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Large volume injection of 1-octanol as sample diluent in reversed phase liquid chromatography: Application in bioanalysis for assaying of indapamide in whole blood

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ABSTRACT

Large volume injection of samples in strong diluents immiscible with the mobile phases used in reversed phase liquid chromatography (RPLC) has been recently introduced in practice. In the present work, the potential of the technique has been evaluated for bioanalytical applications. The process consists of the liquid–liquid extraction of indapamide from whole blood into 1-octanol, followed by the direct injection from the organic layer into the LC. Detection was made through negative electrospray ionization (ESI) and tandem mass spectrometry (MS^2). The method was developed, validated, and successfully applied to a large number of samples in two bioequivalence studies designed for indapamide 1.5 mg sustained release and 2.5 mg immediate release pharmaceutical formulations. The performance of the analytical method is discussed based on data resulting from the validation procedure and the completion of the bioequivalence studies.

1. Introduction

The main purpose of bioanalysis is the determination of selected compounds in biological matrices [1,2]. Two challenging problems relate to the sample preparation step in bioanalytical processes: elimination of the biological matrix to sustain method's selectivity and enrichment of the target compounds to achieve low quantitation limits. Protein precipitation methods and extraction processes are more often used to support the above mentioned goals.

Liquid–liquid extraction readily isolates analytes in water immiscible solvents. The sample transfer to the chromatographic system is usually preceded by the removal of the extraction solvent (under gas stream, eventually thermally assisted) and re-dissolution of the dry residue in a solvent compatible with the mobile phase, to further support a large injected volume. Evaporation step may add random errors to the experimental results and seriously lengthens the duration of the analytical process. It would be highly preferable to inject large volumes of the organic phase directly to the chromatographic column. However, it is generally accepted that if the injection solvent is stronger than the mobile phase, the chromatographic peaks will be broadened and/or distorted [3–8]. Some practical solutions to accommodate stronger diluents to large volume injection – reversed phase liquid chromatography (RPLC) through application of pulsed elution gradients have been proposed [9]. Recent studies demonstrated that band broadening/peak distortion does not occur if the dilution solvent has an increased retention compared to target compounds [10]. This also applies for dilution solvents, which are not miscible with the mobile phase and exhibit enhanced affinity for the stationary phase compared to target analytes [11,12]. The reduction of the retention factors characterizing the target compounds should be considered, because the highly retained dilution solvent "saturates" a proportional amount of the stationary phase [11]. The use of water-immiscible solvents as diluents in RPLC has been recently highlighted for the assay of related impurities in active ingredients [13], antioxidants in pharmaceutical formulations [14] and ginkgolic acid in standardized extracts [15]. Additional phenomena related to similarity/dissimilarity of the viscosities characterizing injection solvent and the chromatographic eluent, namely the viscous fingering problem, should also be taken in consideration under these particular conditions [16]. Accordingly, if the injection solvent is less viscous than the eluent, chromatographic peaks tend to have fronting. By the opposite, a more viscous injection solvent should be fingered by the backward eluent, leading to peak tailing [17,18].

The basic phenomena relating to large volume injection of diluents non-miscible with the mobile phase, more precisely

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n-alkanes, were described earlier by our group [11], as a completion of studies carried out with strong miscible diluents (such as acetonitrile, methanol, *i*-propyl alcohol and tetrahydrofuran) in 100% aqueous mobile phases, discussed by Loeser and Drumm [10]. Some theoretical aspects are further discussed in the present work, in Section 3. These theoretical assumptions were previously verified for injection of relative polar compounds such as isosorbide-2 and -5 nitrates, pentoxifylline, tropicamide and methyl-*p*-hydroxybenzoate in *n*-hexane, *n*-heptane and *i*-octane [12]. We have also applied this approach for the assay of traces of butylated hydroxyanisole used as antioxidant in statines formulations [14]. Another interesting application referred to the assay of ginkgolic acids in standardized Ginkgo biloba extracts, which avoided the tedious sample preparation procedure described in the compendial specific monograph [15]. Recently, Loeser et al. [13] extended the application field through using ethyl acetate, *i*-propyl acetate and methyl-*i*-butylketone as diluents for the assay of polar related impurities in a relatively non-polar active pharmaceutical ingredient during the drug development phase. Although this approach has been used in these applications, its advantages in being used within the framework of large scale studies, where robustness is crucial, have not been yet verified. As liquid–liquid extraction in water immiscible solvents is largely used in sample preparation processes related to bioanalysis, we considered that the development, validation and application of this approach for an LC/(-)ESI/MS^2 method designed for a bioequivalence study would be specifically interesting. Our choice was oriented toward a difficult biological matrix, the whole blood. Such a matrix, if inadequately processed, may induce serious interferences in the ion source of the mass spectrometric detector, leading to reduced precision and accuracy. Indapamide, a moderate lipophilic molecule ($\log K_{ow} = 2.66$), was selected as a target compound, owing its particularity of binding on the surface of the red cells, inducing the objective need of its assay directly in blood (and not in plasma or serum). The isolation of the compound was achieved through liquid–liquid extraction, from whole blood to octanol. The extraction solvent was 1-octanol because it fulfills all appropriate conditions needed for large volume injection of samples in diluents non-miscible with the mobile phase (in relation to the target compound indapamide and internal standard in use), as it results from Refs. [10,11] and all argumentation presented later. A large volume (75 μl) from the organic layer was injected into the LC operated under reverse phase conditions. The method was successfully validated and used for two consecutive bioequivalence studies: a multidose bioequivalence study for a sustained release pharmaceutical formulation (coated tablets) containing 1.5 mg of indapamide and a single dose bioequivalence study for an immediate release pharmaceutical formulation (coated tablets) containing 2.5 mg of indapamide. The reliability of the method when used for such large scale applications and the intrinsic quality of the experimental results are discussed further in the present work.

2. Experimental

2.1. Reagents

All solvents (methanol, acetonitrile) were HPLC gradient grade from Merck (Darmstadt, Germany). Water for chromatography (resistivity minimum 18.2 MΩ and TOC maximum 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument and used during experiments. Reference standards were obtained from European Pharmacopoeia (Council of Europe, Strasbourg, France), batch no. 4 for indapamide (4-chloro-*N*-[(2-methyl-2,3-dihydro-1*H*-indol-1-yl]-3-sulphamoylbenzamide) and batch no. 1c for 5-chloro-2-methoxy-*N*-[2-(4-sulphamoyl phenyl)ethyl]benzamide, used as internal standard (IS). 1-Octanol and formic acid were extra pure grade from Merck. Sodium chloride from Merck was pro-analysis grade.

2.2. Equipment

Experiments were performed on a system built up from Agilent series 1200 modules (Agilent Technology, Waldbronn, Germany) as following: degasser (G1322 A); binary pump SL (G1312 B); thermostated autosampler (G1367 C); column thermostat (G1330 B). Detection was made through a MS/MS triple quadrupole detector (G2571 A) using an atmospheric pressure electrospray ion source (ESI), operated under negative mode. System control, data acquisition and interpretation were made with the Agilent Mass Hunter software version B 01.00 (B48). Alternative diode array detection (DAD) and refractive index detection (RID) modes used to asses effects arising on large volume injection of the non-miscible diluent were achieved by means of the Agilent 1200 SL series (G1315C) and Agilent 1100 G1362 modules, respectively.

2.3. Chromatographic method

The chromatographic separation was carried out on a Zorbax SB C18 Rapid Resolution, 50 mm length × 4.6 mm internal diameter × 1.8 μm particle size column, thermostated at 40 °C. Such a short column was preferred to increase the method's throughput (considering the increased number of samples being analyzed). Elution conditions were optimized in order to conserve adequate chromatographic resolution and to control matrix effects arising within the ion source of the mass spectrometer. A Phenomenex C18 guard cartridge (2 mm length, 4 mm internal diameter) was used to protect the column inlet. The column was operated under gradient conditions, at a flow rate of 0.8 ml/min. The components of the mobile phase were aqueous 0.1% formic acid and a mixture acetonitrile/methanol in 1/1 volumetric ratio. The following gradient profile (including the re-equilibration step) was applied:

Time (min)	Organic modifier (%)	Flow rate (ml/min)
0	5	0.8
2	45	0.8
5.5	45	0.8
5.51	100	0.8
6.0	100	0.8
6.50	100	1.2
6.51	5	1.2
7.5	5	1.2

The flow rate increase at the end of the gradient is however needed for a faster elimination of 1-octanol from the column. A higher flow rate over the whole gradient profile would produce enhanced throughput through shortening the run (without a sensible loss in term of efficiency, as in such conditions, the van Deemter plot for a 1.8 μm particle size stationary phase is almost flat). However, flow rate limitation up to 0.8 ml/min was required by the proper functioning of the ESI source.

The injection volume was 75 μl. An accurate and reproducible injection process was obtained only through reducing the dispensing speed of the autosampler from the normal speed of 1000 down to 100 μl/min, to compensate for the high viscosity of 1-octanol. Additional column re-equilibration (at 0.8 ml/min and 5% organic modifier) is obtained during the injection process, which takes about 2 min.

Investigation of effects produced through injection of large volumes of octanol in the column was carried out under isocratic elution conditions, consecutively using acetonitrile, methanol or a mixture acetonitrile/methanol (1:1 (v/v)) as organic modifier in