Selectively multiplexed LC-MS/MS analysis of steroids for the clinical laboratory

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INTRODUCTION

Liquid chromatography–tandem mass spectrometry (LC–MS/MS) is the gold-standard technique for steroid analysis in clinical samples, because of greater sensitivity and/or specificity compared with immunoassays (1).

LC-MS/MS ready-to-use commercial methods are present in the market that enable multiplexed determination of many steroids simultaneously in approximately 15 minutes per run. These “widely multiplexed” (WM) methods are very attractive, in principle. In practice, however, since LC-MS/MS is an expensive technology and since used in this way it can only process 20-30 samples/day, the WM strategy can result in very low sample throughput and associated high costs/analysis.

The WM methods can therefore be unsuitable in clinical laboratories where usually a limited number of LC-MS/MS analyzers are present. In most cases , however, simultaneous analysis of many steroids is unnecessary to address specific clinical hypotheses.

Taking into account this practical issues, we went to customize a WM commercial method (capable of 10 steroids) to a “selectively multiplexed” (SM) much faster method , specific for only two steroids of interest in our current laboratory practice: dehydroepiandrosterone (DHEA) and 17-hydroxy progesterone (17-OHP).

The SM method greatly helped us sustaining routine analysis of these two steroids with the very sensitive, accurate and precise LC-MS/MS technique.

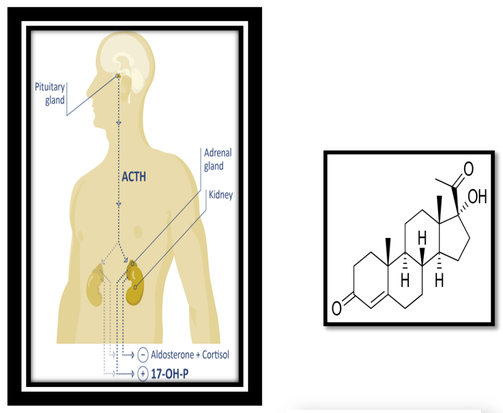


Fig. 1 The analyte structures and sketch of applications

METHODS AND MATERIALS

A WM commercial method was kindly provided by BSN s.r.l. This method involves a unique sample treatment procedure for extraction of all analytes. In brief:

-mixing 400 uL of serum with 600 uL of a deproteinizing solution containing deuterated internal standards;

-vortexing for 30 sec

- centrifuge for 5 min at 12000 rpm;

-evaporate to dryness of 700 uL of the supernatant

-reconstitution with 50 uL of methanol of the dried extracts

-add 50 uL of a derivatizing reagent, left reacting at room temperature for 30 minutes.

Derivatized extracts are then analyzed by an LC-MS/MS instrument (we used a Nexera 2 LC (Shimadzu, JAP) coupled to an API 5500 mass spectrometer (Sciex, CAN, see image):

Injection volume is 20 uL

chromatographic separation is performed by a binary gradient with dedicated mobile phases on a dedicated column (phase B gradient from 5 to 95% in 15 min)

-tandem mass spectrometry detection was in MRM mode with two specific transitions for each analyte.

The modified SM method involves:

- the same sample treatment

- A shorter LC chromatography (phase B gradient 50-95% in 4 min) tandem mass spectrometry detection in MRM mode with only 4 selected transitions (2 for each analyte)

Data analysis was performed with Analyst (Sciex, Toronto, CAN)and MedCalc®, for statistics.

RESULTS

Fig.2, ion extraction chromatograms for the WM method and the new SM method. To note, run time is much shorter with the SM method (4 vs 14 min). Very favorable Signal to noise is observed for both methods but separation of many analytes require much longer run time for the WM method.

In Tab. 1, intra-day and inter-day precision and accuracy for DHEA and 17-OHP with the new SM method are reported.

Sensitivity was also determined and is extremely high (LOQ = 0,05 ng/ml and 0,01 ng ml, for DHEA and 17-OHP, respectively).

Sample treatment, was not re-validated for the new SM method, since it is analog to the already validated commercial WM method.

Method comparison was performed by analyzing 100 clinical serum samples at various concentration levels of both steroids with the WM and the SM methods. In Fig. 3, statistical correlations performed with the Passing-Bablock algorithm built into the MedCalc software are reported.

Control sample (nominal value ng/ml)	Intra-day (N = 10)		Inter-day (N=10)	
	C.V. %	BIAS %	C.V. %	BIAS %
QC 1 (0.8)	9.5	102	9.8	105
QC 2 (5.1)	7.8	95	9.3	94
QC 3 (30.6)	7.5	98	8.6	99

Control sample (nominal value ng/ml)	Intra-day (N = 10)		Inter-day (N=10)	
	C.V. %	BIAS %	C.V. %	BIAS %
QC 1 (15)	6.5	95	8.4	92
QC 2 (50)	5.9	96	8.3	95
QC 3 (150)	5.5	97	8.1	99

Table 1. Intra-day and inter-day precision and accuracy
of the SM method for DHEA (upper box) and 17-OHP (lower box).

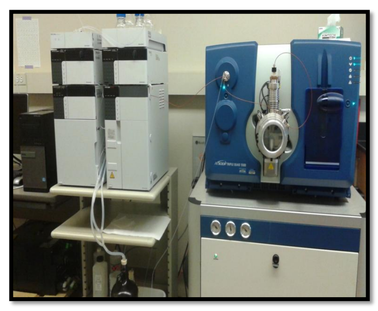
DISCUSSION

The new SM method showed very good sensitivity in the determination of the two steroids of interest in serum samples. Precision and accuracy were also fully appropriate for clinical investigations.

Statistical method comparison (Fig. 3), performed on the basis of 100 clinical serum samples at various concentration levels , both with the WM method and the new SM method, essentially indicated method interchangeability for the two analytes of interest.

The new SM method, however, was characterized by much faster run times and consequent much higher sample throughput. In fact, 1400 min (24 hours) were required to run 100 samples with the WM method, whereas only 400 min (less than 7 hours) were required to run the same samples with the SM method, instead.

Much faster run time determines significant advantages in the routine clinical laboratory. In fact, faster runs are much easier to manage under many practical points of view. High throughput analytics greatly helped sustain routine activity with as many as 200 samples week .



The instrument employed in this work

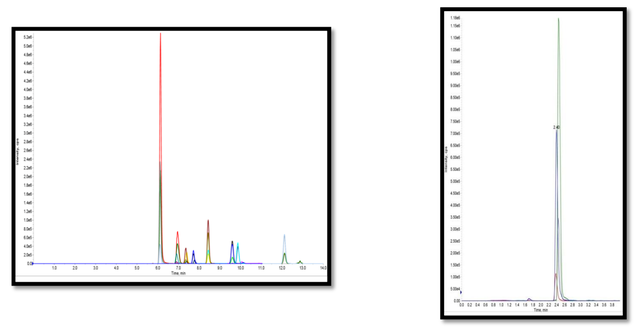


Fig.2 Ion extraction chromatograms
A calibrator sample containing 10 steroids run with the WM method (left) and the SM method (right).

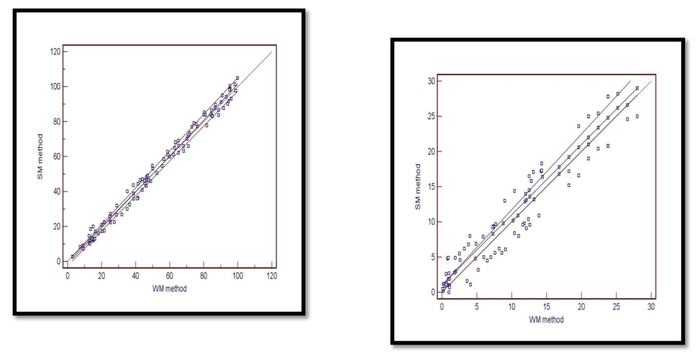


Fig.3 Passing-Bablok correlations (N=100)
Graphical rehpresentation of the Passing-Bablock method comparison performed by MedCalc with SM method data on the Y axis and WM method data on the X axis, for 17-OHP (left) and DHEA (right).

CONCLUSIONS

LC-MS/MS analysis of steroids in selectively multiplexed panels, customized to laboratory needs, could be a practical helpful strategy to support the introduction of LC-MS/MS steroid analysis in clinical laboratories.

REFERENCES

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