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DETERMINATION OF ALPIDEM AND ITS METABOLITES IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND FLUORIMETRIC DETECTION

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SUMMARY

A high-performance liquid chromatographic method has been developed for the simultaneous determination of alpidem and its metabolites in human plasma. The method involved a single extraction of the parent drug and metabolites into diethyl ether from alkalinized plasma, evaporation of the organic solution and chromatography of the extracts on a C_{18} column coupled to a fluorimetric detector. An internal standard was used for the quantitative determination of the compounds. The method was selective for alpidem and three of its metabolites and has a limit of detection of less than 1 ng ml^{-1} for all the compounds. Since the chromatographic run took more than 20 min, the chromatographic process was fully automated and performed overnight.

INTRODUCTION

Alpidem (SL 80.0342, Fig. 1) is a new imidazopyridine derivative possessing anxiolitic and anticonvulsant properties in animals, similar to those of diazepam and chlordiazepoxide [1]. However, alpidem differs from these benzodiazepines in having no sedative and myorelaxant effects in animals [2,3] and in inhibiting the binding of [^{3}H]diazepam to its receptor sites. Alpidem, after oral administration to humans, is actively metabolized, and three circulating metabolites have been identified (Fig. 1). They originate from the following metabolic reactions: (1) N-dealkylation, with loss of a propyl group to give metabolite 1; (2) oxidation

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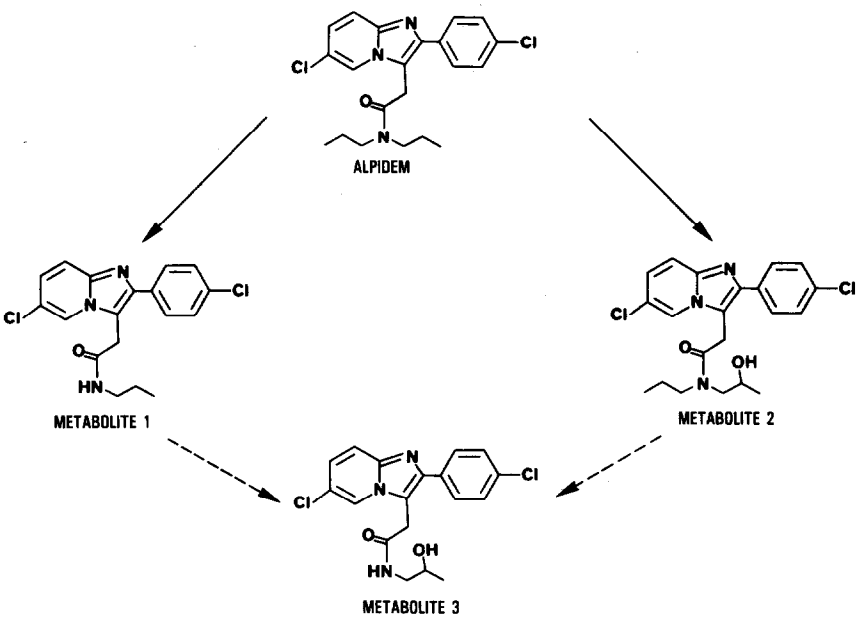

Fig. 1. Metabolic pathway of alpidem in humans; structures of metabolites.

of a propyl group to give metabolite 2; (3) oxidation of a propyl group and loss of the other propyl group to give metabolite 3.

It is likely that the pharmacological activity observed after oral administration to humans is the sum of the activities of all these circulating active metabolites [2] and of the parent compound. We considered it necessary, therefore, to develop an analytical high-performance liquid chromatographic (HPLC) method suitable for the simultaneous determination of alpidem and its metabolites.

EXPERIMENTAL

Reagents and solvents

A 1-l volume of 0.025 *M* potassium dihydrogen phosphate (pH 4.8) was prepared from 3.4 g of analytical grade salt; 0.1 *M* sodium hydroxide was prepared from analytical-grade reagent sodium hydroxide; nanograde diethyl ether, acetonitrile and HPLC-grade methanol were all obtained from J.T. Baker (Deventer, The Netherlands).

The water used for the preparation of these reagent solutions was HPLC grade, produced by the Milli Q-4 system (Millipore, Bedford, MA, U.S.A.).

Alpidem, metabolite 1, metabolite 2, metabolite 3 (Fig. 1) and the internal standard (I.S.), 6-chloro-2-(3,4-dimethoxyphenyl)-N,N-dipropylimidazo[1,2-*a*]pyridine-3-acetamide, were of pharmaceutical grade and provided by the LERS Chemistry Department (Paris, France).

Standard solutions

Stock solutions of alpidem, its metabolites and the I.S. were prepared in methanol at a concentration of 0.5 mg ml^{-1}. Standard solutions were prepared from

TABLE I

STANDARD SOLUTIONS USED FOR ANALYSIS

Standard solution	Concentration (ng per 20 μl)				Internal standard (ng per 30 μl)
	Alpidem	Metabolite 1	Metabolite 2	Metabolite 3	
1	1	1	1	1	30
2	2	2	2	2	30
3	10	10	10	10	30
4	50	50	50	50	30
5	100	100	100	100	30
6	300	300	300	300	30
7	0	0	0	0	30

stock solutions by suitable dilutions with methanol (Table I). Stock solutions were stable for at least one month if stored at 0–5 °C.

Chromatographic system

The determinations were carried out using the following chromatographic system: Model 414-T constant-flow pump and a Model SFM 23/B spectrofluorimetric liquid chromatography detector (Kontron, Zürich, Switzerland), with excitation set at 255 nm and emission at 423 nm; a Sedex 100 automatic sample injector (Sedere, Vitry-sur-Seine, France) with an automatic valve and a loop capacity of 50 μl; an analytical column (25×0.46 cm I.D.) filled with 5-μm Supelcosil® LC-18-DB, maintained at ambient temperature; and a guard column (2×0.46 cm I.D.) filled with the same packing (Supelco, Bellefonte, PA, U.S.A.). The mobile phase was acetonitrile–methanol–0.025 *M* potassium dihydrogen phosphate pH 4.8 (45:10:45, v/v). The flow-rate was 1.5 ml min^{-1} (at ca. 100 bar).

The detector was coupled to an SP 4270 chromatographic integrator (Spectra-Physics, San Jose, CA, U.S.A.) for the determination of peak height. The calculations were performed according to the "Internal Standard Method" using a multi-point linear calibration. Under these conditions the approximate retention times of the compounds were as follows: metabolite 3, 3.5 min; metabolite 1, 7 min; metabolite 2, 8 min; internal standard, 10 min; alpidem, 24 min.

Extraction from human plasma

A 30-μl volume of I.S. solution (Table I, solution 7) was transferred to a screw-capped test-tube (PTFE-lined caps, Sovirel 15) for each unknown sample; 1 ml of biological fluid was added and the samples were mixed well. A separate set of standards was prepared by transferring 20-μl aliquots of mixed standard solutions (Table I, solutions 1–6) into separate screw-capped test-tubes; 1 ml of control (pre-dose) plasma was added and mixed well. To all samples, 0.5 ml of 0.1

M sodium hydroxide were added and mixed well, then 7 ml of diethyl ether were added and all the tubes were shaken on a tumble extractor (10 min at 40 rpm).

The samples were then centrifuged at 1500 *g* for 3 min at 5 °C in a refrigerated centrifuge Model K110 (Jouan, Saint-Nazaire, France). The aqueous phase was then immediately frozen by dipping the tubes into a cryogenic bath (−75 °C). The supernatant was transferred to conical test-tubes (Sovirel 13) and evaporated to dryness under a gentle stream of pure nitrogen in a thermostated water-bath at 37 ± 2 °C.

The residues were dissolved in a suitable volume of HPLC mobile phase (150–1000 μl, depending on the expected concentration of the compounds to be assayed), aliquots were transferred to conical vials (autosampler vials, Cat. No. 3-3208, Supelco). An aluminium disk was crimped into each vial and all samples were set in a sample tray for automatic injection.

Extraction from human urine

The procedure was similar to that described for plasma.

Calculation

Peak-height ratios between the four different compounds and the I.S., obtained from plasma extracts plotted versus their concentrations, were used to generate the linear least-squares regression line. The concentrations of alpidem and its three metabolites in the unknowns were determinated by interpolation from the calibration curve using peak-height ratios obtained from unknown specimens.

RESULTS

Linearity

A linear correlation between peak-height ratios of the four different compounds and the I.S. versus their concentrations was found in the range 1–200 ng ml^{-1} of plasma. The linear least-squares regression performed on the peak-height ratios versus concentrations gave the following equations: for alpidem: $y=88.91x-0.18$, $r^2=1$, $n=6$; for metabolite 1: $y=22.20x+0.10$, $r^2=1$, $n=6$; for metabolite 2: $y=41.04x+0.17$, $r^2=1$, $n=6$; for metabolite 3: $y=15.60x-0.12$, $r^2=1$, $n=6$.

Statistical validation of the method

Before performing the statistical evaluation of the method, by using the I.S., the absolute recoveries of all five compounds from control spiked plasma were investigated, and found to be between 90 and 98% for all the compounds over a wide range of concentrations.

Intra-assay precision studies were performed on control plasma spiked with different amounts of alpidem and its three metabolites, processed as described under *Extraction from human plasma.* Intra-assay precision (within-day) was obtained by replicate analysis of plasma samples on the same day (Tables II–V). Inter-assay precision (day-to-day) was determined by analysing the standard samples on various days over five weeks (Tables II–V). The results demonstrated

TABLE II

REPRODUCIBILITY FOR PLASMA SAMPLES SPIKED WITHIN ALPIDEM

Amount added (ng ml^{-1})	n	Amount found (mean ± S.D.) (ng ml^{-1})	Recovery (%)	Coefficient of variation (%)
Within-day				
1	7	1.14 ± 0.13	114.0	11.3
10	7	9.58 ± 0.39	95.8	4.0
50	7	52.74 ± 2.80	105.5	5.2
200	7	190.52 ± 10.20	95.3	5.3
Mean				6.4
Day-to-day				
2	7	2.25 ± 0.07	112.0	3.2
10	10	10.55 ± 0.68	105.0	6.4
50	10	48.13 ± 2.90	96.2	6.0
100	10	97.05 ± 3.68	97.0	3.8
300	4	299.39 ± 19.60	99.8	6.5
Mean				5.2

acceptable precision of the method over the concentration ranges investigated. Fig. 2B shows a relevant chromatogram of this experiment.

Limit of detection

The limit of detection of the method was ca. 1 ng ml^{-1} of plasma for alpidem, 0.2 ng ml^{-1} for metabolite 3, 0.5 ng ml^{-1} for metabolites 1 and 2, with a signal-

TABLE III

REPRODUCIBILITY FOR PLASMA SAMPLES SPIKED WITH METABOLITE 1

Amount added (ng ml^{-1})	n	Amount found (mean ± S.D.) (ng ml^{-1})	Recovery (%)	Coefficient of variation (%)
Within-day				
1	7	1.12 ± 0.08	112.0	7.0
10	7	10.13 ± 0.19	101.3	1.9
50	7	54.50 ± 1.82	109.0	3.3
200	7	191.93 ± 10.88	96.0	5.7
Mean				4.5
Day-to-day				
2	7	2.02 ± 0.20	101.0	9.8
10	10	10.00 ± 0.58	100.0	5.8
50	10	49.18 ± 1.99	98.4	4.0
100	10	96.58 ± 4.08	96.6	4.2
300	4	297.24 ± 4.48	99.0	1.5
Mean				5.1

TABLE IV

REPRODUCIBILITY FOR PLASMA SAMPLES SPIKED WITH METABOLITE 2

Amount added (ng ml^{-1})	n	Amount found (mean ± S.D.) (ng ml^{-1})	Recovery (%)	Coefficient of variation (%)
Within-day				
1	7	1.09 ± 0.12	109.0	11.5
10	7	9.58 ± 0.33	95.8	3.4
50	7	52.06 ± 0.98	104.0	1.9
200	7	184.28 ± 7.96	92.1	4.3
Mean				5.3
Day-to-day				
2	7	1.93 ± 0.25	96.7	13.0
10	10	9.89 ± 0.79	98.9	7.9
50	10	46.96 ± 3.61	94.0	7.7
100	10	90.84 ± 4.95	90.8	5.4
300	4	281.76 ± 18.70	94.0	6.6
Mean				8.1

to-noise ratio of ca. 3:1.

Selectivity

Several blank plasma and blood samples from different subjects were tested for the absence of interfering endogenous compounds. In no case was any endogenous chromatographic interference found at the retention time of any of the

TABLE V

REPRODUCIBILITY FOR PLASMA SAMPLES SPIKED WITH METABOLITE 3

Amount added (ng ml^{-1})	n	Amount found (mean ± S.D.) (ng ml^{-1})	Recovery (%)	Coefficient of variation (%)
Within-day				
1	7	1.09 ± 0.04	109.0	3.9
10	7	9.92 ± 0.24	99.2	2.4
50	7	52.55 ± 2.01	105.0	3.8
200	7	186.78 ± 9.80	93.4	5.2
Mean				3.8
Day-to-day				
2	7	1.99 ± 0.25	99.6	12.0
10	10	10.64 ± 0.77	106.0	7.0
50	10	50.73 ± 2.44	101.5	4.8
100	10	103.02 ± 4.65	103.0	4.5
300	4	308.42 ± 12.40	102.8	4.0
Mean				6.5

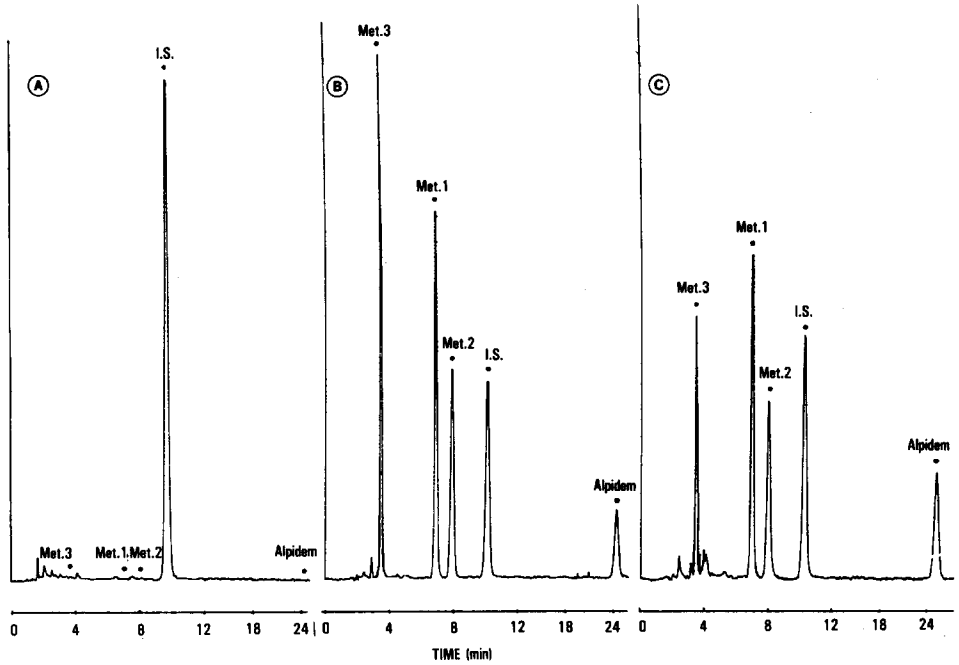

Fig. 2. (A) Chromatogram of pre-dose plasma extract containing the I.S. (B) Chromatogram of authentic standards recovered from pre-dose plasma spiked with alpidem and its three metabolites. Nominal concentration was 30 ng ml^{-1} of plasma for each compound. (C) Chromatogram of plasma extract from a subject administered orally with 50 mg of alpidem (single administration). Sample taken 1 h after drug intake. Chart speed was 0.5 cm min^{-1} from 0 to 12 min, then 0.25 cm min^{-1}.

interested compounds. Fig. 2A shows a typical chromatogram of a drug-free plasma extract containing the I.S.

Interferences

The chromatographic interferences of other drugs that could be co-administered were checked. No interferences were found when control plasma spiked with all the five compounds and some benzodiazepines or tricyclic antidepressants were processed according to this method. The drugs tested were diazepam, bromazepam, flunitrazepam, nordiazepam, amitriptyline, nortriptyline, imipramine and desipramine.

Application of the method to biological specimens

The method described was applied to the determination of alpidem and its metabolites in plasma of healthy volunteers who had received a single oral dose of 50 mg of alpidem. The mean plasma concentration–time course of alpidem and its metabolites, in healthy subjects, is shown in Fig. 3, and a typical chromatogram is shown in Fig. 2C. From Fig. 3 it appears that the parent drug is extensively metabolised into the three metabolites.

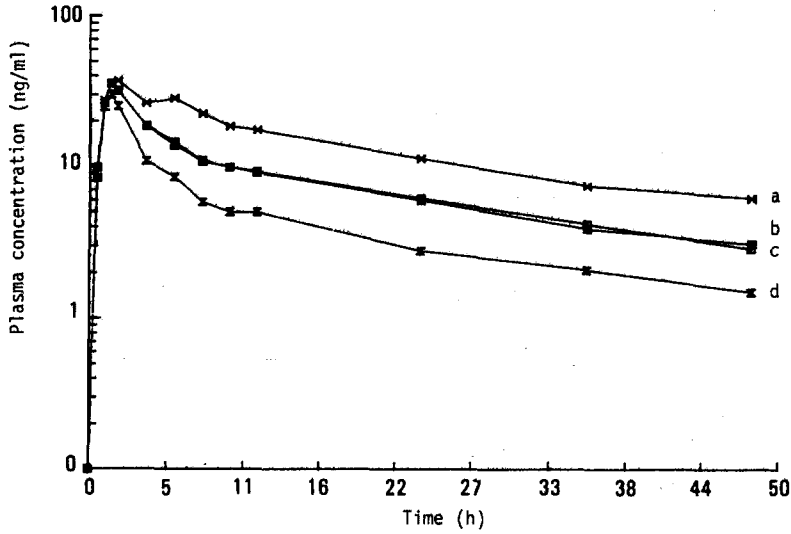

Fig. 3. Mean plasma concentration–time course of alpidem and its metabolites in ten adult humans administered orally with a 50-mg dose of alpidem (single administration). (a) Metabolite 3; (b) metabolite 2; (c) alpidem; (d) metabolite 1.

DISCUSSION

This simple HPLC method allows the simultaneous determination of alpidem and three of its circulating metabolites using an I.S. The separation between metabolites 1 and 2 (see Fig. 1) could be achieved only when methanol was present in the mobile phase. If methanol was replaced by acetonitrile the separation between metabolites 1 and 2 was incomplete. The chromatographic run took ca. 25 min, which can be considered as a long time, but it must be remembered that five different compounds are being separated isocratically. Moreover, the utilization of an automatic sampler allowed overnight injection. If alpidem plasma levels only are required, a more rapid and sensitive HPLC method is available [4].

The method, used for pharmocokinetics and monitoring studies, was tested on more than 500 biological samples and it produced satisfactory and reproducible results.

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