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