



Pushing quantitation limits in micro UHPLC–MS/MS analysis of steroid hormones by sample dilution using high volume injection

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ABSTRACT

Ultratrace analysis of sample components requires excellent analytical performance in terms of limits of quantitation (LoQ). Micro UHPLC coupling with sensitive tandem mass spectrometry provides state of the art solutions for such analytical problems. Decreased column volume in micro LC limits the injectable sample volume. However, if analyte concentration is extremely low, it might be necessary to inject high sample volumes. This is particularly critical for strong sample solvents and weakly retained analytes, which are often the case when preparing biological samples (protein precipitation, sample extraction, etc.). In that case, high injection volumes may cause band broadening, peak distortion or even elution in dead volume. In this study, we evaluated possibilities of high volume injection onto microbore RP-LC columns, when sample solvent is diluted. The presented micro RP-LC–MS/MS method was optimized for the analysis of steroid hormones from human plasma after protein precipitation with organic solvents. A proper sample dilution procedure helps to increase the injection volume without compromising peak shapes. Finally, due to increased injection volume, the limit of quantitation can be decreased by a factor of 2–5, depending on the analytes and the experimental conditions.

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1. Introduction

In the past few years micro liquid chromatography (μ LC) has become a common technique in many applications, including pharmaceutical, proteomics, environmental, clinical and food analysis [1–5]. The main reason of using μ LC is to gain lower limits of quantitation by preventing sample dilution within the chromatographic column. Reducing column internal diameter leads to increase analyte concentration in the chromatographic band. Moreover, decreased flow rate may result in higher ionization yield and reduced ion suppression/enhancement effects [6–8]. On the other hand, such reduction of column i.d. decreases kinetic efficiency [9–11] and injection volume. Loading large sample volume onto μ LC columns may cause difficulties: wide, distorted bands or if sample solvent strength is too high even analyte breakthrough can

be observed, especially in the case of weakly retained compounds [12,13]. Distorted peaks imply complications in peak integration, decrease sensitivity and cause poor resolution [14].

Some recent studies presented practical insight into the relation of injection volume, elution program, column volume and sample solvent strength [15–17]. Injectable volume strongly depends on column dead volume, on the retention factor compared to mobile phase of the solute within sample solvent and mobile phase, and their mixing before reaching the chromatographic bed. Even particle type has an effect on injectable volume, core-shell particles possess superior efficiency over fully porous particles, higher kinetic efficiency provides narrower analyte bands, but due to reduced porous volume, they are slightly more sensitive to sample overload [18]. The effect of injection volume on kinetic efficiency of Kinetex UHPLC columns with different lengths (50, 100 and 150 mm) has been reported and shorter columns (decreased column volume) were found to be prone to lose their efficiency earlier than longer ones [19].

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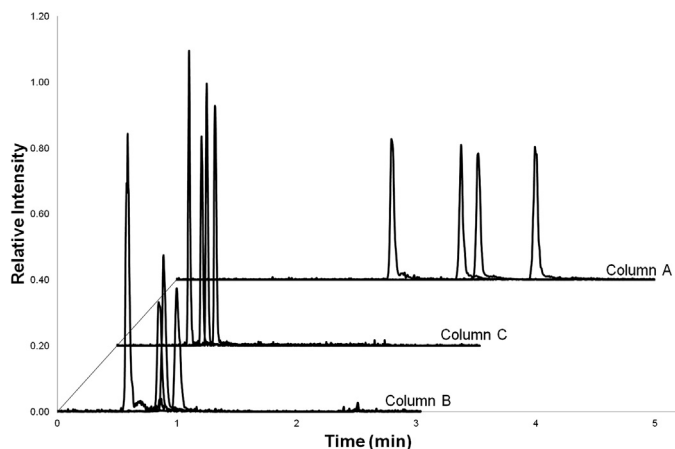


Fig. 1. Comparison of different chromatograms injecting 500 nl study solution from water. Every peak was divided with peak area. Column A: Eksigent, 3 μ m, ChromXP C18CL, 120 Å, 150 $\times$ 0.3 mm; Column B: Eksigent, 2.7 μ m, HALO Fused-Core C18, 90 Å, 50 $\times$ 0.3 mm; Column C: Eksigent, 2.7 μ m, HALO Fused-Core Phenyl Hexyl, 90 Å, 50 $\times$ 0.5 mm.

Injection volume can be increased with on-column focusing, where the sample solvent has lower elution strength than the mobile phase [20–23]. Other possible solution was described by Gritti et al. Sample injection followed by water plug focuses the

analytes at the front of the column, causing band compression [24]. Performance Optimizing Injection Sequence (POISe) technique gives special solution over conventional injection methods; the sample and a weak solvent mix while flowing through the injector and before entering the column. If the weak solvent follows the sample band through the injection system in the appropriate volume ratio, significant decrease in peak widths can be observed [25]. D'Orazio et al. packed capillary columns with two different stationary phases, the first short part of the column was intended for focusing the sample components, while the second zone was to use for their separation [26]. Properly set gradient elution [27] and pulse gradient [28] methods result also in band focusing, when injection of the sample occurs into a low elution strength mobile phase which enables to focus the band while entering the column.

Even with the previous applications, injecting sample in strong solvent of higher volume may cause peak distortion. If the sample solvent is strong enough and the injection volume is large enough, the mobile phase before and after the injection plug is not able to mix well and dilute sample solvent to prevent band broadening. Solute molecules in the sample plug possess lower retention and move more quickly through the column than others being present in diluted sample band where molecules slow down and travel with the normal migration rate [15]. Because of their stronger affinity to the stationary phase, molecules with higher retention factor are less sensitive for this effect. This means, that longer retained compounds allow larger injection volume [17,29,30].

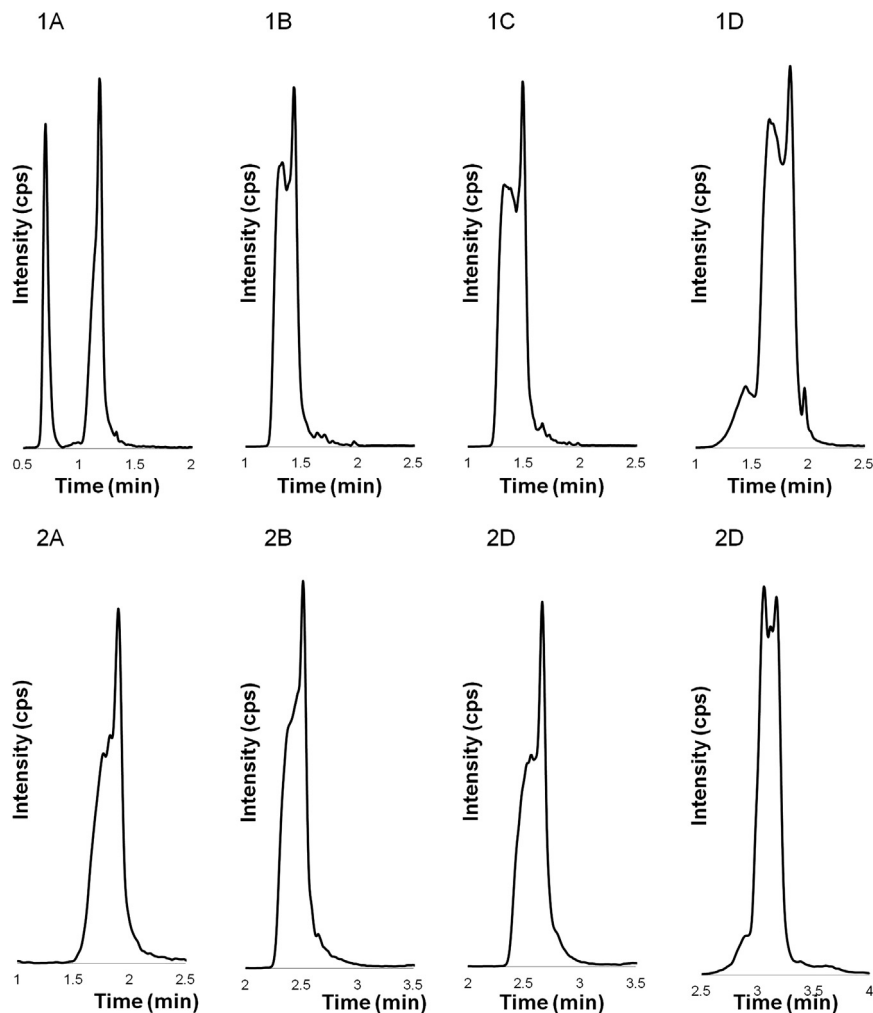


Fig. 2. Analyte's peak shapes injecting 2 μ l study solution from MeOH. On the top (1) gradient time was 0.6 min, on the bottom (2) it was 1.8 min. A: cortisol, B: testosterone, C: androstenedione, D: progesterone.

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