ELSEVIER

Journal of Chromatography A, 668 (1994) 403–411

JOURNAL OF CHROMATOGRAPHY A

Determination of alpidem, an imidazopyridine anxiolytic, and its metabolites by column-switching high-performance liquid chromatography with fluorescence detection

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Abstract

Alpidem, 6-chloro-2-(4-chlorophenyl)-N,N-dipropylimidazo[1,2-*a*]pyridine-3-acetamide, is an anxiolytic imidazopyridine that undergoes a first-pass elimination after oral administration to humans; it is actively metabolized and three circulating metabolites have been identified in plasma due to N-dealkylation, oxidation or a combination of both processes. For the determination of the unchanged drug and its metabolites in human plasma, a column-switching HPLC method was developed. The method, based on solid-phase extraction (performed on-line), involves the automatic injection of plasma samples (200 μl) on to a precolumn filled with C_{18} material, clean-up of the sample with water in order to remove protein and salts and transfer of the analytes to the analytical column (after valve switching) by means of the mobile phase. All the processes were performed in the presence of an internal standard, a compound chemically related to alpidem. During the analytical chromatography, the precolumn was flushed with different solvents and after regeneration with water, it was ready for further injections. The analytical column was a C_8 type and the mobile phase was acetonitrile–methanol–phosphate buffer solution (45:15:45, v/v/v) at a flow-rate of 1.5 ml min^{-1}. The column was connected to a fluorimetric detector operating at excitation and emission wavelengths of 255 and 423 nm, respectively. The limits of quantitation of alpidem and three metabolites were 2.5 and 1.5 ng ml^{-1}, respectively, in human plasma.

1. Introduction

In the last few years, interest in solid-phase extraction (SPE) has grown considerably as an alternative to the traditional liquid–liquid extraction, which has some disadvantages such as low selectivity, emulsion formation, extensive solvent use and waste disposal and non-automation [1]. In particularly, automatic on-line SPE with HPLC, known as column-switching HPLC, offers a simple solution to the complex problem of sample purification and preconcentration of drugs and metabolites in biological fluids [2–5]. The automation allows the sample throughput to be increased [6,7] and human errors to be avoided.

Alpidem, 6-chloro-2-(4-chlorophenyl)-N,N-dipropylimidazo[1,2-*a*]pyridine-3-acetamide, is a non-benzodiazepinic anxiolytic that has an interesting anxiolytic profile at both animal and clinical levels [8,9]. The drug, after oral administration to humans, is actively metabolized, and three circulating metabolites have been identified

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0021-9673/94/$07.00
SSDI 0021-9673(93)E1056-6

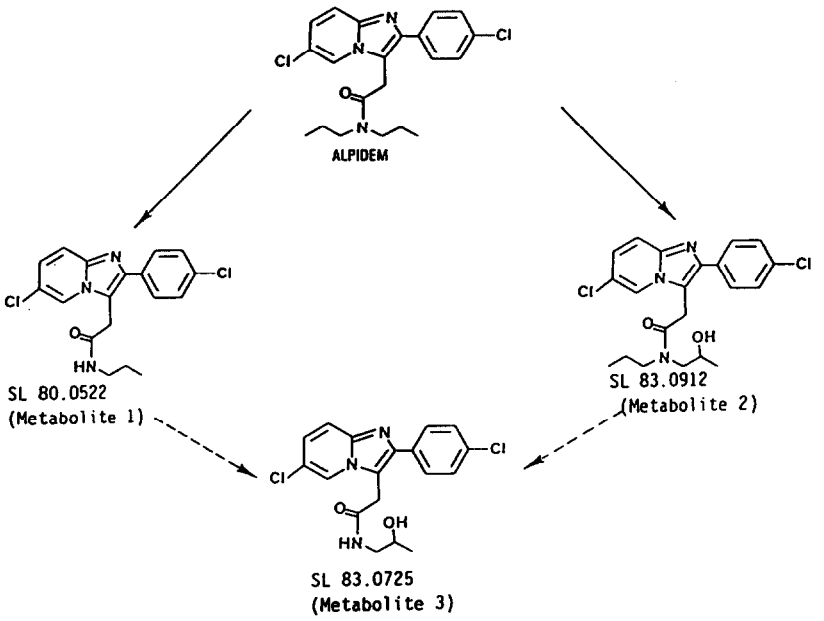

Fig. 1. Metabolic pathway of alpidem in humans and structures of metabolites.

(Fig. 1). They originate from N-dealkylation, with loss of a propyl group to give metabolite 1, oxidation of a propyl group to give metabolite 2 and oxidation of a propyl group and loss of the other propyl group to give metabolite 3. For the determination of alpidem and its metabolites in human plasma a method has been published [10] that is based on liquid–liquid extraction and HPLC with fluorescence detection; here we propose a method based on column-switching HPLC that does not require any manipulation of the biological sample before injection.

2. Experimental

2.1. Chemicals, reagents and standards

Methanol and acetonitrile were of HPLC grade (E. Merck, Darmstadt, Germany) and potassium dihydrogenphosphate was of analytical-reagent grade (E. Merck). The water used for the preparation of the buffer solutions and chromatographic eluent was of HPLC grade, produced from tap water by a two-step process: prepurification through a Milli-RO 60 Plus and then through a Milli-Q4 system (Millipore, Bedford, MA, USA).

The 0.025 *M* phosphate solution used for the preparation of the eluent mixture was prepared by dissolving 137 g of anhydrous potassium dihydrogenphosphate in water and diluting to 1 l, then further diluting 25 ml of this solution to 1 l. The mobile phase was prepared by mixing 450 ml of the 0.025 *M* phosphate solution, 400 ml of acetonitrile and 150 ml of methanol, then filtering through a 0.22-μm filter membrane by means of a solvent clarification kit (Millipore). Alpidem, SL 80.0522 (metabolite 1), SL 83.0912 (metabolite 2), SL 83.0725 (metabolite 3) (Fig. 1) and SL 80.0633, 6-chloro-2-(3,4-dimethoxyphenyl)-N,N-dipropylimidazo[1,2-*a*]pyridine-3-acetamide (Internal Standard), were of pharmaceutical grade and were provided by Synthélabo Recherche (Bagneux, France).

2.2. Standard solutions

Stock standard solutions of alpidem, the metabolites and the internal standard were prepared in methanol at a concentration of 1 mg

ml^{-1}. Fresh stock standard solutions were prepared every 2 months and stored at 0–5°C. Working standard solutions were prepared from the stock standard solutions by suitable dilutions with methanol (see Table 1). Fresh working standard solutions were prepared every 2 weeks and stored at 0–5°C.

The standard solutions were used for the preparation of the plasma standards used for calibration.

2.3. Sample preparation

Aliquots (20 μl) of methanolic standard solutions (Table 1) were added to drug-free plasma (calibration samples), and 20 μl of internal standard solution (Table 1) were added to all samples (calibration samples and unknowns). All the samples were transferred into Eppendorf centrifuge vials (with caps), the vials were centrifuged on an Eppendorf-type centrifuge at 11 000 *g* and the supernatant was transferred to conical autosampler vials for automatic sample injection. The scheme of the operations is illustrated in Fig. 2.

2.4. Basic chromatographic system

The basic chromatographic system consisted of a Model 420 constant-flow pump (Kontron, Milan, Italy), an SFM 23/B spectrofluorimetric HPLC detector (Kontron) operating at excitation and emission wavelengths of 255 and 423 nm

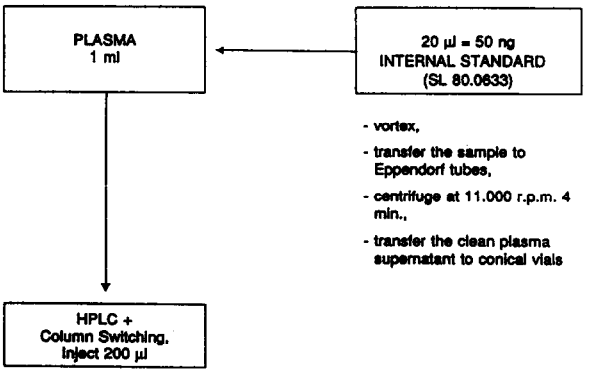

Fig. 2. Scheme of sample preparation. Internal standard = 6-chloro-2-(3,4-dimethoxyphenyl)-N,N-dipropylimidazo[1,2-*a*]pyridine-3-acetamide.

respectively, a Model 460 automatic sample injector (Kontron) provided with a six-port automatic valve and an external 250-μl loop and an analytical column (15 cm × 0.46 cm I.D.) packed with 5-μm Hypersil C_8 BDS (Shandon, Runcorn, UK) provided with a guard column (2 cm × 0.46 cm I.D.) packed with 40-μm Pelliguard LC_8 (Supelco, Bellefonte, PA, USA).

The mobile phase was acetonitrile–methanol–0.025 *M* phosphate solution (pH 4.5) (40:15:45, v/v/v) at a flow-rate of 1.5 ml min^{-1}.

2.5. Extended chromatographic system

The apparatus required for automatic column-switching HPLC was obtained by implementing

Table 1
Standard solutions used for daily calibration

Standard solution	Alpidem (ng per 20 μl)	Metabolite 3 (ng per 20 μl)	Metabolite 1 (ng per 20 μl)	Metabolite 2 (ng per 20 μl)	Internal standard (ng per 20 μl)
A	100	200	100	100	–
B	50	100	50	50	–
C	25	50	25	25	–
D	10	20	10	10	–
E	5	10	5	5	–
F	2.5	5	2.5	2.5	–
G	–	–	–	–	50

the basic apparatus with a precolumn (7.5 cm × 0.21 cm I.D.) for on-line purification, dry-packed with 30–40-μm Perisorb C_{18} (Merck), a valve-switching apparatus provided with a Tracer Model MCS-670 six-port solvent selector (Kontron), a programmer for the management of the valves and a Programmer 200 solvent selector (Kontron) and finally a Model 410 HPLC single-piston pump (Kontron) used for the clean-up of the processed plasma samples. The solvents used for precolumn clean-up and back-flushing were water, acetonitrile–water (50:50, v/v), acetonitrile, methanol–water (50:50, v/v) and water, each supplied at a flow-rate of 2 ml min^{-1}. The extended apparatus is shown in Fig. 3.

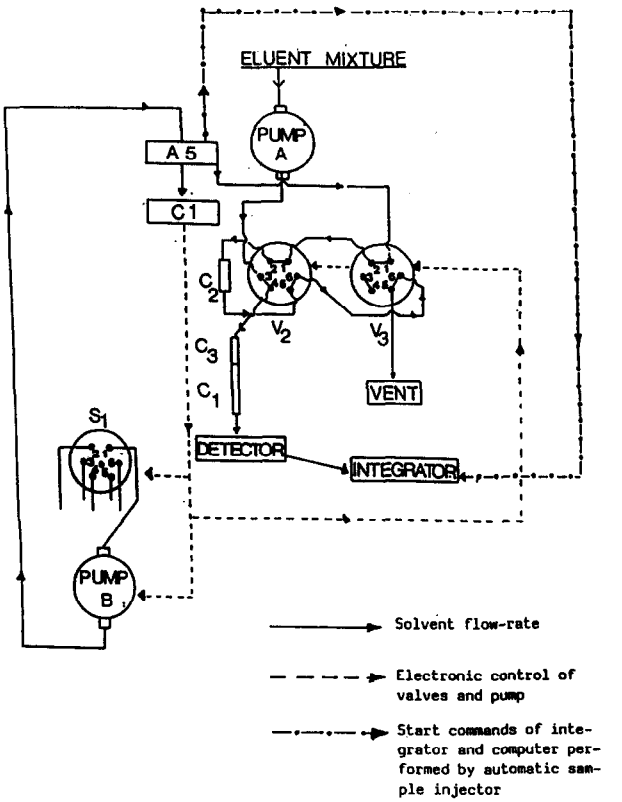

Fig. 3. Scheme of extended chromatographic apparatus. C_1 = Analytical column; C_2 = precolumn; C_3 = guard column; V_2 = valve for back-flushing; S_1 = solvent selector; A 5 = automatic sample injector; C 1 = computer.

2.6. *Operating conditions for on-line clean-up and column-switching HPLC*

The operations of fluid sampling, clean-up and enrichment on the precolumn, transfer of the analytes from the precolumn to the analytical column and analytical chromatography with simultaneous precolumn back-flushing and regeneration are performed automatically as depicted in Figs. 4–7. The programmes of on-line clean-up and column switching can be summarized as follows: after the injection of a human plasma sample (200 μl) on to the precolumn, the latter is flushed for 2 min with pure water; alpidem, metabolites and internal standard are retained, while proteins and salt are removed, then the precolumn is connected to the analytical column for 1.5 min in order to transfer the

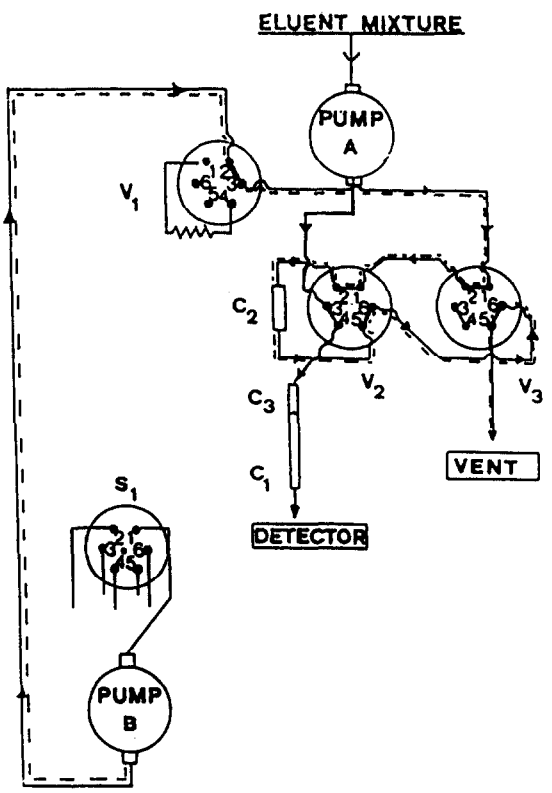

Fig. 4. Equilibrium preceding sample injection.

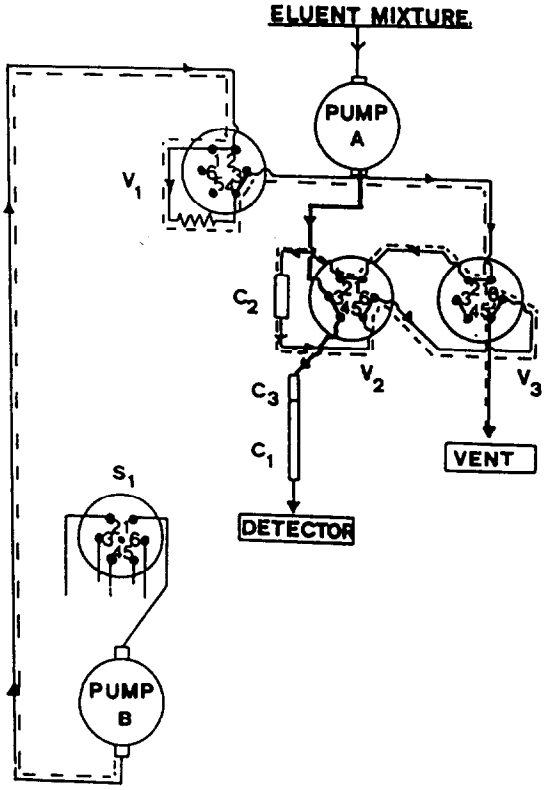

Fig. 5. Loading and clean-up of the sample on the precolumn.

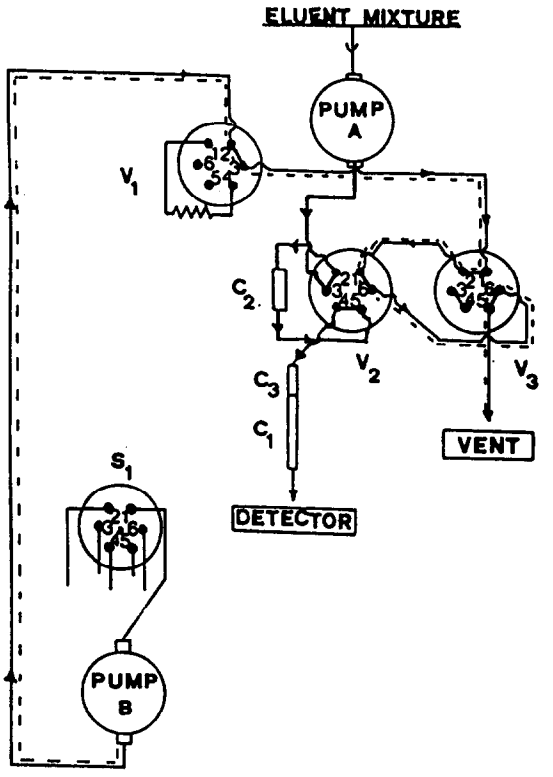

Fig. 6. V_2 switching and elution of sample from the precolumn to the top of the analytical column.

analytes to the analytical column by means of the mobile phase. While the analytical chromatography takes place, the precolumn is back-flushed with acetonitrile–water (50:50, v/v), acetonitrile, methanol–water (50:50, v/v) and water.

3. Results

3.1. Stability

As far as the stability of alpidem and its metabolites in different media is concerned, the results indicate that all the compounds were stable in methanol (stock standard solutions) for at least 2 months if maintained at 0–5°C and the methanolic working standard solutions were stable for at least 2 weeks if maintained under the same conditions.

Concerning the stability of the compounds in human plasma samples, when maintained under ordinary laboratory conditions no variations were found for all the investigated compounds during 24 h in comparison with freshly prepared and immediately analysed plasma samples (processed according to the described method). Also, no significant variations were found in human plasma samples when submitted to three freeze–thaw cycles in comparison with freshly prepared plasma samples that were immediately analysed.

The stability study was performed at levels of 8 and 80 ng ml^{-1} in human plasma for both alpidem and its metabolites, in triplicate, in the presence of 50 ng ml^{-1} of internal standard.

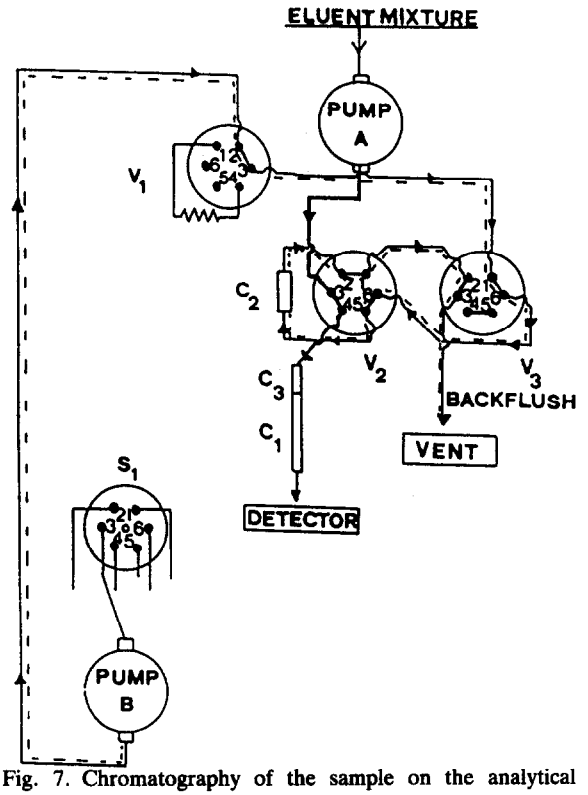

Fig. 7. Chromatography of the sample on the analytical column and back-flushing of the precolumn.

3.2. Selectivity

Several drug-free human plasma samples from different subjects were tested for the absence of interfering compounds; in no case was any chromatographic interference found at the retention times of alpidem, its metabolites and the internal standard (Fig. 8A).

Some drugs that could be co-administered with alpidem were checked for the possibility of giving chromatographic interferences. Diazepam, nordiazepam, lorazepam, nitrazepam, amitriptyline, chlomipramine (and their demethylated metabolites), ranitidine and cemetedine do not interfere chromatographically as they give no response to the fluorescence detector; trazodone responds to the detector but is well separated from the compounds of interest; zolpidem, another imidazopyridine, interferes with the more polar metabolite of alpidem (metabolite 3), but the metabolites of zolpidem do not interfere.

3.3. Recovery

The absolute recovery of alpidem and its metabolites was evaluated by comparing the chromatographic response of aqueous standard solutions (in physiological fluid) directly injected on to the HPLC column (by-passing the precolumn) to the response of plasma standards processed according to the described method. The recovery of alpidem was about 77%, for all three metabolites it was about 80% and for the internal standard it was about 73%.

3.4. Linearity

A linear correlation was found in human plasma between the ratio of peak height of alpidem to that of the internal standard and the concentration of alpidem in the range 2.5–100 ng ml^{-1}, in the same range for metabolites 1 and 2 and in the range 5–200 ng ml^{-1} for metabolite 3 (Fig. 9A–D).

3.5. Limit of quantification

The limit of quantification (LOQ) of the method, calculated on a peak with a signal-to-noise ratio of about 3, was 2.5 and 1.5 ng ml^{-1} in human plasma for alpidem and its metabolites respectively (the limit was calculated from the chromatogram reported in Fig. 8B).

3.6. Precision and accuracy

The method was validated according to an internal protocol that involved two analysts working on the same chromatographic apparatus (on different days); each analyst, after the daily calibration (performed in quadruplicate), analysed five times each two quality control specimens, of low and medium concentrations, over a 2-day period. The precision and accuracy results were evaluated during a day (intra-day) and during different days (inter-day); the cumulative results (Table 2) show that the described method

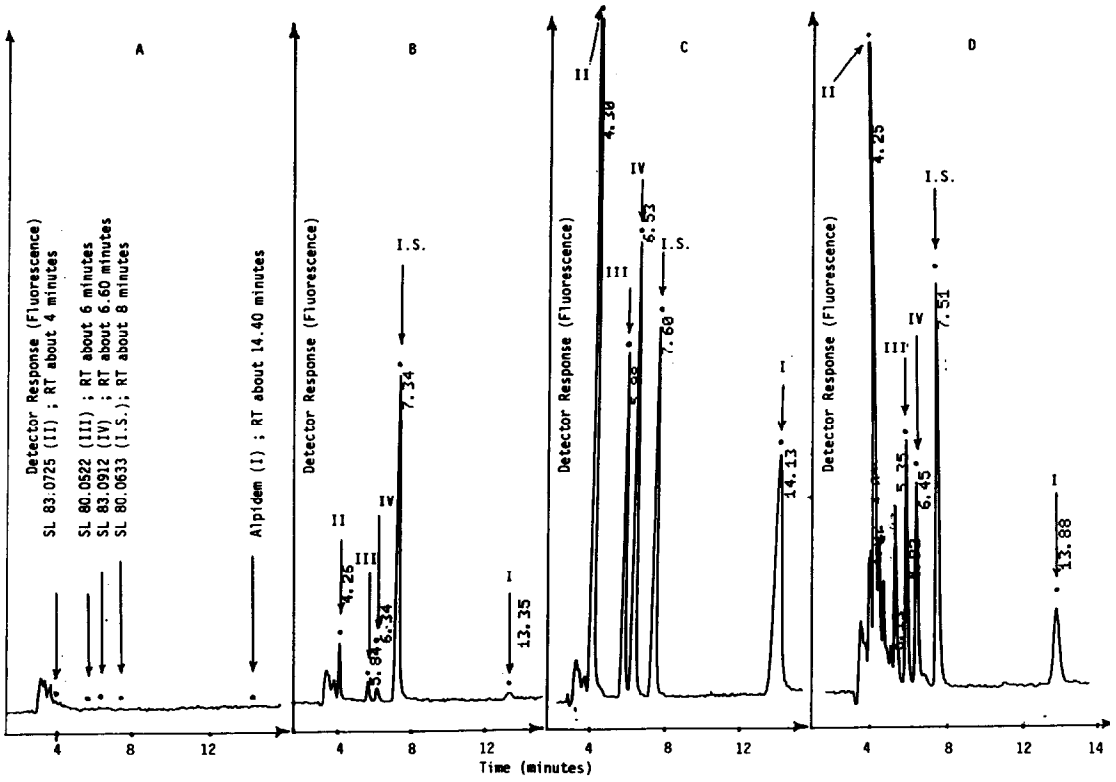

Fig. 8. (A) Chromatogram of drug-free plasma. (B) Chromatogram of a plasma standard containing metabolite 3 at 5 ng ml^{-1}, other compounds at 2.5 ng ml^{-1} and internal standard at 50 ng ml^{-1}. (C) Chromatogram of a plasma standard containing metabolite 3 at 160 ng ml^{-1}, metabolite 1 at 40 ng ml^{-1}, metabolite 2 and alpidem at 80 ng ml^{-1} and internal standard at 50 ng ml^{-1}. (D) Chromatogram of a plasma sample from a subject administered orally with alpidem with a 50-mg dose daily for 8 days; sample taken on day 7, 4 h after drug intake. RT = Retention time.

possesses sufficient precision and accuracy to be used for pharmacokinetic studies in humans. Fig. 8C shows a typical chromatogram.

3.7. Application of the method to specimens from in vivo studies

The proposed method has been extensively used for the determination of alpidem and its active metabolites in plasma from subjects treated orally with alpidem by single and/or repetitive oral administration during pharmacokinetic investigations and in clinical studies (phase 2 to phase 4) for assessing patient compliance. The method gave results comparable to those obtained by the conventional liquid–liquid extraction method [10] but it is simpler, being completely automated. Fig. 8D shows a typical chromatogram.

4. Discussion

The stability study performed on human plasma samples spiked with alpidem and its metabolites confirmed that there was no degradation of any compound, parent drug and/or metabolites, thus allowing us to leave plasma samples on the rack of the automatic sample injector (preinjection conditions) for up to 24 h; this is an extreme condition, since during 24 h it is possible to process 94 plasma samples. The precolumn, the core of the purification and extraction process, is usually replaced after about 250–300 plasma

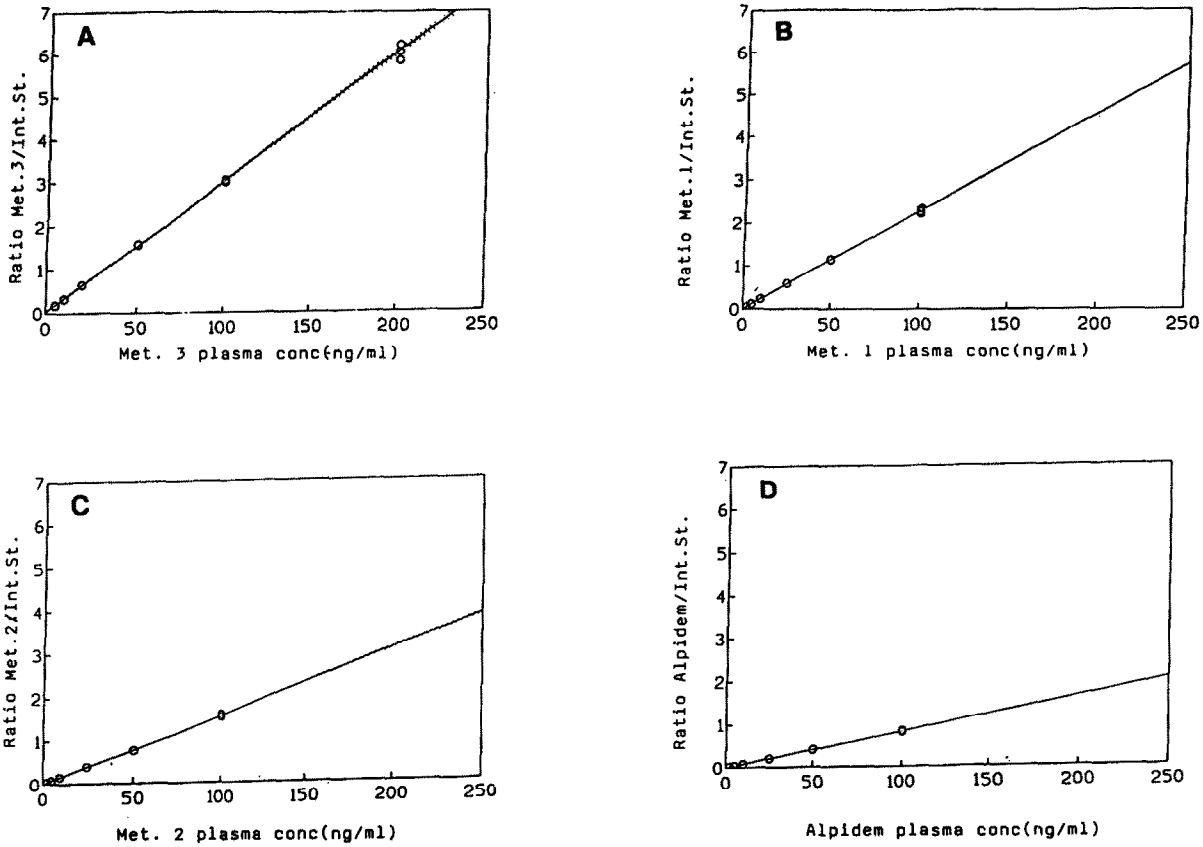

Fig. 9. (A) Regression line for metabolite 3. Equation: $y = 32.978\ x - 0.91$ ($n = 24$, $r = 0.9995$). (B) Regression line for metabolite 1. Equation: $y = 43.599\ x - 0.073$ ($n = 24$, $r = 0.9997$). (C) Regression line for metabolite 2. Equation: $y = 64.839\ x - 0.311$ ($n = 24$, $r = 0.9994$). (D) Regression line for alpidem. Equation: $y = 122.526\ x - 0.228$ ($n = 24$, $r = 0.9994$).

injections (or when the back-pressure exceeds 30 bar).

The efficiency of the analytical column is not affected by the plasma injections on to the precolumn; with the same column it was possible to analyse more than 1500 samples; there is an increase in the throughput of the samples (in comparison with the traditional extraction method) with continuous running outside of the normal working periods, and the extensive automation of the system allows minimum sample handling, removes the need for an extraction solvent and is subject to a minimum of human errors and contamination.

Selectivity with regard to endogeneous compounds and co-administered psychotropic drugs was very good and the sensitivity, precision and accuracy of the method, which has been used routinely without any problems, are high. As the only potentially crucial part of the whole chromatographic system is the precolumn (with the risk of precipitation of biological material and clogging), it was necessary to ensure that in case of an accident and a subsequent high back-pressure of the pump, a simple security apparatus will stop the pump and reset the automatic sample injector.

Finally, for laboratories that do not have a switching apparatus similar to ours, there is the possibility of utilizing the several different devices available on the market or of assembling in-house a cheap system consisting of an isocratic pump, a solvent selector and two six-port switching valves; the valves and solvent selector can be

Table 2
Precision and accuracy results

Parameter	Metabolite 3		Parameter	Metabolite 1	
	160 ng ml^{-1}	16 ng ml^{-1}		40 ng ml^{-1}	4 ng ml^{-1}
Precision:			Precision:		
R.S.D. intra (%)[a]	1.1	1.3	R.S.D. intra (%)[a]	1.0	3.4
R.S.D. inter (%)[b]	0.9	0	R.S.D. inter (%)[b]	0.4	0
R.S.D. total (%)	1.4	1.3	R.S.D. total (%)	1.0	3.4
LS[c]	2.3	1.8	LS[c]	1.5	4.8
Accuracy[d] (%)	98.0 ± 1.0	98.2 ± 0.6	Accuracy[d] (%)	103.0 ± 0.6	100.9 ± 0.5
	Metabolite 2			Alpidem	
	80 ng ml^{-1}	8 ng ml^{-1}		80 ng ml^{-1}	8 ng ml^{-1}
Precision:			Precision:		
R.S.D. intra (%)[a]	1.0	2.7	R.S.D. intra (%)[a]	1.2	4.2
R.S.D. inter (%)[b]	1.0	1.3	R.S.D. inter (%)[b]	1.7	4.0
R.S.D. total (%)	1.4	3.0	R.S.D. total (%)	2.1	5.8
LS[c]	2.5	4.3	LS[c]	4.3	9.9
Accuracy[d] (%)	103.5 ± 1.2	103.5 ± 1.8	Accuracy[d] (%)	102.9 ± 1.9	98.4 ± 4.4

Precision and accuracy of the method for the determination of metabolite 3, metabolite 1, metabolite 2 and alpidem in human plasma evaluated by analysing quality control samples ($n = 20$ for each concentration, for all compounds).
[a] Within-day relative standard deviation.
[b] Between-day relative standard deviation.
[c] 95% upper confidence limit for the R.S.D.
[d] Calculated as (concentration found/nominal concentration) · 100 at the 95% confidence limit.

managed through the "event time" section of an integrator connected by means of an interface, or by the auxiliary outputs, nowadays available, in several automatic sample injectors. If the precolumn cleaning is performed with normal-flush washings, only one switching valve is required; however, in our experience, back-flush washing is more effective in precolumn regeneration.

5. References

[1] N. Simpson, *Int. Chromatogr. Lab.*, 11 (1992) 7.
[2] W. Voelter, K. Zech, P. Arnold and G. Ludwig, *J. Chromatogr.*, 199 (1980) 343.
[3] W. Roth, K. Beschke, R. Jank, A. Zimmer and F.W. Koss, *J. Chromatogr.*, 222 (1981) 475.
[4] R.D. McDowall, G.S. Murkitt and J.A. Walford, *J. Chromatogr.*, 317 (1984) 475.
[5] V. Ascalone and L. Dal Bo', *J. Chromatogr.*, 423 (1987) 239.
[6] M.J. Koenigbour and R.E. Majors, *LC · GC Int.*, 3, No. 9 (1990) 10.
[7] A. Dale, *Eur. Chromatogr. Anal.*, 3 (1991) 5.
[8] M. Dimsdale, J.C. Friedman and B. Zivkovic, *Drugs Future*, 13 (1988) 106.
[9] B. Zivkovic, E. Morel, D. Yoly, G.H. Perrault, D.J. Sanger and K.G. Lloyd, *Pharmacopsychiatry*, 23 (1990) 108.
[10] V. Ascalone, P. Catalani, L. Dal Bo', N. Deschamps and P. Guinebault, *J. Chromatogr.*, 414 (1987) 101.