Catrin Goebel, Graham Trout, Ray Kazlauskas

Corticosteroids by LC/MS/MS - a minimalist approach

Australian Sports Drug Testing Laboratory (ASDTL), National Measurement Institute (NMI), Sydney, Australia

Introduction

All World Anti-Doping Agency (WADA) accredited laboratories face the problem of having to modify their methods each year to keep up with the changes in compounds to be detected as specified in the WADA Prohibited List (WADA 2005). The 2004 and 2005 Lists have introduced over 30 new compounds including a new class of compounds namely the glucocorticosteroids. Some compounds are relatively simple to add to existing screening procedures whilst others require the development and implementation of new methods. The laboratories face a common problem of having to extend their capabilities without adequate provision of additional resources. The addition of corticosteroids to the 2004 WADA Prohibited List meant that our laboratory needed to quickly develop a method which could be implemented with minimal cost impact. To meet our goals it was highly desirable that the new method:-

- use an existing extraction method so that no additional sample preparation costs are involved.
- use an existing derivatisation procedure or not require derivatisation.
- use an extension or variation of an existing analysis method.

A number of methods have been published for the detection of corticosteroids in human urine (Fluri et al 2001 and Deventer and Delbeke 2003) but none met the criteria we had set for simplicity and cost effectiveness.

Prior to 2004 our laboratory ran the following chromatographic screens :-

- Anabolic steroids using C18 Empore SPE and analysis of MSTFA/TMSI/thiol derivatives by GC/MSD and GC/HRMS. One vial two injections.

- Diuretics using Varian Nexus SPE cartridges and analysis by LC/MS/MS.
- Stimulants using extraction with tertiary butyl methyl ether and analysis by GC/NPD and confirmation by GC/MSD.
- Narcotics using extractive alkylation and analysis by GC/MSD.

We found that the diuretics method could be modified with the least effort to include the detection of corticosteroids.

Experimental

All reagents were of AR or HPLC grade. Water was from a Milli-Q water purification system. The corticosteroids were obtained from the Chemical Reference Materials Section of NMI, Sigma-Aldrich (Sydney NSW Australia), Steraloids (Newport R.I. USA) or from the manufacturers.
The sample extraction procedure was:

- To each 2.5 mL of urine was added 1.5 mL of pH 7 acetate buffer, 100 uL of mefruside/methyltestosterone internal standard (10/0.8 ug/mL) and 40 uL b-glucuronidase E coli (03 707 601 001, Roche, Sydney NSW Australia). The tubes were vortexed and incubated at 50 ^{o}C for 30 minutes and the analytes extracted by passage of the sample through a Varian ABSELUT Nexus SPE column (60 mg, 3 mL). The cartridge was washed with 1 mL water and 1 mL 25% methanol in water, prior to elution of the analytes with 2 mL methanol.
- The methanolic extract was evaporated to dryness under nitrogen and reconstituted in 200 uL of 50% methanol in water.

The HPLC separation was carried out using a Waters 2795 Alliance separation module with a C18 column (Alltech Prevail, 50 mm × 2.1 mm × 3 um) protected by a C18 guard column. The following ternary mobile phase gradient was formed by solvent A (2% aqueous formic acid), solvent B (water), and solvent C (acetonitrile) at a flow rate of 0.2 mL/min: constant 10% A, 0 to 30% C (0 to 0.5 min), 30% C (0.5 to 5 min), 35 to 80% C (6.5 to 7 min), 80% C (7 to 7.5 min), 80 to 0% C (7.5 to 8 min) and 0% C (8 to 11 min).

The MS-MS detection was carried out using a Micromass Quattro Micro with a Z spray API interface. The spray conditions of the interface were: desolvation temperature 200 ^{o}C, desolvation gas flow 550 L/hr, and cone gas flow 50 L/hr. The capillary was set at 3.5 kV and the substance specific cone voltage and collision energy were optimised for each compound. Argon was used as the collision gas at a pressure of 3.3×10^{-3} mbar. Multiple reaction monitoring (MRM) was carried out at the optimum settings using both positive and negative ion modes. The specific conditions used for each analyte are shown in Table 1.

Results and Discussion

As the corticosteroids have similar chemical properties to the anabolic steroids they are extracted in our normal steroid method using C18 Empore cartridges (Kazlauskas et al 1999). However, the derivatising agent used in this method did not give suitable results, producing multiple derivatives and low sensitivities. We investigated the use of an alternative derivatising agent using microwave digestion (Amendola et al 2003) but were unable to obtain reproducible results. We then investigated whether the corticosteroids were being extracted in any of our other routine screens and found that they were also present in the Varian Nexus extract used for the LC/MS/MS diuretics screen (Goebel et al 2004). A standard containing several corticosteroids was analysed using the same chromatographic conditions as that used for our routine diuretics screen and the results obtained are shown in Figure 1. It can be seen that the corticosteroids are readily detected but they are eluting in a very narrow time window with several coeluting. It was not possible to include the corticosteroids in the existing diuretics LC/MS/MS analysis method because more MRM switches were required than the instrument could achieve. There were two obvious ways of accommodating the analysis of the corticosteroids. The first was to modify the diuretics LC/MS/MS method so that it had a much longer run to allow the detection of both diuretics and corticosteroids in one analysis, whilst the second was to develop a new separate analysis method for corticosteroids. The second option was chosen because whilst all our samples are analysed for diuretics, only 30% (the in-competition samples) are analysed for corticosteroids. The optimised method is described in the experimental section.

Analysis of spiked urine samples using this method showed that all the required parent drug analytes could be detected at levels of at least 10 ng/mL. Figure 2 shows the results obtained from a standard mix spiked into urine at a concentration of 30 ng/mL. All the compounds added were readily detected. Recovery experiments demonstrated that the analytes spiked into urine at 30 ng/mL were recovered with efficiencies ranging from 61% to 104%. The results from a positive sample are shown in Figure 3. The subject had been administered budesonide and the results clearly show the detection of the parent compound at low concentration along with a higher concentration of the metabolite 16α OH prednisolone.

The method satisfactorily resolves all but two of the analytes namely betamethasone and dexamethasone. These two compounds differ only in the orientation of a methyl group and have essentially the same MS/MS spectrum and almost identical retention times on the C18 column. To separate these two compounds a separate LC analysis is carried out using a Hypercarb column (Thermo Electron 30 mm x 2.1 mm x 5 um) using an isocratic separation at 90% acetonitrile. Under these conditions betamethasone and dexamethasone are fully resolved.

Conclusions

We are able to screen for the corticosteroids specified by WADA using LC/MS/MS analysis of our existing "diuretics" SPE extract with detection levels lower than the 30 ng/mL proficiency level required (WADA 2004). All compounds have sufficient ions for confirmation by LC/MS/MS.

References

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ANALYTE	MS RUN CONDITIONS					
	Ion Mode	RRT	Precursor Ion	Product Ion	Cone Voltage (V)	Collision Energy (eV)
Beclomethasone	–	1.05	453.1	377.1	25	15
Betamethasone	–	0.97	437.0	361.0	45	15
Budesonide	+	1.17	431.1	413.1	20	15
Desonide	+	1.09	417.0	323.1	20	15
Dexamethasone	–	0.97	437.0	361.0	45	15
Fludrocortisone	–	0.79	425.1	349.1	27	20
Flumethasone	–	0.97	455.0	379.0	30	20
Flunisolide	+	1.08	435.0	321.0	20	15
Fluticasone	+	1.04	397.0	356.9	20	15
Fluticasone propionate	+	1.19	501.1	313.0	20	15
Fluocortolone	+	1.11	377.0	303.0	20	12
Methylprednisolone	–	0.95	419.2	343.1	30	15
Prednisolone	–	0.77	405.0	294.9	30	15
	+		361.0	147.0	40	20
Prednisone	–	0.77	327.1	149.0	50	30
Triamcinolone	–	0.64	393.1	345.2	25	15
Triamcinolone acetonide	+	1.08	435.0	415.2	20	12
16a-OH-prednisolone	+	0.64	377.0	323.0	20	10
Mefruside (I.S.)	+	1.00	383.0	98.8	28	16

Table 1. Conditions used for the analysis of the corticosteroids.

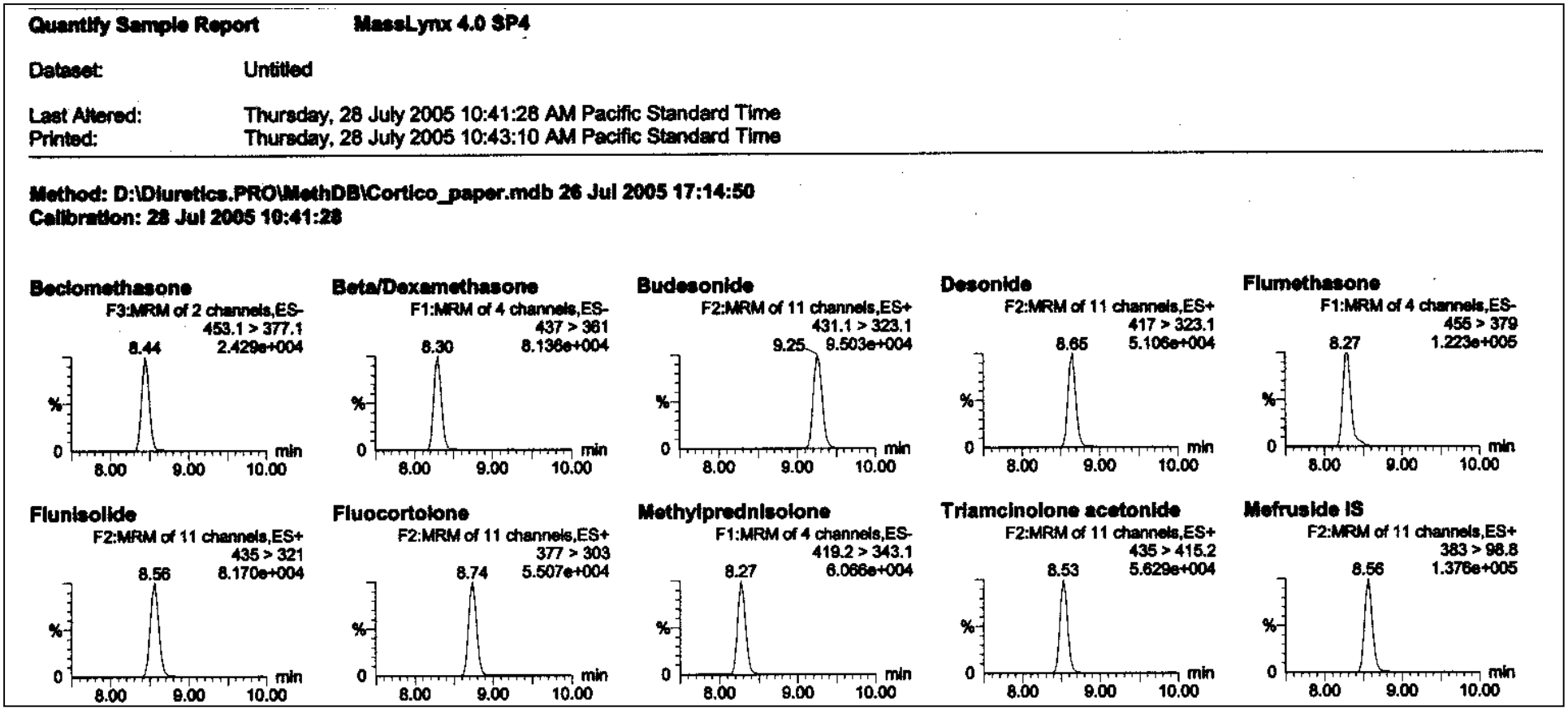

Figure 1. Example of corticosteroids run using the diuretics LC method.

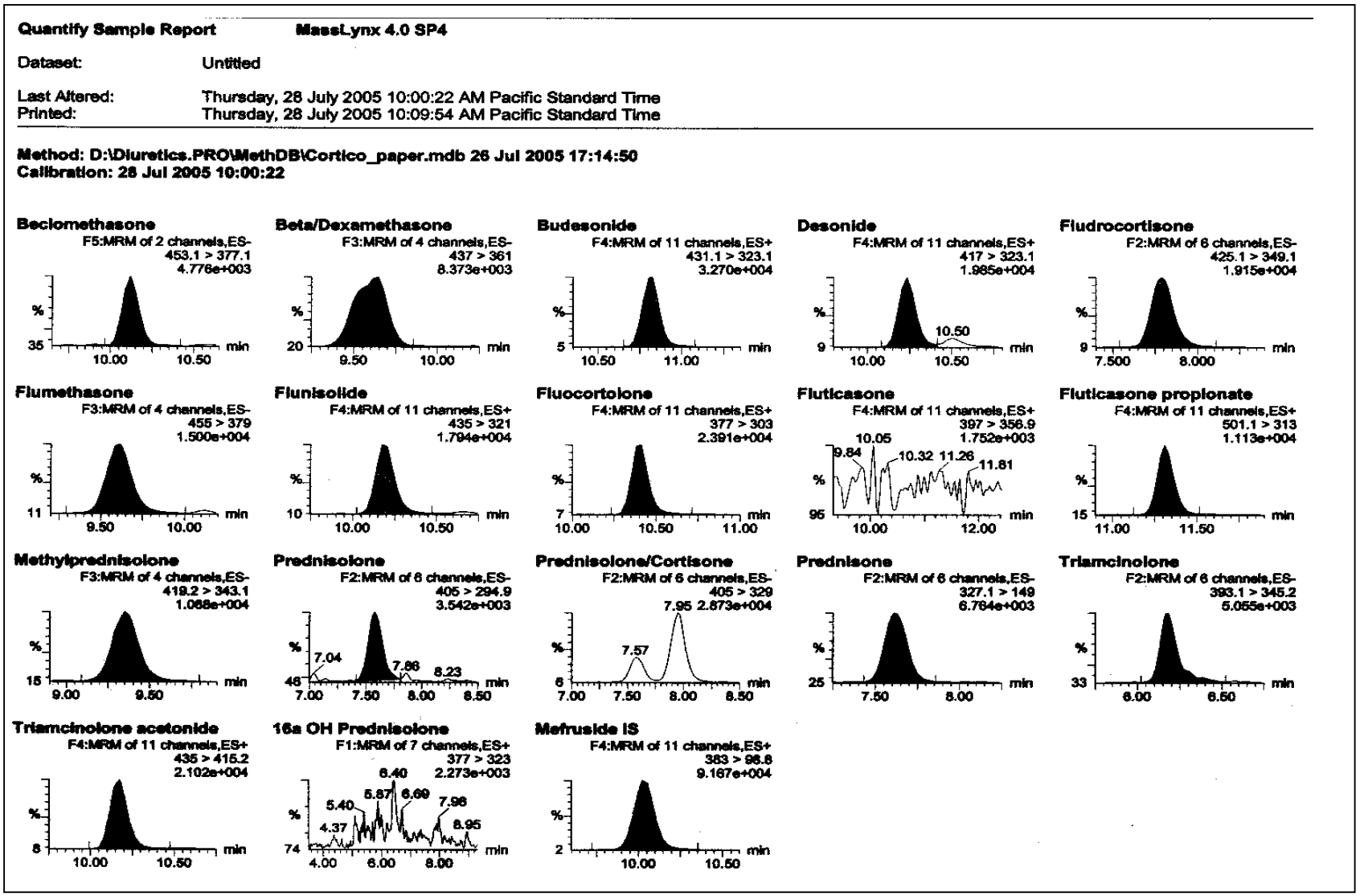

Figure 2. Detection and separation of corticosteroids standards spiked into urine at 30 ng/mL.

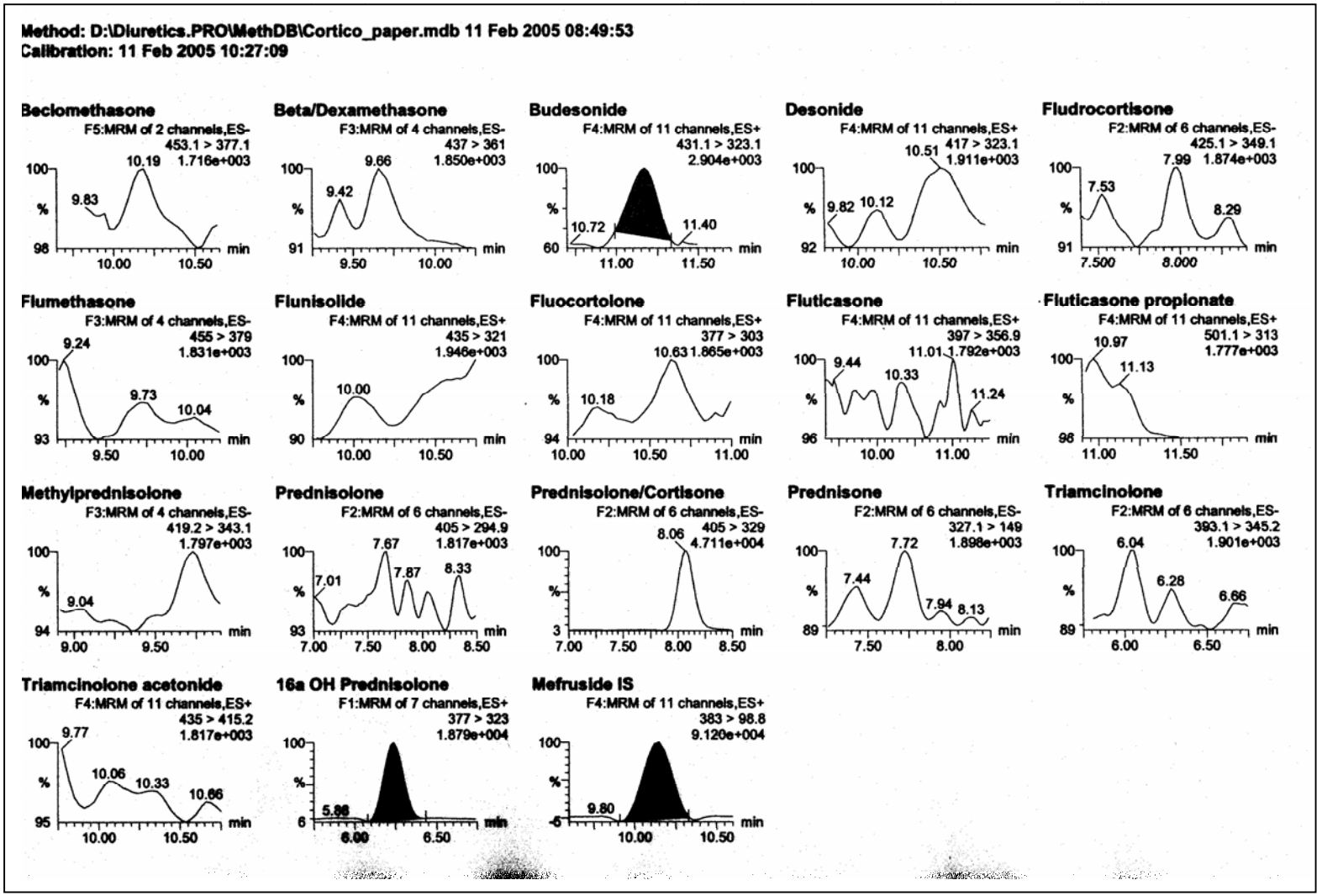

Figure 3. A urine sample positive for budesonide.