Research article

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Use of a green (bio) solvent – limonene – as extractant and immiscible diluent for large volume injection in the RPLC-tandem MS assay of statins and related metabolites in human plasma

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ABSTRACT: Limonene, considered a green solvent, was successfully used to extract simvastatin, lovastatin, and their hydroxy-acid metabolites from human plasma samples. The extraction process was followed by the direct injection of a large volume aliquot (100 µL) from the limonene layer into a Zorbax SB-C_{18} Rapid Resolution chromatographic column (50 mm length 4.6 mm i.d. × 1.8 µm d.p.), operated under gradient elution reversed-phase separation mechanism. Tandem mass spectrometry operated under the multiple reaction monitoring mode was used for detection, providing low quantitation limits in the 0.25–0.5 ng/mL concentration interval. This method was validated and used for quantitation of simvastatin and its hydroxy acid metabolite in incurred plasma samples obtained from two volunteers participating in a bioequivalence study, using lovastatin and its hydroxy analog as internal standards. The results were statistically compared with those produced by means of an alternative RPLC-tandem MS using protein precipitation with acetonitrile. The quality attributes of the two methods are comparatively discussed. The agreement between the quality characteristics of the two methods and the experimental results obtained on real samples may be considered as a consistent basis for the simultaneous use of limonene as extraction medium and injection diluent for hydrophobic compounds in bioanalytical approaches. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: limonene; green solvents; immiscible diluents; large volume injection; statins; bioanalysis

Introduction

Liquid–liquid extraction (LLE), as a classic approach (Rydberg *et al.*, 2004), or as micro-experimental versions (Andruch *et al.*, 2012), remains one of the most used procedures for simultaneous isolation and concentration of analytes from complex samples, including pharmaceuticals in biological matrices (Kole *et al.*, 2011). A wide variety of water-nonmiscible solvents are readily available for sample preparation in bioanalysis (Chang *et al.*, 2007). The LLE procedure is usually followed by a chromatographic separation. The compatibility between the two analytical stages is obtained through the back-extraction of analytes in an aqueous medium, or by evaporation of the organic phase followed by the re-dissolution of the dry extract in suitable solvents or solvent mixtures. All of these additional sample manipulation steps increase the overall complexity of the analytical process, inherently affecting the method's throughput, as well as the accuracy and precision of the results, and raise serious safety concerns about the impact on the health of the laboratory personnel and environmental pollution (De la Guardia and Armenta, 2011).

Injection of an aliquot from the organic layer directly into the chromatographic column would certainly simplify the experimental approach. Large volume injection of samples obtained in diluents stronger than and not miscible with the mobile phase is strictly forbidden by the commonly accepted practices in liquid chromatography (Dolan, 2011). However, recent publications (Medvedovici *et al.*, 2007; Udrescu *et al.*, 2008, 2010) indicate that such an approach is feasible if some conditions are fulfilled. Applications of the concept in bioanalysis are already available for determination of indapamide in whole blood (Udrescu *et al.*, 2011) and fenspiride in plasma (Medvedovici *et al.*, 2011). In these particular cases, 1-octanol was used as extraction solvent and also as sample diluent for injection into the chromatographic column.

Solvents considered as 'green' alternatives are the most desirable in practice, as long as their low toxicity reduces health hazards and the environmental impact. The use of 'green' solvents in analytical chemistry has only recently been considered. Among them, ionic liquids are frequently used as mobile phase additives in separation techniques (Marszall and Kaliszan, 2007) and in modern microextraction (Sun *et al.*, 2011). 'Green'

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Abbreviations used: DAD, diode array detection; L, lovastatin; LA, hydroxy acid; LLE, liquid–liquid extraction; LVI, large volume chromatographic injection; MF, matrix factors; PP, protein precipitation; RID, refractive index detection; S, simvastatin; SA, simvastatin hydroxy acid.

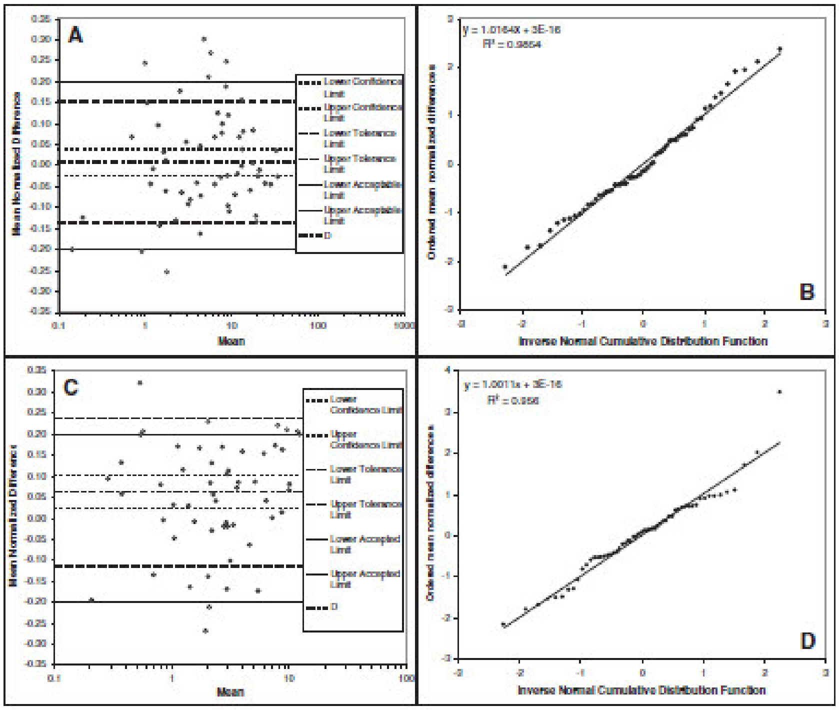

Figure 7. Bland–Altman approach applied to differences normalized to the mean of concentration data obtained from incurred samples through consecutive application of the PP and LLE analytical methods (A, C). Probability plots were used to evaluate the normal distribution of errors obtained between the two alternative methods (LLE vs PP) used to assay simvastatin (S) and simvastatic hydroxy acid (SA) in real plasma samples (B, D).

data pairs outside the ±20% limit (data not shown), meaning that the small systematic positive error does not count for the accuracy of the method with respect to SA; the coefficient of variation ($CV = s_D/\sqrt{2}$) is 10.8%, slightly higher than the value obtained for S. From the probability plot in Fig. 7(D), the errors between methods were normally distributed (the slope of the linear regression in Fig. 7D is 1.0011, and the correlation coefficient is 0.9778).

As a general conclusion, the results obtained through application of the LLE alternative (using limonene as extractant) were practically identical to the results produced through the 'classic' PP approach. This statistical comparison between results obtained on incurred samples reinforces the findings observed during the comparative evaluation of the quality attributes of the methods produced over the validation process.

Finally, trials using racemic limonene instead of D-limonene failed, although their declared purity grades were similar (>97%). More precisely, when using racemic limonene, an additional column cleaning procedure was necessary to conserve a constant retention during consecutive chromatographic runs, meaning that another impurity with increased apolar character accumulated within the stationary phase upon repetitive injections.

Conclusions

Extraction of apolar compounds from biological matrices using limonene as the extractant phase is feasible. High volume injection of aliquots from the organic layer directly to the chromatographic column is also achievable, as long as all the conditions needed for diluents nonmiscible with the mobile phase are simultaneously fulfilled. The two features taken together may represent a straightforward and enhanced-sensitivity approach for sample preparation in bioanalysis. The use of fast gradient elution profiles in RPLC may successfully produce high-throughput, convenient elimination of the solvent plug and of the residual matrix. The quality attributes of a method based on LLE in limonene and LVI of the organic layer are quasi-similar to those characterizing classical approaches in bioanalysis, such as PP by means of organic solvent addition followed by RPLC-MS/MS. The comparison by means of the Bland–Altman approach between the assay of the target compounds in incurred samples resulting from application of the two alternative methods (LLE and PP) leads to the conclusion that data are similar. For the moment, the single major drawback relating to the simultaneous use of limonene as extracting solvent from biological matrices and LVI diluent in RPLC refers to the commercial unavailability of a convenient purity-grade item.

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