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Determination of verapamil and norverapamil in human plasma by liquid chromatography: comparison between a liquid–liquid extraction procedure and an automated liquid–solid extraction method for sample preparation*

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Abstract: A conventional liquid–liquid extraction (LLE) procedure with high-performance liquid chromatography (HPLC) has been developed for the determination of verapamil and its main metabolite, norverapamil, in plasma. After addition of the internal standard, plasma samples were basified with phosphate buffer (pH 9.0) and extracted with a mixture of cyclohexane–dichloromethane. After centrifugation, the organic layer was separated and the analytes were extracted back into a 0.1 N sulphuric acid solution containing 2-aminoheptane. An aliquot of this aqueous phase was then injected directly onto the HPLC column. This LLE procedure has been compared with an automated liquid–solid extraction (LSE) method that has been developed in parallel. Good linearity was obtained using both extraction methods. The absolute recoveries for the two analytes were *ca* 95% with the automated LSE procedure and slightly lower (*ca* 84%) for the LLE method. The automated method gives better results with respect to detectability and precision, but the LLE procedure is simpler to develop, requires much less expensive equipment, and remains a useful alternative when the number of samples to be analysed is limited.

Keywords: *Verapamil; norverapamil; HPLC; liquid–liquid extraction; automated liquid–solid extraction; disposable extraction cartridges.*

Introduction

Several high-performance liquid chromatographic (HPLC) methods for the bioanalysis of the basic drug verapamil and its N-demethylated metabolite, norverapamil, have been published [1–8]. All these methods involve liquid–liquid extraction (LLE). The latter is followed in most cases by a back-extraction to an acidic aqueous solution [1–3, 6, 7].

Such sample preparation techniques are often tedious and time-consuming. The automation of these techniques is clearly of interest when the number of samples to be analysed is relatively large (>100) [9]. The most popular automated techniques for sample preparation are based on liquid–solid extraction (LSE) [9, 10]. The different LSE steps can be performed automatically on disposable extraction cartridges (DECs) by means of a sample processor, such as the ASPEC system [12–14]. In order to facilitate the determination of the hundreds of plasma samples generated by pharmacokinetic studies on verapamil and norverapamil, such an automatic LSE method coupled to HPLC has been recently developed [15].

In the present paper, another method for the HPLC determination of verapamil and norverapamil in plasma, involving a conventional LLE procedure, is described. The principal aim of this paper is to compare this LLE method with an automatic LSE method described recently [15]. The two methods have been validated with respect to recovery, linearity, detectability and precision. The results obtained by using these two sample preparation techniques in combination with HPLC are presented and the advantages and limitations of both methods are discussed.

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Experimental

Apparatus

The HPLC equipment used in the method involving liquid–liquid extraction consisted of a Waters model 590 twin piston pump (Waters Associates, Milford, MA, USA), a Waters sample processor (WISP) model 710 B, a programmable column oven model T6300 from Merck–Hitachi (Darmstadt, Germany) and a Perkin–Elmer LS 4 fluorescence detector (Perkin–Elmer, Watford, UK). Data were collected on a computerized integrator/plotter model 3359A from Hewlett–Packard (San Diego, CA, USA).

The chromatographic equipment for the automated liquid–solid extraction method was an integrated ASPEC (Automatic Sample Preparation with Extraction Cartridges) system [12–16] from Gilson (Villiers-le-Bel, France), to which a model 305 pump (Gilson) and a model F-1050 fluorescence detector from Merck–Hitachi were linked. The ASPEC system consists of three main components: a set of racks for solvents, samples and disposable extraction cartridges (DECs), a dilutor/pipettor and an auto-sampler with an XYZ-motion arm equipped with a needle dispensing the different liquids. A model 02PT923 water-bath from Heto (Birkeröd, Denmark) was used for column temperature control. An IBM compatible computer loaded with GME-714 and GME-718 software packages (Gilson) and a model BD9 recorder (Kipp and Zonen, Delft, The Netherlands) were used for data collection.

A Manu-CART system, which consisted of a LiChroCART analytical column (125 × 4 mm i.d. in the LLE method or 250 × 4 mm i.d. in the automated LSE method) and a short LiChroCART guard column (4 × 4 mm i.d.) from Merck was thermostatted at 30.0 ± 0.1°C (LLE method) or at 35.0 ± 0.1°C (LSE method).

The fluorimeters were used at an excitation wavelength of 275 nm and an emission wavelength of 310 nm [17].

Chemicals and reagents

Verapamil, norverapamil and the internal standard, gallopamil, were kindly supplied by the SMB-Galephar Department of Research and Development (Brussels, Belgium), and used without further purification.

Dichloromethane was a 'Baker analysed' HPLC reagent (Baker, Phillipsburg, NJ, USA). Acetonitrile and methanol were of HPLC grade (Baker; Janssen, Geel, Belgium; Riedel-de Haen, Seelze, Germany). Cyclohexane was a RPE-ACS solvent from Carlo-Erba (Milan, Italy).

Potassium monohydrogen phosphate, potassium dihydrogen phosphate, sodium acetate, sodium hydroxide, glacial acetic acid, phosphoric acid (min. 85%) and sulphuric acid (min. 95–97%) were of p.a. quality from E. Merck (Darmstadt, Germany). 2-Aminoheptane was purchased from Aldrich (Gillingham, UK) and was doubly distilled before use [15].

The deionized water used in all experiments was of Milli-Q quality from Millipore (Bedford, MA, USA).

Bond-Elut DECs (1-ml capacity) packed with 50 mg of cyanopropyl endcapped phase (CN^{EC}) with a particle size of 40 μm were used as supplied by Analytichem (Harbor City, CA, USA) [15].

The LiChroCART analytical column was packed with a Superspher 100 RP-18 bonded phase (particle size: 4 μm), whereas the LiChroCART guard column was filled with LiChrospher 100 RP-18 (particle size: 5 μm) from Merck.

Chromatographic method

The mobile phase comprised acetate buffer (pH 3.0)–acetonitrile–2–aminoheptane (72:28:0.5, v/v/v) [18] and the flow rate was 1.0 ml min^{-1}, as described in the USP.

The pH 3.0 acetate buffer was obtained by mixing 1 l of 0.01 N sodium acetate solution with about 33 ml of glacial acetic acid, and the pH was adjusted with sodium hydroxide as necessary.

Standard solutions

In the LLE procedure, verapamil and norverapamil were dissolved in methanol to give a stock solution of approximately 1 mg ml^{-1} of each analyte. The methanolic solution was first diluted to 10 μg ml^{-1} using Milli-Q water. The latter solution was then diluted either to 1 μg ml^{-1} or to 100 ng ml^{-1} for spiking plasma samples (concentration range 10–250 ng ml^{-1}). The stock solution of internal standard (gallopamil) was prepared similarly to obtain a final concentration of 2.5 μg ml^{-1}.

The preparation of calibration and internal standard solutions for spiking plasma samples in the automated LSE method has been described elsewhere [15].

Liquid–liquid extraction procedure

A 1.0-ml volume of plasma thawed at room temperature was pipetted into a centrifuge tube. The internal standard solution (200 μl) and water (500 μl) were then added and the content of the tube was vortex-mixed for 10 s after each step. After addition of 0.5 ml of 1 M phosphate buffer (pH 9.0) and 6.0 ml of a mixture of cyclohexane–dichloromethane (85:15, v/v), the sample was vortexed for 15 s, shaken mechanically for 10 min and centrifuged at 2500 rpm for 10 min. A 5.0-ml aliquot of the organic phase was transferred to a conical centrifuge tube containing 1.0 ml of a mixture of 0.1 N sulphuric acid and 2-aminoheptane (0.5%) and vortexed again for 1 min. The centrifuge tube was then stoppered and centrifuged for 3 min at 2500 rpm. Afterwards, the aqueous phase was transferred into a WISP limited volume insert and 80 μl was injected into the HPLC system.

The pH 9.0 phosphate buffer used for the extraction of the analytes from plasma samples was prepared in a 100 ml volumetric flask by dissolving 22.8 g of potassium monohydrogen phosphate in 90 ml of water. The pH of the solution was then adjusted to 9.0 by phosphoric acid and the flask was filled to the mark with water.

Automated liquid–solid extraction procedure

After centrifugation of the thawed plasma sample at 6000 rpm for 10 min, a 1.5 ml volume was transferred into a vial placed on the appropriate rack of the ASPEC system. This sample processor was used to perform all LSE operations on disposable extraction cartridges (DECs) and to inject into the HPLC system.

The internal standard solution (0.03 ml of gallopamil at 2.5 μg ml^{-1}) was first added to the sample. The CN^{EC} (50 mg) cartridge was then conditioned with methanol (1.0 ml) followed by pH 7.4 phosphate buffer (1.0 ml). After application of the plasma sample (1.0 ml), the DEC was washed with the same phosphate buffer (1.0 ml). The analytes were eluted with methanol containing 0.2%, v/v, 2-aminoheptane (0.24 ml), and pH 3.0 acetate buffer (0.41 ml) was added to the methanolic eluate. The resultant solution (0.65 ml) was then mixed and an aliquot (250 μl) was injected onto the analytical column.

The minimum dispensing flow rate available (0.18 ml min^{-1}) was automatically selected for the sample loading step [13] and sample preparation was performed in the concurrent mode [13–15].

The pH 7.4 phosphate buffer used in the conditioning and washing steps was prepared in a 1-l volumetric flask by mixing 250 ml 0.1 M potassium dihydrogen phosphate with 195.5 ml 0.1 M sodium hydroxide, water being added to volume.

Results and Discussion

Liquid–liquid extraction procedure

The liquid–liquid extraction of the basic compounds verapamil (pK_a 8.6), norverapamil and gallopamil from plasma is usually performed after increasing the pH of the sample by addition of an appropriate buffer. A phosphate buffer of pH 9.0 was selected for this purpose in the present method as it was found that no further improvement in analyte recoveries was obtained by use of buffers of higher pH. The addition of a small percentage of dichloromethane to cyclohexane had a favourable effect on analyte recovery and did not significantly alter the selectivity of the extraction. Back-extraction into a limited volume of acidic aqueous phase is preferred to an evaporation step, in order to avoid possible analyte losses due to adsorption. 2-Aminoheptane was added to this aqueous phase to minimize differences in composition with the HPLC mobile phase, which also contains this competing amine. Such differences might create disturbances in the HPLC system on injection of large volumes (80 μl) and give rise to peak deformations [19].

Validation and comparison of the two methods

The absolute recoveries of the analytes and of the internal standard were determined by comparing the peak areas obtained by direct injection of aqueous standard solutions to those obtained after plasma treatment using the LLE or the LSE procedures [20]. The results are given in Table 1.

For the LLE method, the analyte recoveries at different concentrations were lower than 90%, but still satisfactory (*ca* 84%). In the automated LSE method all recoveries were

Table 1
Absolute recoveries (%) (n = 3) of verapamil, norverapamil and the internal standard, gallopamil for the liquid–liquid extraction and the liquid–solid extraction methods. NV, norverapamil; V, verapamil; G, gallopamil

Conc. (ng ml^{-1})	LLE						LSE					
	NV	RSD	V	RSD	G	RSD	NV	RSD	V	RSD	G	RSD
100	85	3.5	90	3.6	85	4.0	99	2.1	97	2.0	95	2.6
50	84	3.7	82	4.8	81	4.2	96	2.4	98	3.2	—	—
25	83	6.9	85	7.2	79	5.1	91	5.1	93	4.5	—	—
Mean	84	4.7	86	5.2	82	4.4	95	3.2	96	3.2	95	2.6

Table 2
Validation data for the LLE and LSE methods. NV, norverapamil; V, verapamil

	LLE		LSE	
	NV	V	NV	V
r^2	0.99945	0.99875	0.99999	0.99996
LOD (ng ml^{-1})	2.7	4.1	1.7	1.0
LOQ (ng ml^{-1})	9.1	13.8	3.3	5.8
Reproducibility (100 ng ml^{-1} RSD %)				
Within-day (n = 6)	3.6	2.9	1.9	1.4
Between-day (n = 5)	3.5	3.7	3.0	1.9

greater than 90% (*ca* 95%). Such high recoveries are typical for this kind of LSE method, where each step can be optimized in a more systematic way [21].

Table 2 shows the determination coefficients (r^2) of the regression lines for verapamil and norverapamil in both methods. In the LLE method, linear regression analysis of the peak height ratio (y) vs the concentration (x) in ng ml^{-1}, gave the following equations (conc. range 10–100 ng ml^{-1}; $n = 6$):

$$\text{norverapamil: } y = 0.0066x + 0.0142;$$
$$\text{verapamil: } y = 0.0067x + 0.0134.$$

The regression equations obtained with the LSE procedure [15] were (conc. range 1–500 ng ml^{-1}; $n = 8$):

$$\text{norverapamil: } y = 0.0154x + 0.0135;$$
$$\text{verapamil: } y = 0.0149x + 0.0453.$$

Thus the calibration curves for the two compounds of interest are linear for both extraction methods.

The limits of detection (LOD) and of quantitation (LOQ) [20] for the two analytes were calculated from regression lines [22]. They are presented in Table 2. Higher detectability was obtained with the liquid–solid extraction method. In this method, the apparent improvement in the detection limit is most probably related to the more systematic optimization of the individual LSE steps [15]. In particular, a higher proportion (38.5%) of the final extract from LSE can be introduced into the HPLC system without damaging the analytical column, while in the LLE method, this proportion must be limited to 8%.

Table 2 also gives the within-day and between-day reproducibilities for the two extraction methods. Better precision has been observed in the LSE method, which is to be expected from the use of a fully automated, highly reliable sample handling procedure.

At the retention time of verapamil and norverapamil, the absence of interfering endogenous components is demonstrated in Figs 1 and 2, which show chromatograms obtained on injection of blank and spiked plasma samples, respectively, using the two extraction methods. It is clear that the role of the sample preparation procedure is less critical in this case, due to the highly selective detection mode (fluorimetry) employed.

Conclusions

The method involving liquid–liquid extraction described in this paper is, as expected, somewhat less rapid, less sensitive and less precise than the fully automated

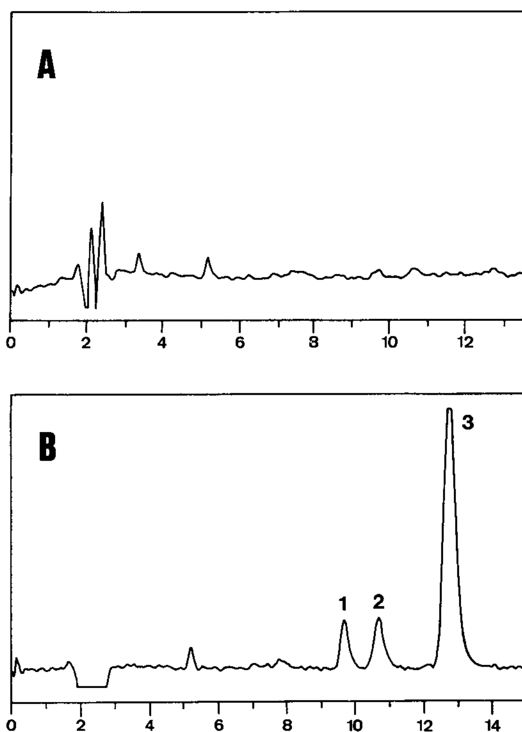

Figure 1
Typical chromatograms obtained by coupling LLE sample preparation to HPLC. (A) Chromatogram of blank plasma; (B) chromatogram of plasma spiked with norverapamil and verapamil (25 ng ml^{-1}). Chromatographic conditions as described in the text with fluorescence detection (excitation wavelength: 275 nm; emission wavelength: 310 nm). Peaks: 1, norverapamil 2 ng (25 ng ml^{-1}); 2, verapamil 2 ng (25 ng ml^{-1}); 3, gallopamil (internal standard).

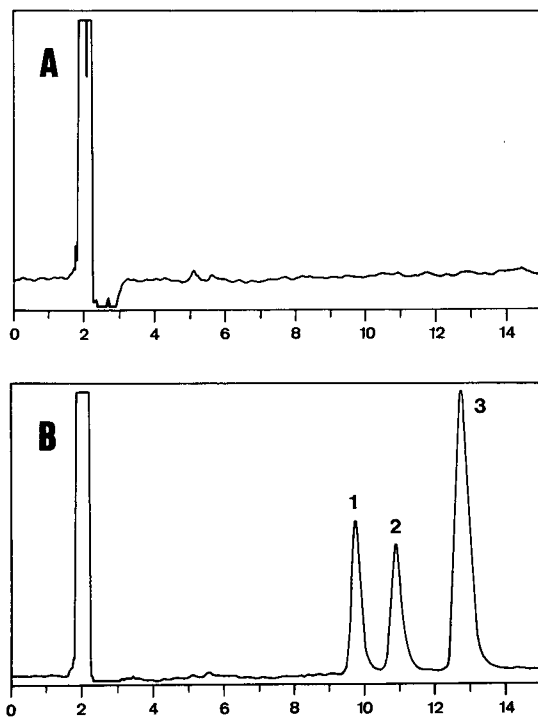

Figure 2
Typical chromatograms obtained by using the automated LSE procedure in combination with HPLC. (A) Chromatogram of blank plasma; (B) chromatogram of plasma spiked with norverapamil and verapamil (25 ng ml^{-1}). Chromatographic conditions as described in the text with fluorescence detection (excitation: 275 nm; emission wavelength: 310 nm). Peaks: 1, norverapamil 9.4 ng (25 ng ml^{-1}); 2, verapamil 9.4 ng (25 ng ml^{-1}); 3, gallopamil (internal standard).

method based on liquid–solid extraction on disposable cartridges [15]. Clearly, the latter method is perfectly suited for the determination of verapamil and norverapamil in large numbers of plasma samples. However, the results obtained with the LLE procedure are acceptable. This method thus appears to be a useful alternative to use when the number of samples to be analysed is small; indeed, it is more straightforward to develop and to handle. It is also much less expensive than the automated method, which requires sophisticated instrumentation.

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