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Development of a coupled-column liquid chromatographic–tandem mass spectrometric method for the direct determination of betamethasone in urine

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Abstract

Different hyphenated liquid chromatographic (LC) and mass spectrometric (MS) techniques were investigated in order to set-up a method for the fast, direct analysis of betamethasone in hydrolysed and non-hydrolysed urine using large-volume sample injection. After the optimisation of the LC parameters using a traditional UV detector and of the thermospray and mass spectrometric parameters by flow injection, urine samples (0.5 ml) were submitted to analysis by either LC combined with tandem mass spectrometry (MS–MS), coupled-column LC (LC–LC) combined with single quadrupole MS, and LC–LC–MS–MS. Both the three-step configurations (LC–MS–MS and LC–LC–MS) did not provide satisfactory results: loss of sensitivity was noted in the case of LC–MS–MS (likely due to reduced efficiency in the ionisation of betamethasone in the thermospray owing to the presence of large amounts of matrix interference), while in the case of LC–LC–MS a high chemical noise resulting in insufficient selectivity of detection was observed. On the contrary, LC–LC–MS–MS analysis proved to meet the demand of high speed of analysis (sample throughput, 4.5 h^{-1}), selectivity, and sensitivity (LOQ, 1 ng/ml; LOD, 0.2 ng/ml). Notwithstanding the complex analytical system adopted, the developed procedure was manageable and very robust, provided that at the beginning of each analytical session the performance of the system was controlled by checking the retention time of the analytes on the first analytical column with UV detection and by optimising vaporiser temperature of the thermospray by flow injection.

Keywords: Mass spectrometry; Betamethasone

1. Introduction

Betamethasone is a synthetic corticosteroid designed to exert a marked glucocorticoid activity. As the free alcohol or as ester, betamethasone finds widespread clinical applications related to its anti-inflammatory and immunosuppressant activity. In spite of the well documented efficacy, the therapeutic uses of this as well as of other corticosteroids have been defined as ''largely empirical'' [1]. Some of the typical effects of betamethasone on nervous system (euphoria), on carbohydrates metabolism (stimulation of gluconeogenesis), on cardiovascular apparatus, and on blood (increase of the hemoglobin and red-cell content) make this drug attractive for doping purposes. The misuse of corticosteroids in sports has indeed been recognised since 1975. Since 1986 the administration of corticosteroids to athletes has been

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banned with the only exception of topic application, intra-articular injection and inhalation with clearly declared therapeutic purposes by written communication to the Medical Commission of the IOC [2]. Illicit use of betamethasone in zootechnics has also been reported. Together with the already described effects, betamethasone is known to exert a growth promoting action, to act as an appetiser, and to exalt the action of β-agonists. The misuse of corticosteroids in livestock production has been banned since 1986 [3].

The extensive use and misuse of betamethasone needs sensitive and selective analytical procedures in order both to monitor clinical administration and to verify compliance with the law. The selective analysis of corticosteroids in biofluids (serum, urine) has been accomplished by gas chromatography–mass spectrometry (GC–MS) after derivatisation [4–12], and by high-performance liquid chromatography (HPLC)–MS [12–16]. Owing to the low polarity and to the neutral character of the molecule, that limit the possibility to obtain an efficient purification from the biological matrix, sample preparation is undoubtedly the critical step in corticosteroids analysis. Excellent purification has been obtained by means of immunoaffinity chromatography [9,10,16] or by combining different extraction methods, e.g., double Chem Elut extraction followed by HPLC fractionation [5], or liquid–liquid extraction with further purification by gel chromatography [11]. However, all these methods have the disadvantage of being labour and time consuming and, consequently, of providing a low sample throughput. Highly sensitive analysis of drug residues in biological matrices has been successfully performed using liquid chromatography (LC) combined with tandem mass spectrometry (MS–MS) [17] or coupled-column liquid chromatography (LC–LC) combined with MS [18]. Applications of LC–LC–MS–MS to the direct analysis of residues of the β-agonists clenbuterol and salbutamol in urine and serum have been recently described [19–22]. In the present paper the feasibility of direct analysis of betamethasone in hydrolysed and non-hydrolysed urine by large-volume sample injection (0.5 ml) was investigated. Different hyphenated LC and MS approaches were evaluated: LC–MS–MS, LC–LC–MS, and LC–LC–MS–MS.

2. Experimental

2.1. Chemicals and reagents

Betamethasone was kindly gifted by Glaxo Wellcome (London, UK), while prednisolone and 6α-methylprednisolone were purchased from Sigma (St. Louis, MO, USA). Stock methanolic solutions (1 mg/ml) of the analytes were prepared and stored in the dark at −20°C. Stock solutions were diluted in HPLC-grade water when needed. A 0.010 mg/ml dilution of 6α-methylprednisolone (internal standard) in water was prepared for the direct addition to urine samples. HPLC-grade acetonitrile, methanol and water were obtained from Carlo Erba (Milan, Italy), Merck (Darmstadt, Germany) and Sigma–Aldrich (Steinheim, Germany), respectively. Ammonium acetate (p.a., 99%) was purchased from Aldrich. β-Glucuronidase from *E. coli* (specific activity: at least 200 U/ml at 37°C with 4-nitrophenyl-β-D-glucuronide as substrate) was obtained from Boehringer Mannheim (Mannheim, Germany).

2.2. Samples

Urine samples collected from betamethasone-free healthy volunteers where used throughout method development. A post-mortem urine sample collected from a patient chronically treated with betamethasone (administration route and doses unknown) and dead from acute respiratory insufficiency during an asthma attack was also analysed.

2.3. Apparatus

The LC–MS instrumentation (Figs. 1 and 2) consisted of the following components: a Rheodyne (Cotati, CA, USA) Model 7725i manual injector, IV, equipped with 20-μl (flow injection analysis, FIA) or 500-μl loop (LC analysis); a Rheodyne Model 7010 switching valve, RV, controlled by a MUST device from Spark Holland (Emmen, The Netherlands); a Valco (VICI, Shenkon, Switzerland) Model EC4W switching valve, VV, controlled by the MS system; a Waters (Milford, MA, USA) Model 6000A isocratic LC pump, IP; a Waters Model 600 MS gradient LC pump equipped with a flow-rate stabilisation option

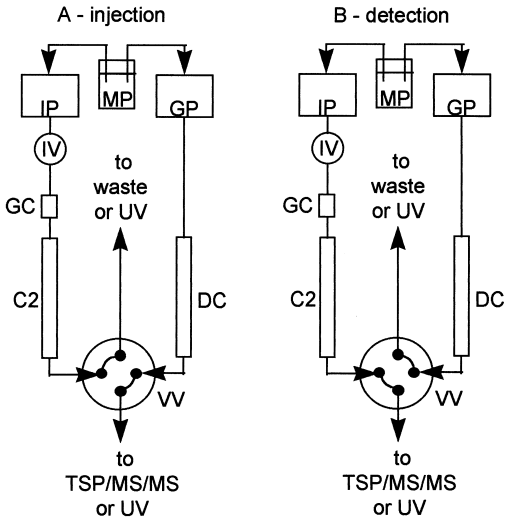

Fig. 1. Scheme of the single-column approach. C2=Analytical column; DC=''dummy'' column; GC=guard column; GP=gradient pump; IP=isocratic pump; IV=injection valve; MP=mobile phase reservoir; VV=Valco switching valve.

and a helium degassing system, GP; a Waters Model 441 UV detector set at 280 nm, UV; a Perkin-Elmer (Norwalk, CT, USA) Model 561 recorder; a Finnigan MAT (San Jose, CA, USA) TSQ 700 tandem mass spectrometer, MS–MS, equipped with a Finnigan MAT TSP-2 thermospray interface, TSP, and with a liquid nitrogen trap system to remove the bulk of the thermospray vapour from the ion source. A 50×4.6 mm I.D. column packed with 3 μm Microspher C_{18} particles from Chrompack (Bergen op Zoom, The Netherlands) was used as the first column (C1). A Chrompack 10×3 mm I.D. RP-guard column (GC) was inserted before C1. A 250×4.6 mm column packed with 5 μm Zorbax TMS particles from DuPont (Wilmington, DE, USA) was used as the second analytical column (C2). A 40×4.6 mm Micro-Guard RP-guard column (Bio-Rad, Hercules, CA, USA) was inserted before C2 in the single-column approach (see Fig. 1). Another column, having the same characteristics as C2, was used as a ''dummy'' column, DC, in order to maintain a constant pressure in the TSP interface while switching VV or RV.

The mobile phase consisted of methanol–water (50:50, v/v) containing 0.1 *M* ammonium acetate (A) or of acetonitrile–water (37:63, v/v) containing 0.15 *M* ammonium acetate (B), or 0.05 *M* ammonium acetate (C). The mobile phase was freshly prepared before use and filtered through a type HA

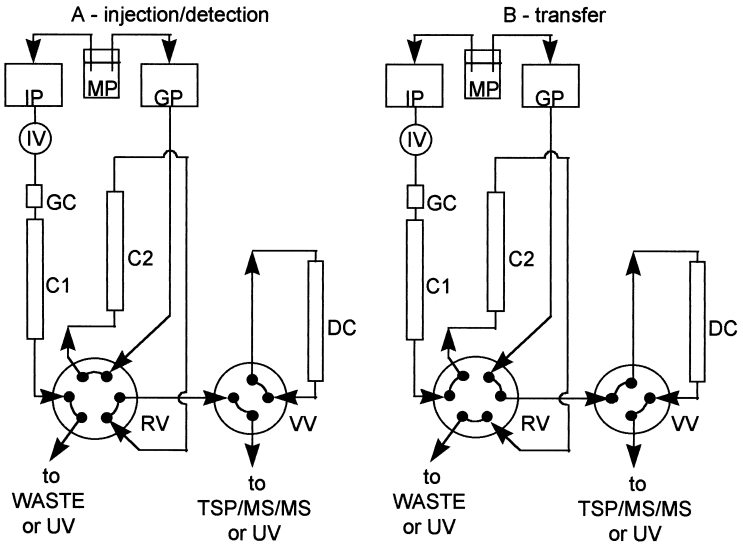

Fig. 2. Scheme of the coupled-column approach. C1=First analytical column; C2=second analytical column; DC=''dummy'' column; GC=guard column; GP=gradient pump; IP=isocratic pump; IV=injection valve; MP=mobile phase reservoir; RV=Rheodyne switching valve; VV=Valco switching valve.

0.45 μm filter (Millipore–Waters). In all cases a 1.3 ml/min flow-rate was used.

2.4. Urine samples pretreatment

Urine samples (7.5 ml) were placed in 10-ml glass tubes and, in the case of LC–LC analysis, 25 μl of internal standard solution was added. After the adjustment of pH to 6–6.5 with 1 *M* NaOH or 1 *M* HCl, 75 μl of the β-glucuronidase solution were added and hydrolysis was performed for 1 h at 45°C. After cooling at room temperature, hydrolysed urine samples were filtered through 0.22 mm Millex-GS filters (Millipore), and 1.5-ml aliquots were injected in the 500-μl injector loop. In some cases the hydrolysis step was omitted and the urine sample was injected after addition of the internal standard, pH adjustment and filtration.

3. Results and discussion

3.1. Set-up of LC conditions

In the single-column approach, chromatographic separation was developed using C2, mobile phase A, and the instrumental configuration illustrated in Fig. 1 (with UV simulating TSP-MS–MS): the sample was injected into C2 and the eluate was sent to waste. Meanwhile, the same mobile phase as that flowing in C2 was pumped by GP, through DC, into UV (Fig. 1, injection). Just before the elution of the analytes, VV was switched in order to allow their transfer into UV (Fig. 1, detection). After the adequate transfer time, VV was switched back to the initial position. Under these conditions, betamethasone exhibited a k' of 3.1, and an excellent peak shape notwithstanding the large amount of sample injected (500 μl). Fig. 3 shows a sequence of injections of a mixture of prednisolone (candidate internal standard) and betamethasone, both at 1 μg/ml, followed by blank hydrolysed urine. As the Figure evidences, column performance remained unaltered after the injection of 0.5 ml of urine (compare first and third standards injections), nonetheless, as expected, the analytes eluted in an extremely ''UV-dirty'' region.

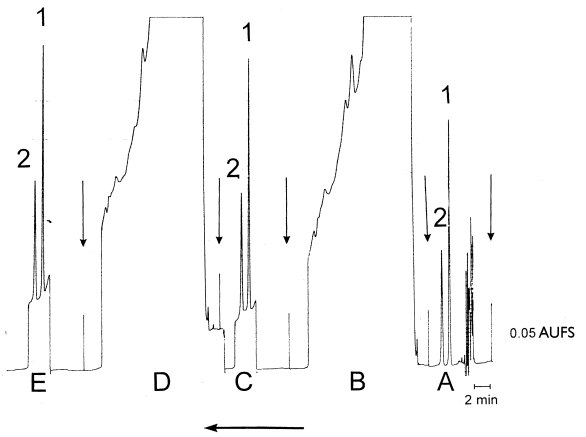

Fig. 3. Single-column (LC) approach. Sequence of injections of a mixture of prednisolone (candidate internal standard) and betamethasone, both at 1 μg/ml (A, C, E), and blank hydrolysed urine (B, D). Switching times for (C) and (E): 0–4.30 min clean-up (eluate sent to waste); 4.30–7.00 min transfer (eluate sent to UV); back to injection position after 7.00 min. (A, B and D): always in detection position (see Fig. 1, no switching applied). Peaks: 1=prednisolone; 2=betamethasone.

3.2. Set-up of LC–LC conditions

In order to reduce the amount of matrix interferences introduced into the TSP, a coupled-column LC approach was investigated. Mobile phase B and the instrumental configuration shown in Fig. 2, with UV simulating TSP-MS–MS were adopted: the sample was injected into C1 and the eluent was sent to waste. Meanwhile, the same mobile phase was pumped by GP, through C2, to UV or TSP-MS–MS (Fig. 2, injection/detection). Just before the elution of analytes, both RV and VV were switched in order to put: (a) C1 and C2 on-line, and (b) DC on-line with UV, thus avoiding the abrupt pressure decrease and the instability of the UV signal during the transfer of analytes from C1 to C2 (Fig. 2, transfer). After the adequate transfer time, the two valves were switched back and the C2 eluent was monitored by UV. The described configuration was preferred to the option of putting on-line C1, C2 and UV during transfer time, avoiding the use of DC and VV. In this case, in fact, the pressure increase in the system would have been too high. For the same reason, viscosity of the mobile phase was reduced by substituting 50% of methanol (mobile phase A) with

37% acetonitrile (mobile phase B). In order to reduce the transfer time from C1 to C2, the internal standard was also changed: 6α-methylprednisolone was chosen as it exhibited a k' quite close to that of betamethasone. Fig. 4 shows a sequence of four consecutive chromatograms: (A) monitoring of C1 eluate with no switching applied after the injection of a mixture of betamethasone and internal standard, both at 1 μg/ml; (B) monitoring of C2 eluate (clean-up time, 1.83 min; transfer time, 0.83 min) after the injection of the same mixture of standards with switching applied; (C) and (D) monitoring of C2 eluate (same switching conditions as B) after the injection of, respectively, hydrolysed and non-hydrolysed urine. Although the two analytes are not completely separated by C2 (Fig. 4B), no further LC optimisation was carried out as the signals of the two analytes can be easily separated by both MS and MS–MS analysis.

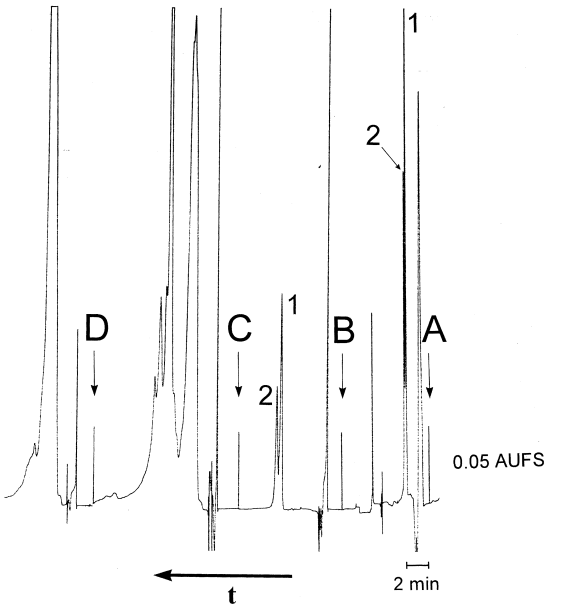

Fig. 4. Coupled-column (LC–LC) approach. Sequence of four consecutive chromatograms: (A) monitoring of C1 eluate, with no switching applied, after the injection of a mixture of betamethasone and internal standard (6α-methylprednisolone), both at 1 μg/ml; (B) monitoring of C2 eluate after the injection of the same mixture of standards with switching applied (clean-up time, 1.83 min; transfer time, 0.83 min); (C) and (D) monitoring of C2 eluate (same switching conditions as B) after the injection of, respectively, hydrolysed and non-hydrolysed urine. Peaks: 1=6α-methylprednisolone; 2=betamethasone.

3.3. Set-up of TSP-MS conditions

The optimisation of TSP-MS as well as of MS–MS parameters was carried out by FIA, with GP pumping the mobile phase, through the IV loop (20 μl), into the TSP, at a flow-rate of 1.3 ml/min. The fragmentation pattern of betamethasone in the TSP ion source operated in the electrolyte-mediated ionisation mode (filament and discharge off) was investigated using different mobile phase compositions (mobile phases A, B and C). Vaporiser and source temperature were set at 100 and 220°C, and repeller voltage was set at 60 V. No differences were observed in the positive ions (PI) fragmentation pattern of betamethasone using different organic modifiers (methanol or acetonitrile) or different concentrations of ammonium acetate (0.05 or 0.15) (Fig. 5). In all cases, in fact, betamethasone exhibited an extensive fragmentation, with four prominent ions: $[MH]^+$ at m/z 393; $[MH-H_2O]^+$ at m/z 375; $[MH-CH_2O]^+$ at m/z 363; and $[MH-C_2H_4O_2]^+$ at m/z 333 (base peak). A similar fragmentation pattern was observed in the case of 6α-methylprednisolone (Fig. 6). Extensive fragmentation, though a favourable condition for identification purposes in the MS mode, is by converse unfavourable in the MS–MS mode where most of the signal should be carried by the parent ion in order to get high sensitivity. Therefore, attempts were made to reduce fragmentation by decreasing the repeller voltage at 30 and 15 V, but no improvement was obtained.

FIA experiments were also performed in order to optimise vaporiser temperature and repeller voltage with both mobile phases A and B. First, vaporiser temperature was increased from 90 to 115°C at 5°C steps, and betamethasone was injected three times at each step (repeller voltage was set at 60 V) measuring the absolute peak areas obtained by monitoring the signal at m/z 333. Similarly, repeller voltage was optimised by increasing its value from 15 to 90 V at 15-V steps (vaporiser temperature set at 100°C). Maximum sensitivities were obtained at 98°C, 60 V and 103°C, 60 V for mobile phases A and B, respectively. According to these result, an acquisition method was prepared for the analysis of urine

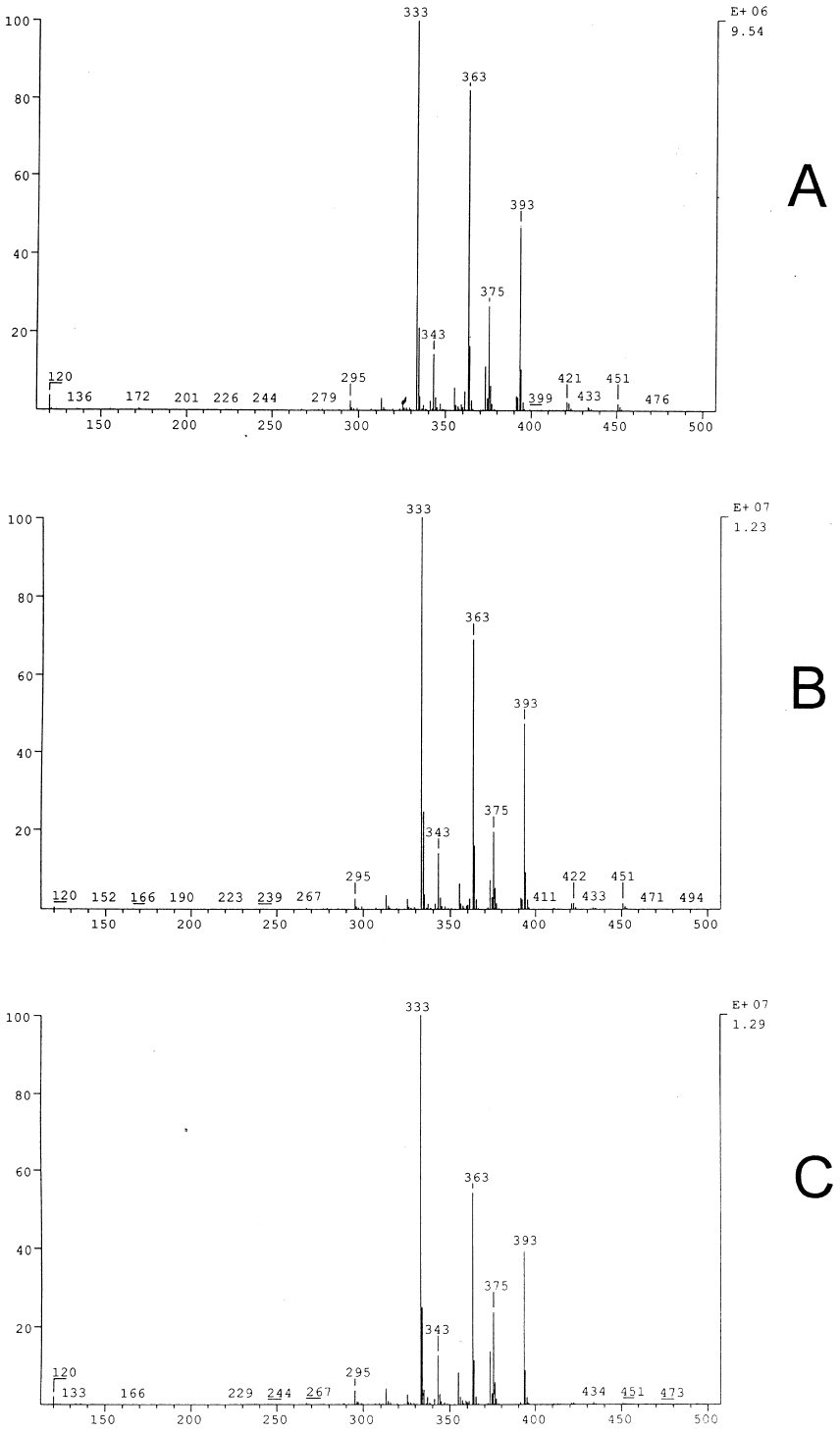

Fig. 5. Fragmentation pattern of betamethasone by FIA–TSP-MS (PI mode) using different mobile phases: methanol–water (50:50, v/v) containing 0.1 *M* ammonium acetate (A); acetonitrile–water (37:63, v/v) containing 0.15 *M* ammonium acetate (B), or 0.05 *M* ammonium acetate (C).

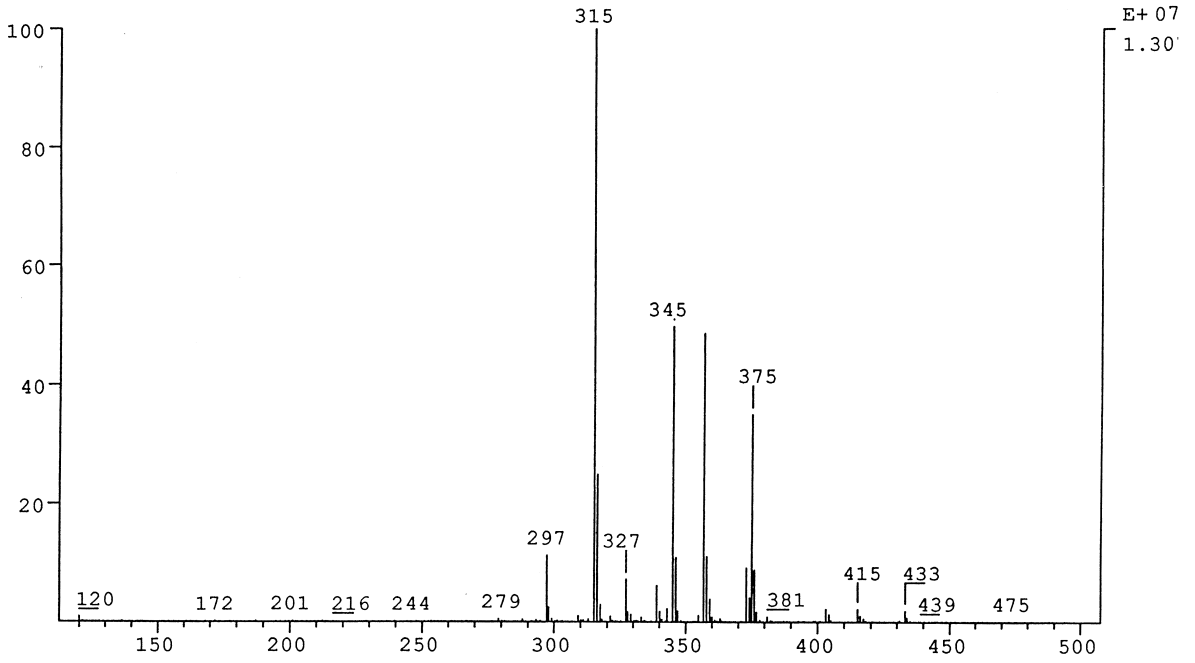

Fig. 6. Fragmentation pattern of 6α-methylprednisolone (internal standard) by FIA–TSP-MS (PI mode) using mobile phase B.

samples after LC–LC separation in selected ion monitoring (SIM) mode. Four ions for betamethasone (*m/z* 393, 375, 363, 333) and four ions for the internal standard (*m/z* 375, 357, 345, 315) were selected, with a total scan time of 1.2 s/scan.

3.4. Set-up of MS–MS conditions

The base peaks in the PI mass spectra of both betamethasone and internal standard were selected as parent ions (*m/z* 333 and 315). The daughter ions mass spectra of both analytes by collision with argon (collision pressure at −1 mTorr; 1 Torr=133.322 Pa) were recorded at different collision energies (from −5 to −45 eV, with 10 eV steps) (Fig. 7). At low collision energies minimal fragmentation of the parent ion occurred in the case of betamethasone: at −15 eV, in fact, the parent ion at *m/z* 333 was the base peak in the spectrum and two main fragments were observed at *m/z* 313 and 295 resulting from the loss of HF and from the combined loss of H_2O and HF, respectively (Fig. 7A). By increasing the collision energy, however, extensive fragmentation of *m/z* 333 occurred with an overall loss of sensitivity (compare the intensity of *m/z* 295 in the spectrum at −15 eV with that of *m/z* 171, base peak in the spectrum at −35 eV shown in Fig. 7B). Therefore, the transition *m/z* 333→*m/z* 295 at −15 eV was selected in order to maximise sensitivity. The daughter ion spectrum of 6α-methylprednisolone significantly differed from that of betamethasone owing to the absence of fluorine in the molecule: at −15 eV the daughter ion resulting from the loss of water (*m/z* 297) was the more intense, and a lower ion at *m/z* 187 was present in the spectrum (Fig. 7C). Despite the higher abundance of *m/z* 297, the transition *m/z* 315→*m/z* 187 was preferred as it was considered to be more selective than that resulting from loss of water. A further experiment was carried out to optimise collision gas pressure. By monitoring the transition *m/z* 333→*m/z* 295 (with collision energy set at −15 eV), betamethasone standard was injected by FIA at three different collision pressures: 0.5, 1 and 2 mTorr. Maximum sensitivity was achieved at 1 mTorr. According to these results, a SRM acquisition method monitoring the transitions *m/z* 333→*m/z* 295 for betamethasone and *m/z* 315→*m/z* 187 for the internal standard (total scan

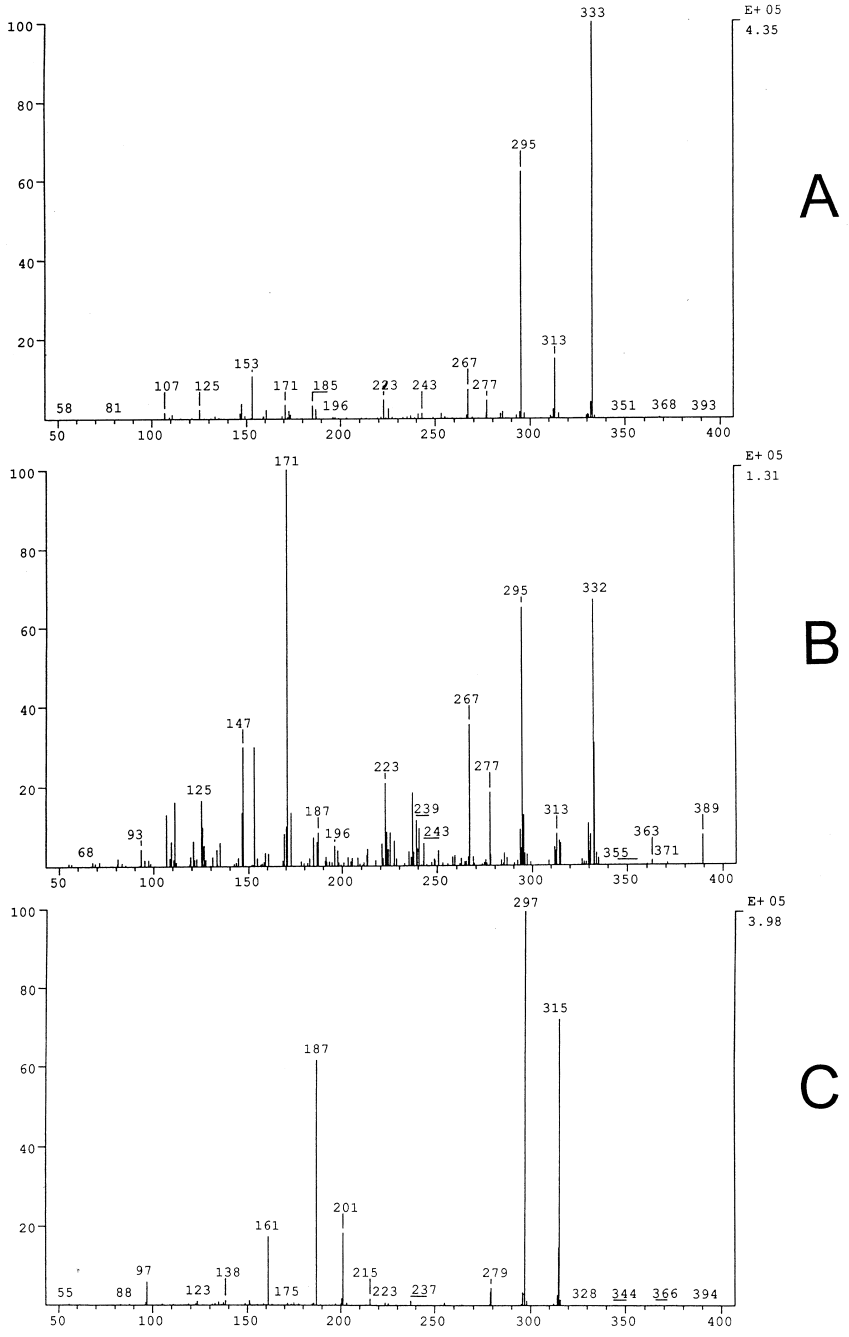

Fig. 7. Daughter ions mass spectra of m/z 333 (parent ion for betamethasone) and of m/z 315 (parent ion for internal standard, 6α-methylprednisolone) by collision with argon (collision pressure, ~1 mTorr) at different collision energies: (A) daughters of m/z 333 at −15 eV; (B) daughters of m/z 333 at −35 eV; (C) daughters of m/z 315 at −15 eV.

time, 1.2 s/scan) was prepared for the MS–MS analysis of urine samples after LC or LC–LC separation.

3.5. LC–MS–MS approach

Notwithstanding the large amount of interferences coeluting with betamethasone in the single-column approach (Fig. 3), MS–MS was expected to provide enough selectivity of detection after LC separation (see Section 3.1). The presence of DC in the adopted instrumental configuration (Fig. 1) reduced the change in pressure accompanying the switching of VV from 2000 to about 100 p.s.i. (1 p.s.i.=6894.76 Pa). Actually, sudden large pressure changes may damage the TSP interface. Nonetheless, GP took about 15 s to stabilise at the new pressure value after the switching of VV, thus obliging to switch 30 s before betamethasone began to elute from C2 in order to perform MS–MS detection under constant pressure conditions. The consequent drawback was to increase the amount of matrix transferred into TSP when urine was injected. Fig. 8 shows the analysis of water (A) and hydrolysed urine (B) both containing 50 ng/ml of betamethasone. VV was switched 5.25 min after injection, and a transfer time of 1.66 min was applied. Compared to the injection of the standard solution of betamethasone, signal abundance was about 80% lower when urine containing the same concentration of betamethasone was analysed. The phenomenon was likely to be a matrix effect on ionisation efficiency of the analyte. Although the ion chromatogram was free from interference peaks, the limit of detection was estimated to be about 20 ng/ml. Owing to the previously discussed reasons the LC–MS–MS approach was considered as not suitable for the direct analysis of betamethasone in urine.

3.6. LC–LC–MS approach

The modifications introduced in the mobile phase (from 50% methanol to 37% acetonitrile) while passing from the single-column to the coupled-column approach were decided not only with respect to chromatographic aspects (see Section 3.2), but also to meet the requirements for optimal TSP-MS detection. It is known, in fact, that sensitivity in the electrolyte-mediated ionisation mode is reduced at the increase of the organic character of the mobile phase [23]. Ammonium acetate concentration was also increased in the attempt to prevent further matrix effects on ionisation efficiency of analytes in the TSP ion source. The mass chromatograms at m/z 357 (internal standard) and m/z 393 (betamethasone) obtained for the analysis of water (A) and hydrolysed urine (B) both spiked with betamethasone at 50 ng/ml and of blank hydrolysed urine (C) are shown in Fig. 9. Despite the high signal abundance and an apparent good selectivity, chemical noise was still remarkable, as evidenced by the mass chromatograms of blank urine. In the adopted conditions a detection limit of about 10 ng/ml could be achieved.

3.7. LC–LC–MS–MS approach

A four-step approach was then attempted by combining the LC–LC settings described in Section 3.2 with the MS–MS settings described in Sections 3.3 and 3.4. Preliminary experiments showed that the addition of a fourth analytical step resulted in a substantial improvement in selectivity due to the effective removal of chemical noise. Furthermore, no loss of signal due to matrix effect was observed when the signal abundance given by the same amount of betamethasone added to water or to hydrolysed urine, respectively, were compared. The analyte's signal appeared in a region of the chromatogram where chemical noise is extremely low, thus increasing the S/N ratio (Fig. 10). Therefore, the four-step approach was chosen in the final procedure.

Some example LC–LC–MS–MS chromatograms of urine samples are shown in Fig. 10.

The linearity of the method was tested in the range 1–50 ng/ml with the random analysis of blank hydrolysed urine samples spiked with betamethasone at different concentrations. The correlation coefficient of the analyte/internal standard ratio was 0.9951. The precision and accuracy data are shown in Table 1. The lower limit of the calibration plot was established as the quantification limit at 1 ng/ml ($S/N>10$), while the limit of detection ($S/N=3$) was of 0.2 ng/ml.

Notwithstanding the complex analytical system adopted, the procedure proved to be manageable and

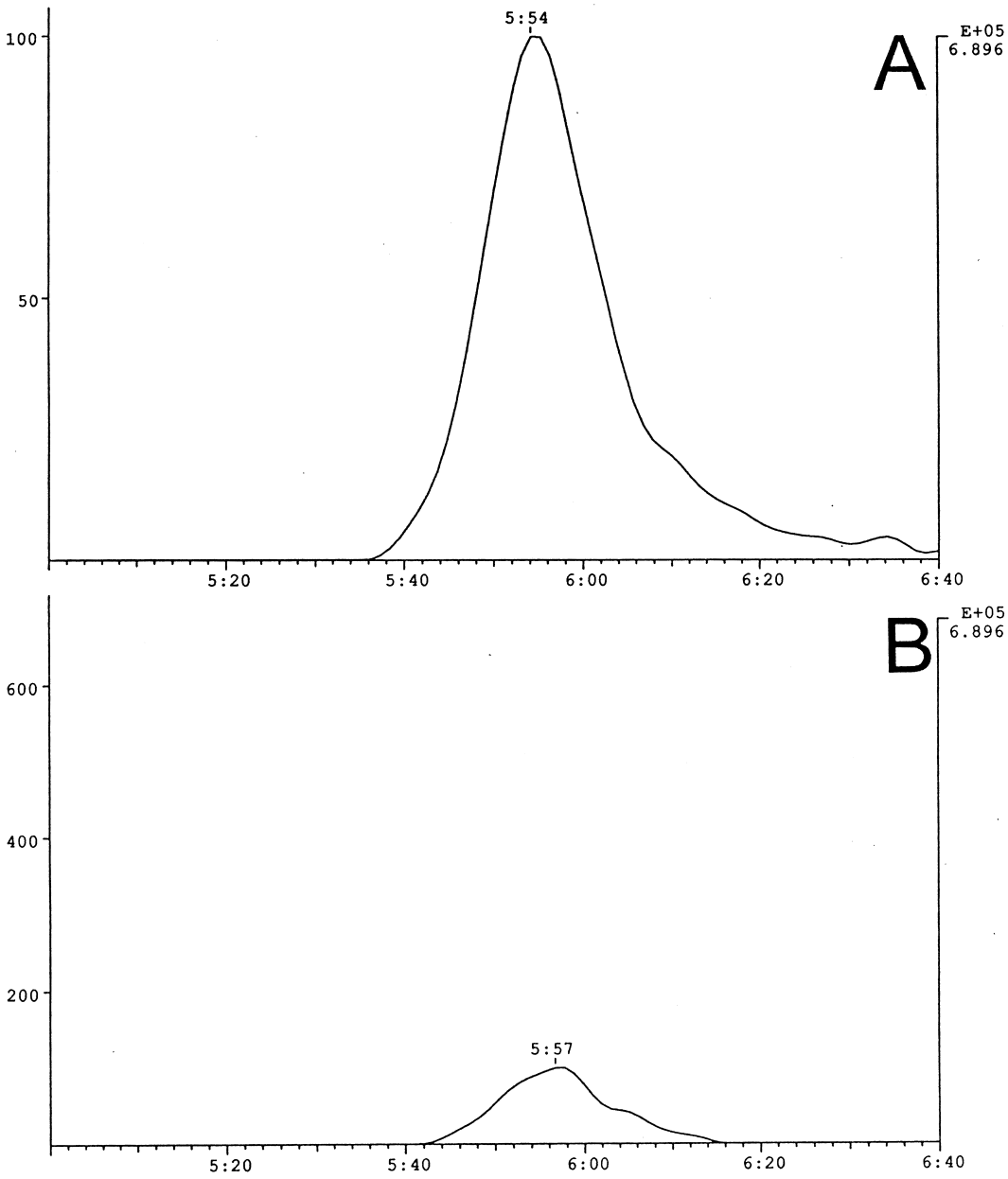

Fig. 8. LC–MS–MS approach. Analysis of water (A) and hydrolysed urine (B) both containing 50 ng/ml of betamethasone. Clean-up time: 5.25 min; transfer time: 1.66 min.

very robust as verified with the consecutive analysis of 0.5-ml samples of hydrolysed and non-hydrolysed urine. After sessions of more than 20 injections no increase in pressure of the LC system, no clogging of the TSP interface and no decrease in the overall performance of the MS system were observed. The variations in C1 performance owing to the large volume injected were less pronounced than expected and were easily controlled by applying an adequate transfer volume (1.08 ml compared to a total elution

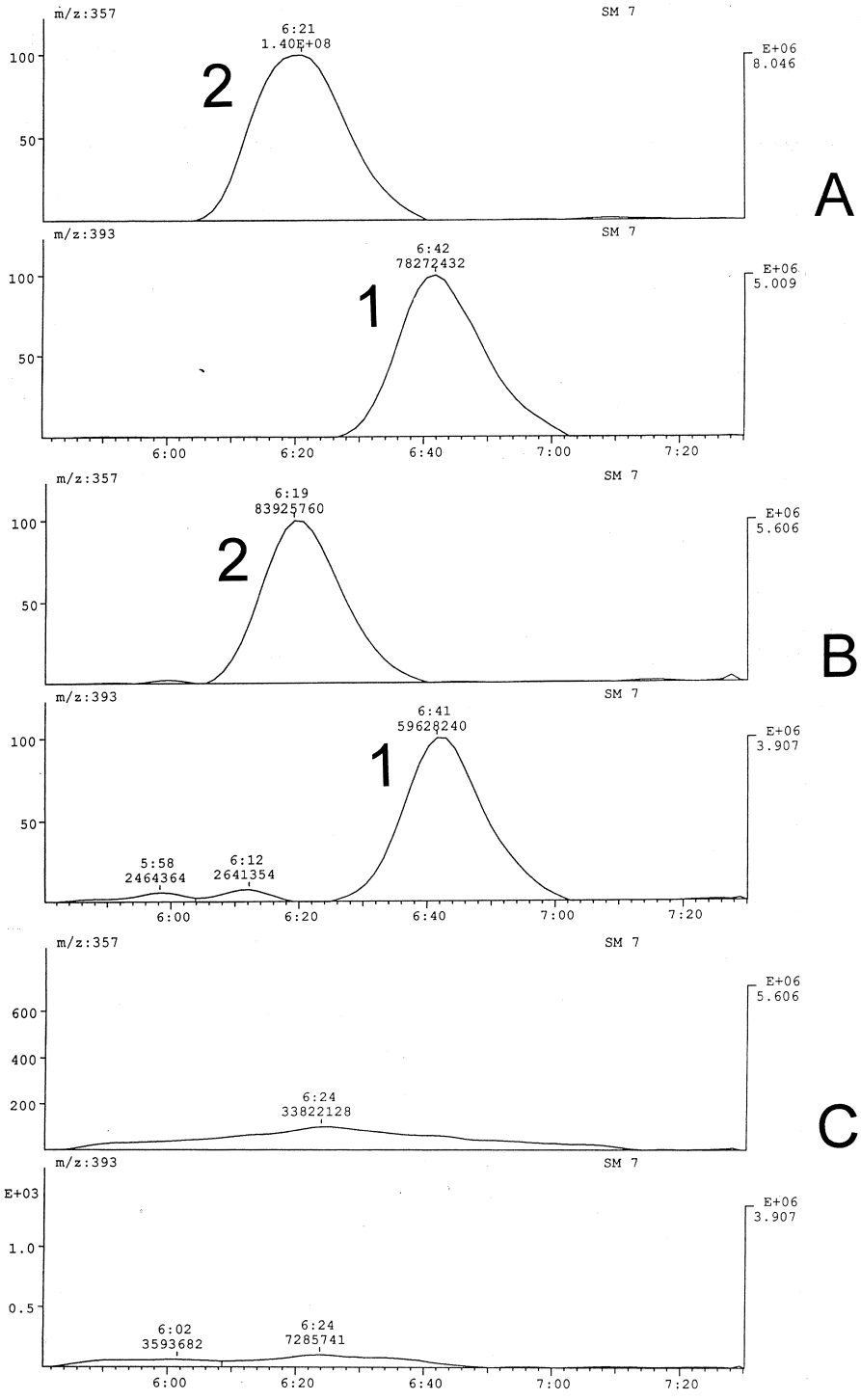

Fig. 9. LC–LC–MS approach. Mass chromatograms at *m*/*z* 357 (internal standard) and *m*/*z* 393 (betamethasone) obtained for the analysis of water (A) and hydrolysed urine (B) both spiked with betamethasone at 50 ng/ml and of blank hydrolysed urine (C). Peaks: 1=betamethasone; 2=internal standard (6α-methylprednisolone).

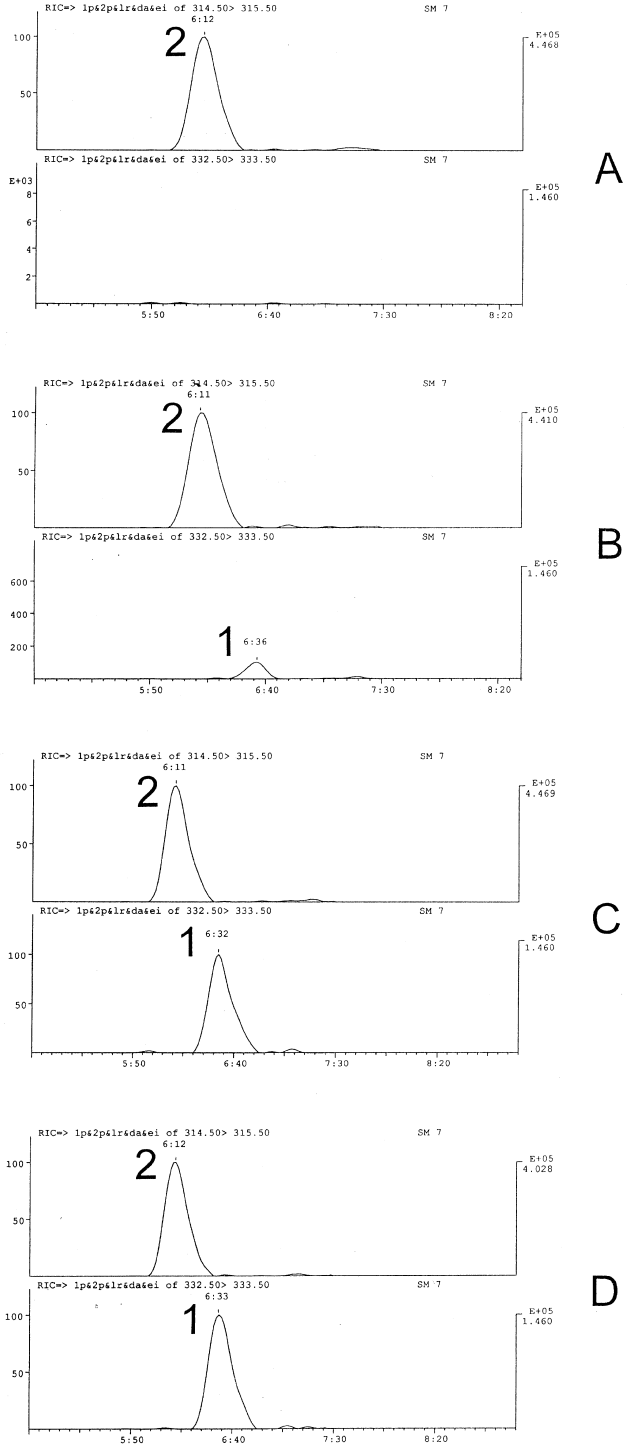

Fig. 10. Example chromatograms obtained using the LC–LC–MS–MS approach: blank hydrolysed urine (A); blank hydrolysed urine spiked with betamethasone at 1 ng/ml (B) and 10 ng/ml (C); hydrolysed urine sample in which a concentration of 13.5 ng/ml of betamethasone was measured (D). Peaks: 1=betamethasone; 2=internal standard (6α-methylprednisolone).

Table 1
Precision and accuracy data for the direct LC–LC–MS–MS analysis of betamethasone in hydrolysed urine

Concentration added (ng/ml)	Concentration found (ng/ml)	Standard deviation (ng/ml)	*n*	R.S.D. (%)
1	0.96	0.21	4	22.26
2	1.96	0.38	4	19.66
5	4.84	0.78	4	16.22
10	10.28	0.19	4	1.93
50	49.95	4.68	4	9.37

volume of the two peaks of 0.32 ml). Moreover, the analysis of an aqueous solution of standards every three injections of urine samples allowed to check for reproducibility of retention times and for column contamination.

At the beginning of each analytical session the performance of the system could be rapidly controlled (a) by checking the retention time of the analytes on C1 with UV detection and (b) by optimising the vaporiser temperature of the TSP interface by FIA of an aqueous standard of betamethasone. At the end of each session the TSP interface was washed by direct connection with GP with 10 ml of water followed by 10 ml of methanol; C1 and C2 were separately cleaned, each with 15 ml of methanol.

The mass chromatograms produced by a sample of hydrolysed urine in which betamethasone was detected is shown in Fig. 10D. In this sample a mean concentration of 13.5 ng/ml of the analyte ($n=2$, 13.3 and 13.8 ng/ml) was measured. The same sample analysed without hydrolysis gave a mean betamethasone concentration of 12.6 ng/ml ($n=2$, 12.6 and 12.7 ng/ml).

4. Conclusions

The LC–LC–MS–MS method here described allowed the sensitive and selective analysis of betamethasone in urine by direct large-volume injection. It proved to be the only among the investigated approaches able to provide adequate performance with respect to the goals of the analysis. In fact, both the three-steps analytical approaches, LC–MS–MS and LC–LC–MS, lacked the required sensitivity and selectivity, respectively. On the contrary, LC–LC–MS–MS, although more complex to assemble and manage than the three-step instrumental configurations, met the requirements of robustness, precision and high speed of analysis. This last feature was considered critical in order to counterbalance the operative costs of the apparatus with a high sample throughput (4.5 h^{-1}, not including the hydrolysis step which can be accomplished on a batch of samples, utilising dead times).

The method is potentially suitable for the target analysis of other corticosteroids as well as of other drugs in urine [19–21] and serum [22]. The needlessness for extensive sample preparation allows an easy and rapid method development, which is limited to the optimisation of the chromatographic parameters with a traditional UV detector on one side, and to the optimisation of the mass spectrometric parameters by FIA on the other.

Other ionisation modes more efficient than thermospray, such as electrospray (ESI) and atmospheric pressure chemical ionisation (APCI) that are rapidly becoming the standard in interfacing LC to MS will be investigated in the future.

The potential of the method in the analysis of betamethasone in serum using an internal surface reversed-phase (ISRP) column [22], in the analysis of betamethasone glucuronide (thus avoiding the hydrolysis step), and its applicability to the screening of urine for corticosteroids will be also matter of future research.

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