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Determination of diltiazem and its main metabolites in human plasma by automated solid-phase extraction and high-performance liquid chromatography: a new method overcoming instability of the compounds and interference problems

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Abstract

An automated sample preparation method, based on solid-phase extraction (SPE) was developed on an ASPEC-Gilson device and combined with HPLC for the determination of diltiazem and three of its metabolites in human plasma (N-desacetylmonodesmethyldiltiazem, N-monodesmethyldiltiazem, O-desacetyldiltiazem). A 1-ml volume of plasma is diluted with 0.5 ml of 0.1 *M* ammonium dihydrogen phosphate and the sample is automatically loaded onto a SPE silica (C_{18}) column (100 mg); the column is flushed with two different solvents, then eluted with 0.5 ml of a 0.1 *M* ammonium dihydrogen phosphate–acetonitrile mixture (20:80, v/v) containing 0.06% of triethylamine. The eluate is evaporated to dryness and the residue reconstituted with a suitable solvent and injected onto a C_8 silica column connected to a UV detector (λ = 238 nm). This method overcomes problems caused by the partial instability of diltiazem and metabolites in human plasma during analysis. There is no chromatographic interference from endogenous compounds.The limits of quantitation (LOQ) are 2.5 and 2 ng ml^{-1} for diltiazem and the metabolites in human plasma, respectively. Linearity between concentrations and detector response for diltiazem and metabolites ranged from 10–200 and 5–100 ng ml^{-1} in human plasma, respectively. The method has been validated.

1. Introduction

1.1. Pharmacokinetics and metabolism of diltiazem

Diltiazem, *d-cis*-3-acetyloxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)one) hydrochloride, is a calcium channel blocker widely employed in the treatment of angina, hypertension and supraventricular tachyarrythmias [1–5]. The drug has a mean biological half-life of 3–6 h in plasma [6]. The absolute bioavailability of the tablets ranges between 30 and 42% largely due to presystemic hepatic metabolism [7,8]. After absorption, diltiazem undergoes extensive hepatic

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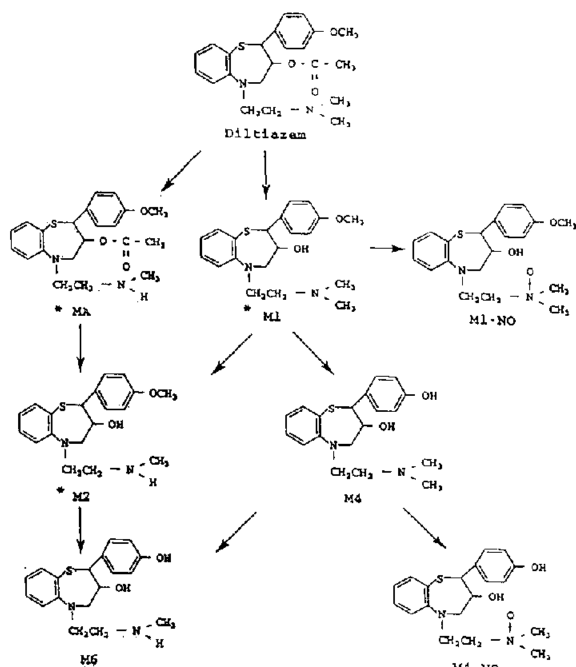

Fig. 1. Chemical structure and proposed metabolic pathways of diltiazem and metabolites in humans.

metabolism through three major metabolic pathways, N-demethylation, O-deacetylation, and O-demethylation [6–9] (Fig. 1) and less than 5% of the administered dose is found as unchanged drug in urine [8]; N-oxidation is a minor metabolic pathway [10] (Fig. 1). Among the known metabolites only N-monodesmethyldiltiazem (MA) and desacetyldiltiazem (M1) have pharmacological activity, with respectively 50 and 20% of the coronary vasodilating potency of diltiazem.

N-monodesmethyldiltiazem is reported to be the major metabolite in humans [11] and accumulates in plasma during chronic treatment [8–12]. It is the main metabolite in young and elderly hypertensives [12]. Desacetyldiltiazem, previously considered the predominant metabolite in plasma after oral dosing [13,14], is more potent with regard to coronary vasodilation and reduction of blood pressure, but N-monodesmethyldiltiazem remains active over a longer period [12,15]. However, the pharmacological activity of these metabolites has not been demonstrated in man [16,17], and metabolites resulting from further biotransformation (Fig. 1) are considered relatively inactive [18].

It is therefore not clear why such effort has been made to develop chromatographic methods to determine diltiazem and six metabolites in human plasma [10,19], when so little, incomplete information is available on only three of them: the N-monodesmethyl, desacetyl and N-desacetyl-monodesmethyl derivatives. The last derivative is important not so much as a metabolite, but as a degradation product of the N-monodesmethyl [20,21] derivative: a high level is a sign of inappropriate treatment of blood samples, and inadequate storage or transport of plasma samples.

Previous methods for the determination of diltiazem in biological fluids were based on GC with electron-capture detection or nitrogen-sensitive detection [22,23]. Recent methods employ liquid–liquid extraction (LLE) [10,21,24] or solid-phase extraction (SPE), HPLC and UV detection [25,26]. The problem of separating diltiazem and its three main metabolites was solved some years ago, but the problem remains to obtain quantitative extraction (LLE) of the drug and metabolites with satisfactory recovery ($\geq 70\%$), and to produce a final extract without chromatographic interferences that may affect quantitation of some of the metabolites. More recent extraction methods are mostly based on manual sample preparation, LLE or SPE, which are time-consuming and subject to human error. Most of these methods are able to measure the three main metabolites. Automated sample preparation is an important goal but not easily performed starting from a LLE method.

SPE, on the other hand, using column-switching HPLC or disposable silica columns, can easily be fully or partially automated [27,28]. Our laboratory has already developed an automated method for diltiazem and its main metabolites which does not require any manual sample manipulation since it is based on SPE and column switching [29,30]. The method has been used for some years for clinical pharmacokinetic studies and for drug monitoring in plasma of patients in clinical trials (Phase 2–4). However,

if this method is applied to very old plasma samples (more than one year at −20°C), badly stored (> −20°C), or badly prepared [20] (from whole blood), it suffers from interferences just as is the case for LLE [10,21,24].

To obtain an automated and robust sample preparation procedure, overcoming the interferences caused by inadequate sample manipulation or storage, we investigated a method based on SPE on a disposable C_{18} column, performed automatically by means of an Aspec device under HPLC conditions previously reported [30]; the sample preparation is, in part, similar to that described by Boulieu *et al.* for a manual SPE procedure [25].

2. Experimental

2.1. Chemicals, reagents and standards

Methanol and acetonitrile were HPLC grade (E. Merck, Darmstadt, Germany), potassium dihydrogen phosphate anhydrous and phosphoric acid were analytical grade (E. Merck), ammonium dihydrogen phosphate was HPLC grade (J.T. Baker, Deventer, Netherlands), triethylamine (TEA) was synthetic grade (E. Merck), pure water used for the reagent preparation and for chromatographic eluent was HPLC grade, produced by the Milli-Q4 system (Millipore, Bedford, MA, USA). The 0.1 *M* ammonium dihydrogen phosphate solution was prepared from 11.5 g of salt by diluting to one liter with water. The 0.05 *M* potassium dihydrogen phosphate solution (pH 2.9) was prepared from 136.1 g of the salt and diluting to one liter to obtain a 1 *M* solution, then 50 ml of this solution were further diluted to give a 0.05 *M* solution, and adjusted to pH 2.9 with phosphoric acid. The eluent mixture was prepared by mixing 600 ml of the 0.05 *M* phosphate buffer (pH 2.9) and 2 ml of TEA, adjusting to one liter with acetonitrile, and filtering the solution through a 0.22-μm membrane filter under vacuum. The HPLC injection solvent was prepared by mixing 80 ml of 0.05 *M* phosphate buffer (pH 2.9) and 20 ml of acetonitrile. The SPE washing solvents were aqueous solutions containing 20% or 40% of acetonitrile (v/v); the SPE eluting solvent was prepared by mixing 80 ml of acetonitrile, 20 ml of 0.1 *M* ammonium dihydrogen phosphate and 60 μl of TEA. It is important to follow the sequential steps and to renew the solution every day.

Diltiazem (DTZ), N-desmethyldiltiazem (MA), desacetyldiltiazem (M1), N-desmethyldesacetyldiltiazem (M2) and propyldiltiazem (internal standard) (Fig. 1) were of pharmaceutical grade, provided by Synthélabo Recherche (L.E.R.S.), Bagneux, France.

2.2. Standard solutions

Standard solutions were used for daily calibration; stock solutions (1 mg/ml) of diltiazem, MA, M1, M2 and internal standard were prepared in methanol. Standard solutions were prepared from stock solutions by dilution with methanol (Table 1) and used for the preparation of the biological standards.

Table 1
Standard solutions used for the analysis of diltiazem and metabolites in human plasma

Standard solution	DTZ (ng/20 μl)	MA (ng/20 μl)	M1 (ng/20 μl)	M2 (ng/20 μl)	I.S. (ng/20 μl)
1	200.0	100.0	100.0	100.0	–
2	100.0	50.0	50.0	50.0	–
3	50.0	25.0	25.0	25.0	–
4	25.0	12.5	12.5	12.5	–
5	10.0	5.0	5.0	5.0	–
6	–	–	–	–	150

2.3. Chromatographic system

The chromatographic system consisted of a double piston pump Model 420 (for 0.01–2 ml min^{-1} flow-rate range) (Kontron, Milan, Italy), a UV detector Model PU 4020 set at 238 nm (Pye Unicam, Cambridge, UK), an automatic sample injector Model 460 (Kontron), an analytical column, 150 × 4.6 mm I.D., filled with 5-μm Hypersil C_8 BDS material (Shandon, Runcorn, UK), a guard-column, 20 × 4.6 mm I.D., filled with 40-μm Pelliguard LC_8 (Supelco, Bellefonte, PA, USA) and an integrator Model Chromjet SP 4400 (Spectra Physics, San Jose, CA, USA).

The pump flow-rate was 1.0 ml min^{-1}, the volume, automatically injected, was 100 μl, the integrator chart speed was 0.5 cm min^{-1} and attenuation was set at 32. Under these conditions, the retention times were *ca.* 3 min for M2, *ca.* 3.5 min for MA, *ca.* 4 min for M1, *ca.* 6 min for DTZ and *ca.* 8 min for the internal standard.

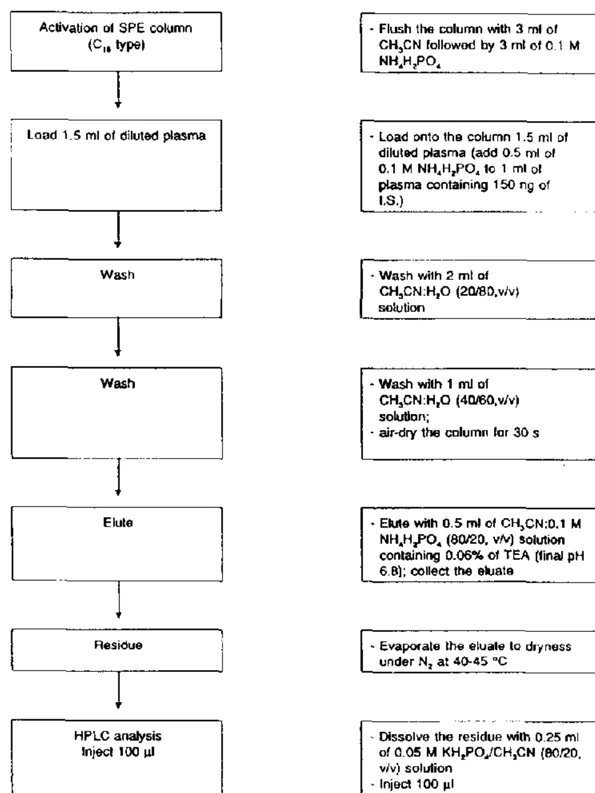

Fig. 2. Sample preparation by SPE.

2.4. SPE and ASPEC system

Disposable SPE columns, C_{18} type, were used, containing 100 mg of silica packing (Lida, Kenosha, WI, USA); the activation of the columns and all the sample preparation and manipulations were done automatically by an ASPEC apparatus (Gilson Biolabo, Middelton, WI, USA) with software comprising several prestored programs for sample preparation.

2.5. Sample preparation

Aliquots (20 μl) of standard solutions were added to 1 ml of pre-dose plasma samples; 20 μl of internal standard were added to all samples (standards and unknown) which were then diluted with 0.5 ml of 0.1 *M* ammonium dihydrogen phosphate solution, and vortex-mixed. The diluted samples (1.5 ml) were loaded onto activated SPE C_{18} type columns and automatically processed by the ASPEC apparatus according to the scheme shown in Fig. 2. The eluates were collected from the SPE column and evaporated to dryness and the residues were reconstituted in 0.25 ml of a mixture of acetonitrile–phosphate buffer (20:80, v/v). The samples were vortex-mixed and 100 μl of the solutions were automatically injected onto the chromatographic column.

3. Results

3.1. Stability

Diltiazem and metabolites were stable for at least 24 h in the diluted plasma (with 0.1 *M* ammonium phosphate), which was longer than in the freshly prepared, immediately extracted and

chromatographed plasma samples. The test, performed in triplicate for one low and one high concentration of each compound, ensures that there was no degradation during the holding step before the fluid is loaded onto the SPE column. In the elution mixture from the SPE column, diltiazem and metabolites were stable for at least 24 h in contrast to freshly prepared, extracted and chromatographed plasma samples. Thus the eluates can be left on the ASPEC rack for up to 24 h before evaporation. Diltiazem and metabolites were stable for at least 24 h in the HPLC injection solvent (pre-injection condition on the automated injection sample rack). Diltiazem and metabolites spiked with human plasma were stable after three freezing-thawing cycles in contrast to freshly prepared, immediately extracted and chromatographed plasma standards.

3.2. *Absolute recovery*

The absolute recovery was evaluated in predose plasma samples spiked with known amounts of the compounds. Two different concentrations (12.5 and 50 ng ml^{-1} for metabolites and 25 and 100 ng ml^{-1} for diltiazem) were added to predose plasma; the sample was extracted according to the method described, and the internal standard (propyldiltiazem) was added to the final injection solvent just before injection. Recovery of the internal standard was evaluated by adding 150 ng ml^{-1} propyldiltiazem to pre-dose plasma using diltiazem as internal standard. The absolute recovery (%) was calculated from the ratio of the amount of the compound extracted from plasma to the amount added to 0.25 ml of HPLC injection solution. Recoveries at 12.5 and 50 ng ml^{-1} were as follows:

–respectively 82.4 and 81.9% for N-desmethyldesacetyldiltiazem;

–respectively 86.9 and 85.1% for N-desmethyldiltiazem;

–respectively 67.2 and 65% for desacetyldiltiazem;

–respectively 73.8 and 68.0% at 25 and 100 ng ml^{-1} for diltiazem;

–78.5% at 150 ng ml^{-1} for propyldiltiazem.

3.3. *Selectivity*

Several pre-dose plasma samples from different subjects were tested for the absence of interfering compounds. No chromatographic interferences were found at the retention time of the compounds under study (Fig. 3a).

3.4. *Linearity*

A linear correlation was found between the peak-height ratio of diltiazem or metabolites and the internal standard and the concentration of diltiazem or metabolites in the range 10–200 ng ml^{-1} and 5–100 ng ml^{-1} in human plasma, respectively. Five concentrations were tested in quadruplicate for each compound. Calibration equations and the relative coefficient of correlation (r) show a good linear correlation between diltiazem and metabolites added to plasma and quantities found. The calibration equations were: $y = 1.0006x - 0.074$, $r = 0.9996$; $y = 1.0048x - 0.256$, $r = 0.998$; $y = 1.008x - 0.349$, $r = 0.9989$; $y = 1.0041x - 0.235$, $r = 0.9973$, respectively for diltiazem (DTZ), N-desmethyldiltiazem (MA), desacetyldiltiazem (M1), N-desmethyldesacetyldiltiazem (M2).

3.5. *Limit of quantitation*

The limits of quantitation (LOQ) for diltiazem and metabolites were respectively 2.5 and 2 ng ml^{-1} for diltiazem and metabolites in human plasma (Fig. 3b).

3.6. *Precision and accuracy*

The method was validated by two analysts working with the same instruments on different days. After the daily calibration (in quadruplicate) each analyst analysed two quality control specimens (low and medium concentrations, in quintuplicate) over a two-day period. The intra- and inter-day precision and accuracy were satisfactory (see Table 2) for diltiazem and metabolites at low and medium concentrations. A typical chromatogram is shown in Fig. 3c.

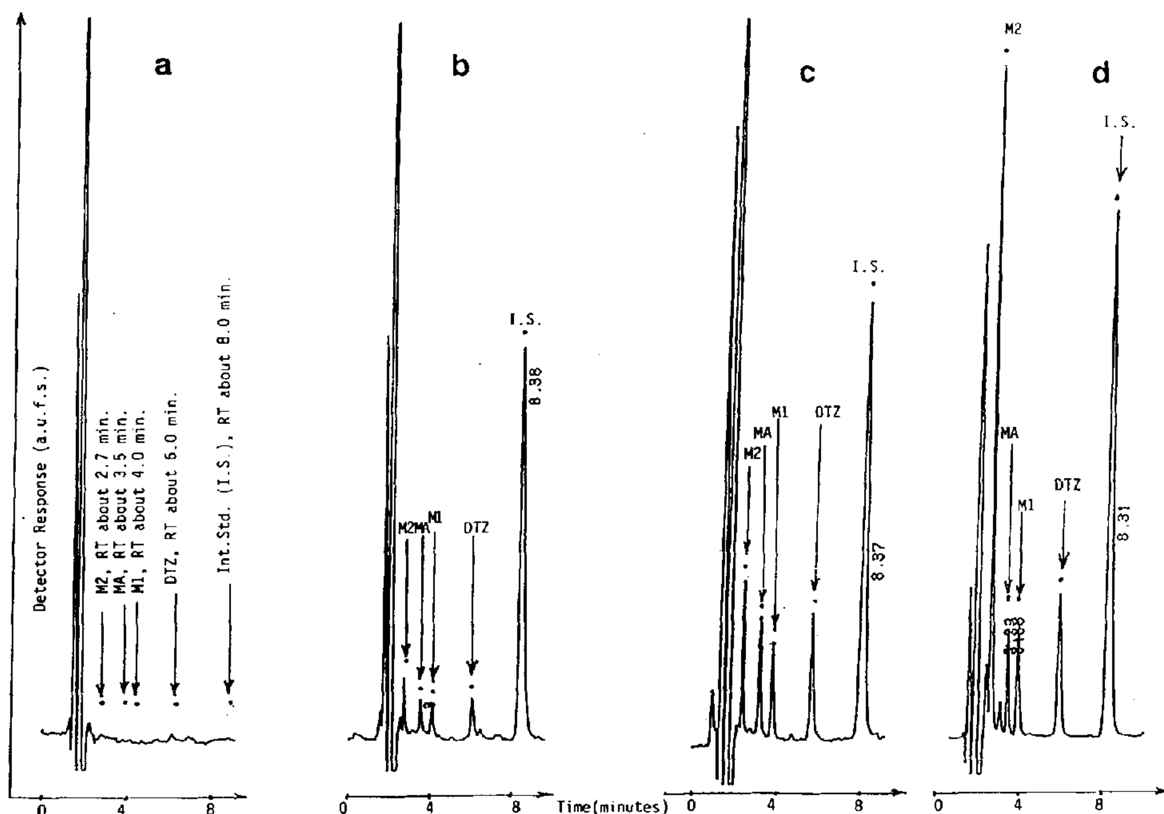

Fig. 3. (a) Chromatogram of pre-dose human plasma sample; N-desmethyldesacetyldiltiazem = M2, N-desmethyldiltiazem = MA, O-desacetyldiltiazem = M1, diltiazem = DTZ, propyldiltiazem (internal standard) = I.S. (b) Chromatogram of authentic standards recovered from pre-dose human plasma sample spiked with 10 and 5 ng ml^{-1} of DTZ and metabolites respectively. (c) Chromatogram of authentic standards recovered from pre-dose human plasma spiked with 30 and 15 ng ml^{-1} of DTZ and metabolites respectively. (d) Chromatogram of a plasma sample from a patient given 120 mg slow-release diltiazem orally; 4 h post-dose sample.

Table 2
Precision and accuracy results obtained from the determination of diltiazem and metabolites in human plasma ($n = 20$ in all the cases and for all the compounds)

	DTZ (ng/ml)		M2 (ng/ml)		MA (ng/ml)		M1 (ng/ml)	
	29.1	143.2	14.9	73.3	15.0	73.8	14.8	72.8
Precision C.V. intra (%)[a]	4.1	1.7	8.1	4.2	6.9	4.8	5.5	2.0
C.V. inter (%)[b]	0.4	1.5	0.0	0.3	1.7	3.5	2.0	1.4
C.V. total (%)	4.2	2.5	8.1	4.2	7.1	5.9	5.9	2.5
LS[c]	5.7	3.7	11.4	6.0	10.0	9.5	8.3	3.8
Accuracy[d]	102.0 ± 1.9	99.1 ± 1.6	99.3 ± 2.6	98.2 ± 1.9	101.8 ± 3.7	98.4 ± 3.8	96.2 ± 3.1	94.6 ± 1.6

[a] C.V. intra = within-day coefficient of variation.
[b] C.V. inter = between-day coefficient of variation.
[c] 95% upper confidence limit for the relative standard deviation.
[d] Recovery (expressed as per cent of the nominal concentration) with 95% as confidence limit.

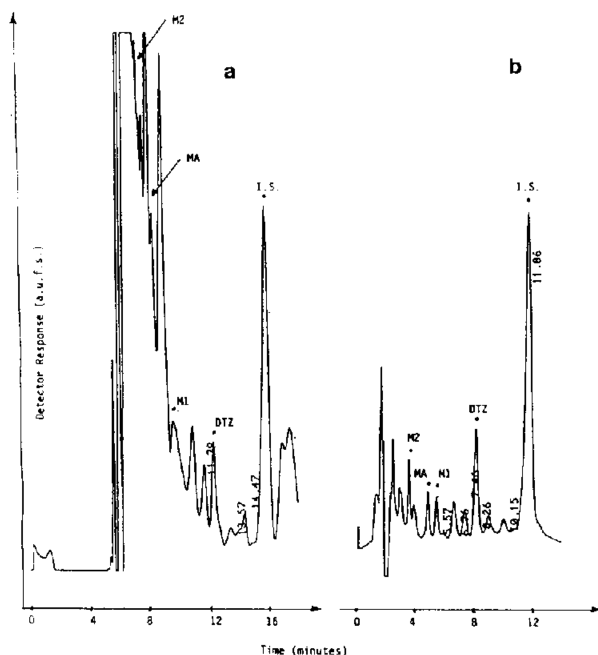

Fig. 4. (a) Chromatogram of a plasma sample from a patient given 200 mg slow-release diltiazem orally; 12 h post-dose sample, 28-month old sample (probably not stored under optimal conditions as it showed a flocculate). Method used: column-switching HPLC. (b) Same sample as above, processed according to the proposed method. In both cases the column was a Supelcosil LC_8 DB.

3.7. *Application of the method to plasma samples (in vivo studies)*

The method was used to measure diltiazem and metabolites in plasma from patients given daily 200 mg slow-release diltiazem orally, over a long period. Even with plasma samples of *ca.* two years old, there was no chromatographic interference at the retention times of diltiazem and metabolites (Fig. 4b), whereas with the column-switching HPLC method [30] it was impossible to quantitate all the metabolites (Fig. 4a). The retention times of the compounds of interest were longer than expected because the column was packed with Supelcosil C_8 DB instead of Hypersil C_8 BDS.

4. Discussion

In our laboratory we usually essay diltiazem and its main metabolites in human plasma by an automated column-switching HPLC method [29,30], which, however, has some limitations. When old or badly stored plasma samples are analysed, some interfering compounds may affect the quantitation of the more polar metabolites. In addition, on account of the partial instability of diltiazem and its metabolites in human plasma at room temperature [20,21], the samples cannot be kept on the autoinjection rack for more than 5–6 h before analysis; this obviously reduces the sample throughput. The proposed automated SPE method overcomes these limitations. Chromatographic profiles of plasma extracts from healthy volunteers or patients treated orally with diltiazem at the usual therapeutic doses, never suffered from interferences, even when the samples were *ca.* two years old.

Dilution of 1 ml of plasma sample with 0.5 ml of 0.1 *M* ammonium dihydrogen phosphate before SPE greatly improves the stability of the compounds in human plasma. Concerning this step, in their original work Boulieu *et al.* [26] diluted 1 ml of plasma with a 0.1 *M* ammonium dihydrogen phosphate–acetonitrile mixture (63:37, v/v) before SPE. When we tried this on the ASPEC system, a gel formed before sample loading (onto the SPE column), preventing aspiration of the sample. In our experience, dilution of plasma with 0.5 ml of ammonium dihydrogen phosphate (without acetonitrile) is sufficient to break the protein binding of diltiazem and metabolites, without causing precipitation.

The method showed very good selectivity for the compounds of interest, good absolute recovery, limits of determination satisfying clinical pharmacokinetic needs and satisfactory precision and accuracy. In only a few plasma samples of patients taking the drug as monotherapy an interfering compound eluted close to the peak of M2 and affected its quantitation. However, quantitation of this metabolite is not important [20,21]. Investigation of the stability of diltiazem and metabolites for 24 h in the solvents used gave good results, enabling the automated preparation of a large number of samples (up to a batch of 72 samples) in a 18-h period, utilizing part of the day and the night. In addition, the

automated sample processing by the ASPEC device and HPLC injection reduces errors, since manual sample processing accounts for *ca.* 30% of the errors generated during an analytical method, and operator errors are responsible for *ca.* 20% [31].

Even though the proposed method seems very robust and specific for diltiazem and some metabolites, it is extremely important to remember that blood handling necessary to obtain plasma, storage and transport of plasma samples before analysis must all be done correctly [20] in order to prevent chemical degradation of the analytes and avoid false results.

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