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Validation of a Qualitative Screening Method for Corticosteroids by Liquid Chromatography Tandem Mass Spectrometry

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1 . Introduction

Corticosteroids are released by the adrenal gland and are involved in many biochemical processes in the human body. They are highly potent in the treatment of inflammations and asthma. Besides, they can alleviate pain in general (1).

As a result therapeutical use of corticosteroids is widespread among sportmen and women. Nevertheless the use of corticosteroids should be controlled as regular use of corticosteroids has physiological and psychological consequences including immunosuppression and euphoria (2).

To control the use of corticosteroids and to prevent their misuse related to euphoria and pain-suppression, the use of corticosteroids is restricted by some sport federations. Systemic use of corticosteroids is forbidden in all circumstances but when medically necessary, local and intra-articular injections are allowed under medical supervision (3).

As GC/MS gives low sensitivity and selectivity due to a required derivatisation step other analytical techniques are preferred. Although HPLC/UV does not need a derivatisation step low sensitivity and selectivity is observed. ELISA is often used to screen urine and plasma for corticosteroids. However this method does not provide the mass spectral data needed for identification . LC/MS seems to be the method of choice. It allows for the direct separation of substances and identification without the need for derivatisation. Moreover it has the required

sensitivity and selectivity. As a result different methods for the screening for corticosteroids with LC/MS have been published (4, 5).

In our laboratory the need for a validated screening method for corticosteroids was high and the resulting method is presented here.

2 . Experimental

Chemicals

Beclomethasone and desoximethasone were bought from Sigma-Aldrich (Bornem, Belgium). Betamethasone and budesonide were a gift from Glaxo-Wellcome (Greenford , Great Britain). Cortisone was purchased from Akzo (Brussels, Belgium) and dexamethasone from Organon (Brussels). Hydrocortisone was obtained from ERFA (Brussels), methylprednisolone from Pharmacia (Diegem, Belgium). Triamcinolone acetonide was a gift from Labaz (Brussels) and the human metabolite of budesonide, 16- α hydroxyprednisolone was obtained from Astra-Zeneca (Lund, Sweden). Potassium carbonate p.a., methanol p.a., diethylether p.a. and acetic acid p.a. were from Merck (Darmstadt, Germany), HPLC grade acetonitrile was purchased from ACROS (Geel).

Excretion study

After oral intake of 1 tablet of Medrol® (Pharmacia) containing 32 mg of methylprednisolone by a female volunteer aged 57, urine samples were collected at 0 h and after 2, 3, 6, 9, 12, 24, 48 and 72 h.

Validation

Validation was carried out following Eurachem validation guidelines (6).

Ten urine samples were spiked at 6 different levels with 7 corticosteroids. Final concentrations were 8,4, 2, 1, 0.5, 0.25 and 0.1 ng/mL, respectively.

Selectivity was tested by analysing several doping agents including beta blockers, narcotics, diuretics and anabolic steroids.

Specificity was tested during the validation procedure. Ten blank urines were extracted and analysed as described above.

Extraction

Internal standard solution (50 µl desoximethasone, 1 µg/mL) was added to 5 mL of urine, followed by addition of 1 g potassium carbonate. Diethylether (5ml) was added and the sample was extracted by rolling for 20 minutes. After centrifugation the organic layer was transferred into a new tube and evaporated to dryness under oxygen free nitrogen (OFN) at 40 °C. The remaining residue was dissolved in 200 µl mobile phase.

Apparatus

Analysis was performed on a Finnigan MAT LCQ-Deca[®] equipped with a 10 cm Nucleosil 5 octadecylsilyl column (id = 3 mm, particle size = 5 µm) (Chrompack; Antwerp, Belgium). The mobile phase consisted of 1% acetic acid (solution A) and acetonitrile. Gradient elution at a flow rate of 0.3 mL/min was used (table 1).

Period (min)	Acetic acid (1%) (%)	Acetonitrile (%)	Flow (ml/min)
0-5	70	30	0.3 ml
5-7	35	65	0.3 ml
7-12	35	65	0.3 ml
12-18	70	30	0.3 ml

TABLE 1: HPLC program

MS/MS and MS³ spectra were determined using direct infusion.

Ionization of analytes was carried out using Electro Spray Ionisation (ESI). The capillary temperature was maintained at 300 °C, the nebulizer gas (nitrogen) was set to 80 units and the auxilliary gas flow was set to 10 units. The capillary voltage was -18 V for the negative ionisation mode and 4 V for the positive ionisation mode.

3. Results and discussion

Good sensitivity for corticosteroids is obtained in both positive and negative electrospray ionisation mode.

Since most abundant ions were observed in negative ionisation mode this ionisation interface was preferred. Corticosteroids were clearly present by the acetate adduct ion $[M+CH_3COO]^-$ in the full scan spectrum. No $[M-H]^-$ was observed.

Tandem mass spectrometry is often used to enhance sensitivity and selectivity, and often results in mass spectra with more diagnostic ions. Moreover product spectra allow for the generally accepted minimum standard of 3 diagnostic ions. As an example the LC/MS and LC/MS/MS spectra of beclomethasone are presented in Figures 1 and 2.

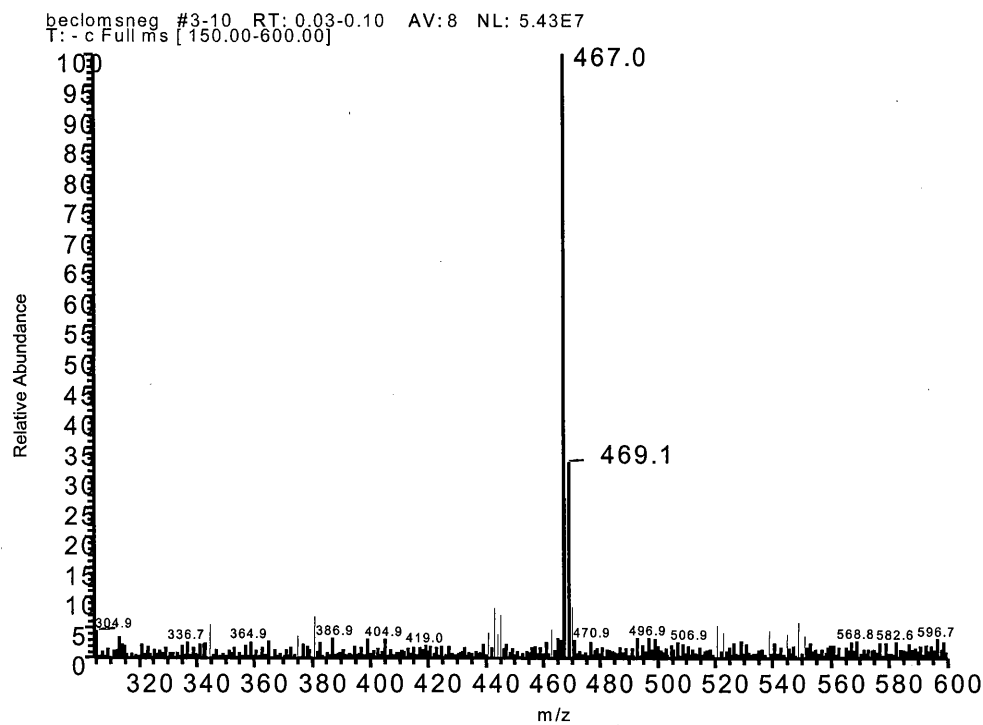


Figure 1: Full scan ESI(-) mass spectrum of beclomethasone (M_w : 408)

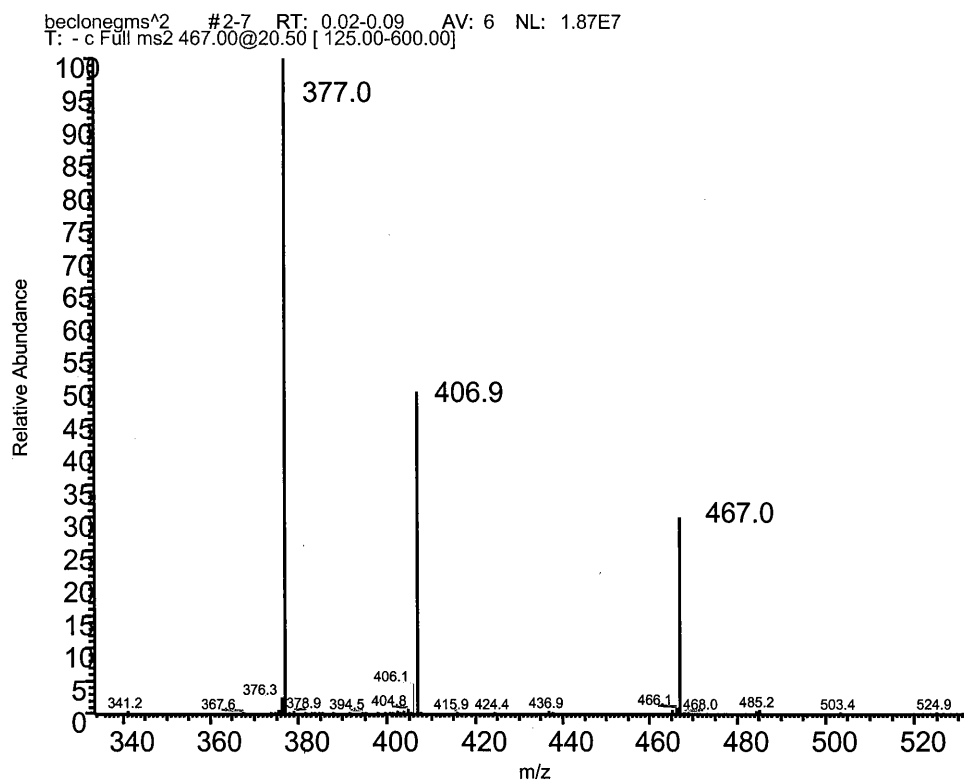


Figure 2 ESI(-) MS/MS spectrum of beclomethasone (precursor: m/z 467)

For all substances two abundant ions were selected from the MS/MS spectrum to evaluate their presence in the urine samples.

Negative ionisation tandem mass spectrometry for 16- α hydroxyprednisolone only resulted in the product ion $[M-H]^-$ and hence positive ionisation tandem mass spectrometry was used for 16- α hydroxyprednisolone resulting in substantial fragmentation. The ions monitored in the screening method are presented in Table 2.

Substance	RRT	M _w	Ionisation mode	precursor ion	diagnostic ions
16 α -hydroxyprednisolone	0.36	376	ESI (+)	377	341, 323
Hydrocortisone	0.55	362	ESI (-)	421	360, 331
Cortisone	0.58	360	ESI (-)	419	358, 329
Methylprednisolone	0.77	374	ESI (-)	433	343, 372
Betamethasone	0.82	392	ESI (-)	451	361, 391
Dexamethasone	0.85	392	ESI (-)	451	361, 391
Beclomethasone	0.93	408	ESI (-)	467	377, 406
Triamcinolone acetonide	0.95	434	ESI (-)	493	375, 413
Desoximethasone (ISTD)	1.00	376	ESI (-)	435	375, 355
Budesonide	1.11	430	ESI (-)	489	357, 339

RRT: relative retention time, M_w: molecular weight

Table 2: RRT, ionisation mode and diagnostic ions

The described method seems to be very selective as no interferences were detected when other doping products including beta-blockers, narcotics, diuretics and anabolic steroids were analysed. In addition, several NSAID's did not interfere with the analysis.

The detection limits are presented in Table 3. Highest sensitivity was observed for triamcinolone acetonide and beclomethasone.

Substance	c (ng/mL)
Desoximethasone*	-
Triamcinolone acetonide	0.5
Dexamethasone	1
Betamethasone	2
Budesonide	4
16 α -Hydroxyprednisolone	4
Methylprednisolone	2
Beclomethasone	0.5
Hydrocortisone	endogenous
Cortisone	endogenous

* ISTD

Table 3: : Detection limits

Specificity was satisfactory as no interfering substances at the appropriate retention times were detected when 10 blank urines were analysed.

Hydrocortisone and cortisone are endogenous corticosteroids and were clearly present in all urine samples.

The method was applied for the detection of methylprednisolone after oral intake of Medrol®. Methylprednisolone was already detected 2 h after intake. The last urine sample in which methylprednisolone could be detected was collected after 48 h. Ion chromatograms and mass spectra of methylprednisolone in the quality control urine and in the urine sample 48 h after intake are presented in Figure 3.

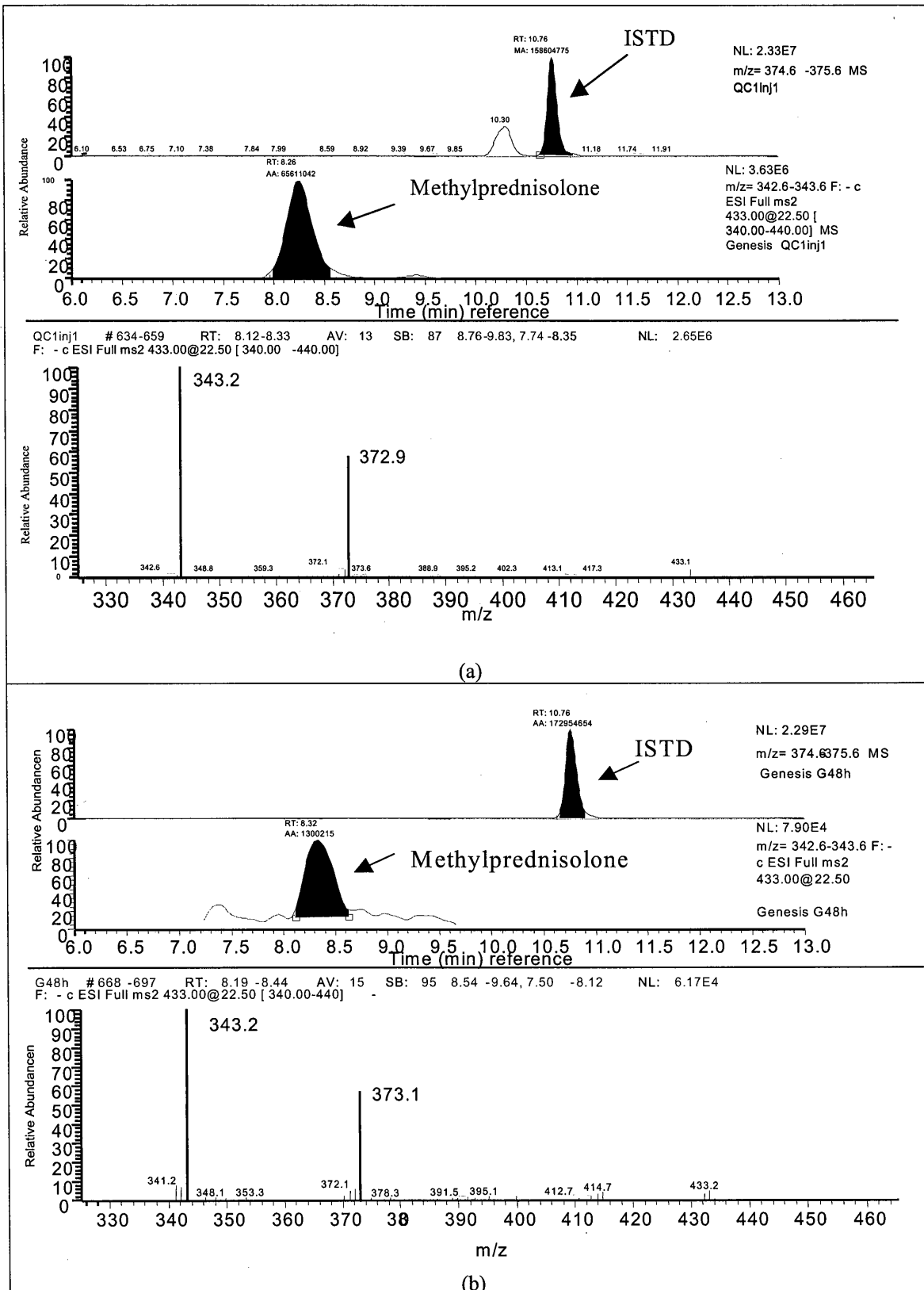


Figure 3: (a) quality control urine spiked at 8 ng/mL

(b) Excretion urine 48 h after administration of Medrol®

4. Conclusion

A screening method for 9 corticosteroids in urine based on LC/ESI/MS/MS was developed and validated.

The method shows to be very sensitive for the detection of corticosteroids after a single liquid-liquid extraction. The detection limits were at least 4 ng/mL for all compounds.

The method was successfully applied for the detection of methylprednisolone after oral intake of one tablet.

5. References

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