Development and Validation of the Simultaneous Measurement of Estrone and 17β-Estradiol in Serum by LC-MS/MS for Clinical Laboratory Applications

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Introduction

Estrogens are sexual steroidal hormones derived from cholesterol, which can exert strong biological effects even at low pg/mL levels. There are two main active estrogens in non-pregnant humans, which are estrone (E1) and 17β-estradiol (E2).

Nowadays, there is an increasing interest in the simultaneous high-sensitivity measurement of these estrogens for both clinical research and routine analysis. The main samples of interest include pediatric, pre-pubertal, post-menopausal and male serum samples. Unfortunately, inmunoassays are not able to reach the very low levels needed in some cases and usually show poor correlation with LC-MS/MS methods, particularly for E1[1,2]. Besides, in the case of E1 determination, the vast majority of routine methods are based on competitive radioimmunoassay (RIA) attained to health and safety risks posed by the use of radiation.

Despite of the known advantages of estrogen measurement by LC-MS/MS, which include increased sensitivity and specificity, these compounds do not contain highly ionizable functional groups. In order to increase the ionization efficiency for these compounds, different derivatization agents have been proposed, including picolinoyl, dansyl chloride or N-methyl-nicotinic acid N-hydroxysuccinimide ester, among others[3].

Therefore, the aim of this work is to establish a rugged and easy-to-handle method for E1 and E2 analysis in serum samples suitable for routine laboratories dealing with low concentration levels and sample volumes.

Experimental

Instrumental conditions

UHPLC: Nexera X2 UPLC (Shimadzu)

- **Column**: a Kinetex C18 100A (2.6 µm, 100 x 3 mm) core-shell column fitted with a C18 AJ0-8775 pre-column (Phenomenex, Torrance, CA, USA) .
- **Flow: 0.3 mL/min**
- **Injection volume: 30 µL**
- **Mobile phases:**
  - **A: H_2O 0.01% HCOOH**
  - **B: Acetonitrile 0.01% HCOOH**

Figure 1. LC-MS/MS system used for E1/E2 determination

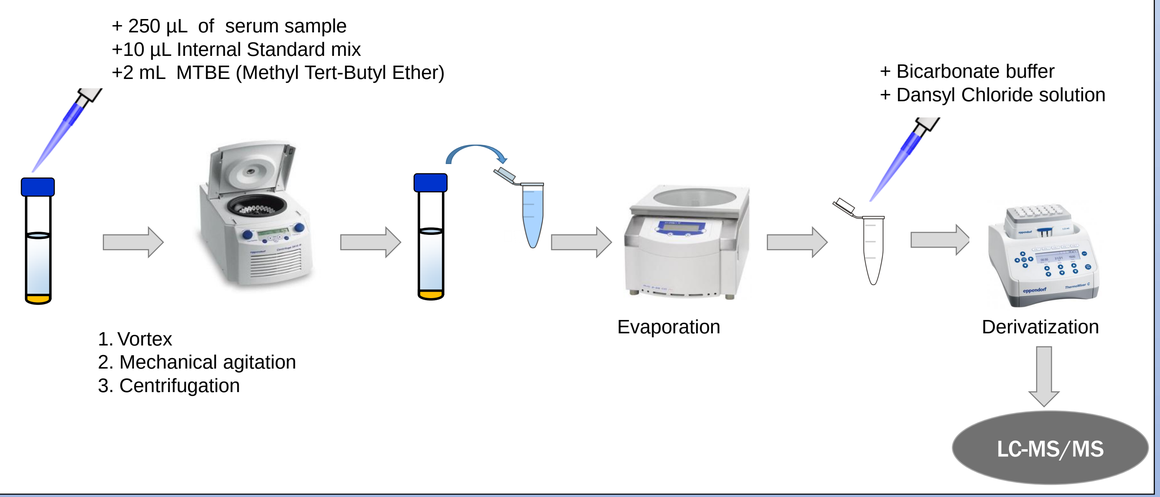
MS: Sciex QTRAP 6500

- Quadrupole-linear ion trap working triple quadrupole mode.
- Positive electrospray Ionization (ESI+).
- Selected Reaction Monitoring (SRM).

For comparsion purposes

- ECLIA: ” electrochemiluminescense immunoassay on modular E170 immunoassay analyzer (Roche Diagnostics, Mannheim, Germany
- RIA : Radioimmuno assay (DIAsource ImmunoAssays, Louvain-la-Neuve, Belgium).

Sample preparation



Conclusions

▪In this work, we have developed and validated an LC–MS/MS method for the simultaneous determination of E1 and E2 at low pg/mL levels in serum samples, according to EMEA validation guidelines.

▪Compared with LC-MS/MS, ECLIA showed a good correlation for E2. On the contrary, RIA showed a marked negative bias for E1 compared with the newly developed LC-MS/MS method. The source of this difference remains uncertain even thought it have been already observed in previous works [1].

▪Finally we have also observed that several non-menopausical patients presented a E1 level under the LOQ of the RIA method, 18 pg/mL. As a consequence, new reference values for E1 must be established by LC-MS/MS.

[[1] T. Fiers, B. Casetta, B. Bernaert, E. Vandersypt, M. Debock, J.M. Kaufman, . J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 893–894 (2012) 57–62.
[2] S. Pauwels, L. Antonio, I. Jans, A. Lintermans, P. Neven, F. Claessens, B. Decallonne, J. Billen, D. Vanderschueren, P. Vermeersch, S. Anal. Bioanal. Chem. 405 (2013) 8569–8577.
[3] W.C. Yang, F.E. Regnier, D. Sliva, J. Adamec. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 870 (2008) 233–240.

Results and Discussion

Method validation

•Validation standards and calibrators were prepared in commercial steroid-free serum materials, spiking with mix solutions of natural analytes prepared gravimetrically.

•Calibration curves were prepared in the range of 5 to 1000 pg/mL while validation standards (or quality controls) were prepared at 5, 10, 200, 400 and 800 pg/mL, following the European Medicines Agency (EMEA) Guideline on bioanalytical method validation.

• For E2 three certified reference materials (CRM) (BCR576, BCR577, BCR578) were also analysed.

Table 1. Validation results for E1 in intra- and inter-assay experiments.

E1	Intra-assay (n=5)		Inter-assay (n=9)	
Conc. (ng/L)	Recovery (%)	CV	Recovery (%)	CV
5	92	13.9%	99	6.3%
10	96	6.5%	102	5.4%
200	113	1.2%	112	1.4%
400	106	2.0%	107	4.6%
800	97	6.3%	96	3.8%

Table 2. Validation results for E2 in intra- and inter-assay experiments.

E2	Intra-assay (n=5)		Inter-assay (n=9)	
Conc. (ng/L)	Recovery (%)	CV	Recovery (%)	CV
5	103	8.3%	106	5.3%
10	102	4.4%	105	2.5%
200	90	4.6%	104	2.6%
400	92	3.1%	105	3.3%
800	99	2.2%	108	2.1%

Table 3. Validation results for E2determination experiments in three certified reference materials.

CRM E2		Intra-assay (n=5)		Inter-assay (n=9)	
Code	Conc. E2 (ng/L)	Recovery (%)	CV	Recovery (%)	CV
BCR576	31.05	97	5.2%	101	3.6%
BCR577	187.94	87	4.0%	93	1.6%
BCR578	364.99	94	3.8%	101	3.7%

Table 4. Matrix effect in different types of serum, obtained by comparison with standards in solvent at the same concentration levels.

Sample type	Matrix Effect (%)			
	E1 at 3xLLOQ[1]	E1 at near ULOQ[2]	E2 at 3xLLOQ	E2 at near ULOQ
Woman Serum	88	88	80	91
Men Serum	95	103	98	104
Hemolized	99	94	171	106
Renal	354	94	118	77
Icteric	170	99	97	99
Lipemic	121	81	97	92

[1]LLOQ: Lower Limit of Quantification
[2]ULOQ: Upper Limit of Quantification

Method comparison

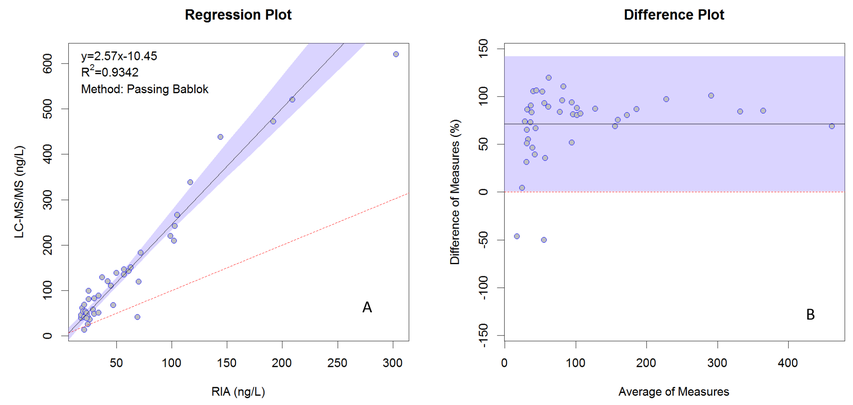


Figure 2. Comparison of E1 results obtained in serum analyzed by LC-MSMS versus RIA (n=41).
A)Passing Bablok regression, B) Difference plot. The horizontal black line represents the mean Difference (71.5%) and the shaded region represents 95% CI of the mean bias.

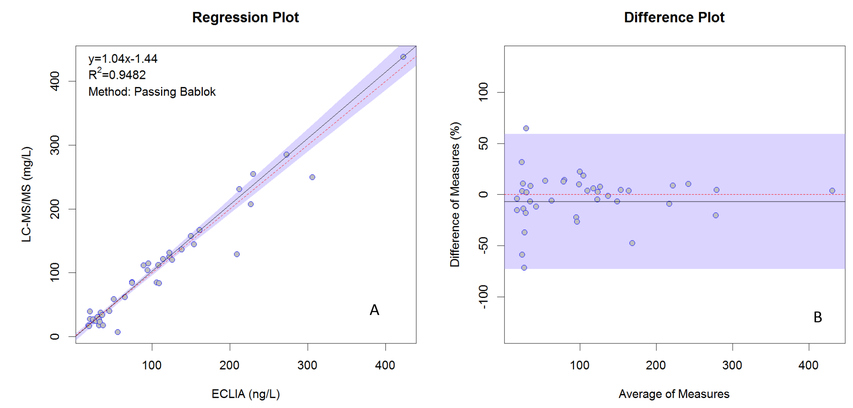


Figure 3. Comparison of estradiol results obtained in serum analyzed by LC-MSMS versus ECLIA (n=41).
A)Passing Bablok regression, B) Difference plot. The horizontal black line represents the mean Difference (-6.66% and the shaded region represents 95% CI of the mean bias.