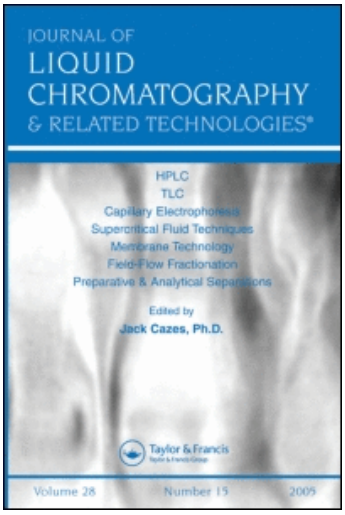

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Simple, Rapid and Specific Identification and Quantification of a Metabolite of Alpidem, a New Imidazopyridine Anxiolytic, in Human Urine, by Direct Injection Into HPLC Column with Fluorescence Detection

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SIMPLE, RAPID AND SPECIFIC IDENTIFICATION AND QUANTIFICATION OF A METABOLITE OF ALPIDEM, A NEW IMIDAZOPYRIDINE ANXIOLYTIC, IN HUMAN URINE, BY DIRECT INJECTION INTO HPLC COLUMN WITH FLUORESCENCE DETECTION

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ABSTRACT

For the investigations of alpidem overdose cases in humans, an HPLC method with fluorescence detection was developed. The method was based on the identification and quantification of an alpidem metabolite (the product of oxidation of the propyl group) in urine, since no unchanged alpidem was found in urine of subjects dosed with the drug. The urinary sample, after deconjugation with β-glucuronidase/ arylsulfatase enzyme and addition of internal standard, was simply diluted (1/1) with pure water and injected onto the chromatographic system connected to spectrofluorimetric detector ($\lambda_1 = 255$ nm, $\lambda_2 = 423$ nm). The analytical column was C_8 type material specially deactivated for basic compounds, the eluent mixture was constituted by a phosphate solution 0.025M (pH=4.6), acetonitrile, methanol, 45/30/15 (v/v/v), supplied at 1.5 ml.min^{-1} flow rate. The determination limit of the method, in human urine, was 20 ng.ml^{-1}; the method was linear in the

range 20–1000 ng.ml^{-1} in urine. The method showed a good selectivity in respect to endogenous compounds and to commonly prescribed psychotropic drugs. The method, validated over a 4 day period, by two analysts, was demonstrated to be precise and accurate.

INTRODUCTION

Alpidem is a new imidazopyridine which has an interesting anxiolytic profile at both the animal and clinical level; in several animal species, alpidem shows anxiolytic–like activities and anticonvulsant properties associated with very weak or no myorelaxant and sedative effects (1,2). In animal models, the drug seems to exert its pharmacological effect by interacting with ω_1 receptors within the GABA supramolecular receptor complex (3). The drug undergoes a first pass elimination, after oral administration to humans (4), it is actively metabolized and three circulating metabolites have been identified in plasma (5), they originate from N–dealkylation, oxidation of one N–propyl group or a combination of both processes (Fig.1).

Alpidem metabolites are mainly eliminated by biliary excretion in the feces, small quantities are eliminated in urine where alpidem unchanged is not found (6).

Since the compound has been available on the French pharmaceutical market for one year, we were requested to develop a simple and rapid analytical method suitable to detect the unchanged compound and/or its metabolites in urinary samples collected from subjects hospitalized after accidental or deliberate drug overdoses.

The determination of alpidem and metabolites in human plasma is performed by HPLC with fluorescence detection method based on a liquid–liquid extraction of the drug and its metabolites (5) from the biological matrix; recently a method was developed in our laboratory

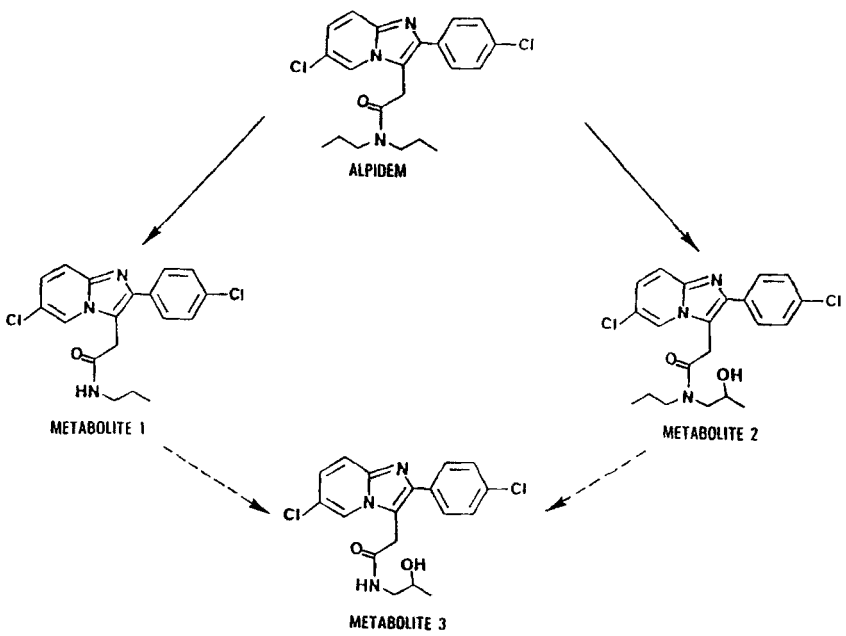

FIGURE 1: Metabolic pathway of alpidem in humans: structures of the principal metabolites

based on the column switching HPLC technique (not yet published). When both the existing methods were tried on urinary samples from subjects treated with alpidem at therapeutic dose, neither unchanged drug nor metabolites were found; however if urine was previously enzymatically deconjugated, a relevant chromatographic peak (corresponding to the retention time of the metabolite obtained from the oxidation of a N–propyl group) was discovered.

This paper describes a single and rapid method of determination of such metabolite in urinary samples by directly injecting the fluid into an HPLC column connected to a fluorescence detector.

MATERIAL AND METHOD

Chemicals

Alpidem (SL 80.0342), metabolite 1 (SL 80.0522), metabolite 2 (SL 83.0912), metabolite 3 (SL 83.0725) (Fig.1) and the internal standard (SL 80.0633) (Fig.2) were of pharmaceutical grade and provided by Synthélabo Recherche (L.E.R.S.), Chemistry Department, Paris (France). HPLC grade acetonitrile and methanol, potassium dihydrogen phosphate anhydrous (pro analysi), β–glucuronidase/ arylsulfatase (extracted from Helix pomatia, β–glucuronidase 30 U/ml, arylsulfatase 20 U/ml) were all obtained from E.Merck (Darmstadt, Germany). Pure water used for HPLC and reagent preparation was obtained from deionized water then purified through Milli Q–system (Millipore, Bedford, MA, U.S.A.).

Reagents

The phosphate buffer 0.025M (pH=4.6) used for the mobile phase was prepared from potassium dihydrogen phosphate by dissolution and dilution with pure water. The mobile phase was prepared by adding to 450 ml of phosphate buffer 0.025M, 150 ml of methanol and 300 ml of acetonitrile, then mixing and filtering on 0.22 µm filter membrane (type HV) with a clarification kit (Millipore).

Standard solutions

Stock solutions (1000 µg/ml) of metabolite 2 (SL 83.0912) and internal standard (SL 80.0633) were prepared monthly in methanol and stored at 0–5 °C conditions. The standard solutions, used for daily calibration, were prepared from stock solutions by suitable dilution with methanol in order to obtain a wide range of concentrations (Table 1).

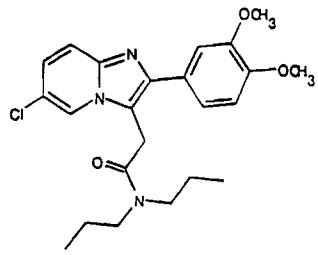

FIGURE 2: Internal standard (SL 80.0633) structure

TABLE 1

Standard solutions used for calibration

Standard solutions	Metabolite 2 concentration (ng/20 µl)	Internal Standard concentration (ng/20 µl)
1	1000	—
2	500	—
3	250	—
4	100	—
5	50	—
6	20	—
7	—	400

The standard solutions were prepared weekly and stored at 0–5 °C conditions.

Quality control samples

Quality control samples were prepared in pre–dose human urine at concentrations of about 300 and 60 ng.ml^{-1} of metabolite 2 by spiking 0.5 ml of methanolic solution of metabolite 2 (at concentration of 14.7 ng.µl^{-1} and 2.95 ng.µl^{-1} respectively) with two 25 ml aliquots of pre–dose urine. 1 ml aliquots of urinary specimens were transferred into screw–capped test tubes and deep–frozen (–20 °C) until analysis.

Chromatography

A Kontron system was used consisting of a double piston pump model 420 with a pump head for 0.01–2 ml.min^{-1} flow rate, the detector was a fluorimetric type model SFM 23 operating at 255 nm, as excitation, and at 423 nm, as emission wavelength; the automatic sample injector was a model 460 provided with a 6 port Valco valve and 100 µl loop. The chromatographic column (15 x 0.46 cm i.d.) was packed with Hypersil BDS, C_8 type material, 5 µm particle size (Shandon, Runcorn, UK); it was provided with a guard column (2 x 0.46 i.d. cm) packed with Pelliguard C_8 material, 40 µm particle size (Supelco, Bellefonte, PA, U.S.A.). The mobile phase consisted in a mixture of aqueous potassium dihydrogen phosphate (0.025M, pH=4.6), acetonitrile and methanol in the ratio 45/30/15 (v/v/v) supplied at 1.5 ml.min^{-1} flow rate (back–pressure about 85 BAR). The quantitative determinations were performed by means of a S.P. model 4290 integrator (Spectra Physics, San José, CA, U.S.A.).

Sample preparation

The frozen urine samples (pre–dose and unknowns) were thawed in a water–bath at 37 °C before pipetting at room temperature; then 1 g of pre–dose urine (for each future urinary standard) and unknown samples were weighed. To the pre–dose urine, 20 µl of the standard solutions were added for each calibration point (Table 1); 50 µl of β–glucuronidase/arylsulfatase solution were added to all samples. All the tubes containing the samples were capped and incubated in a thermostated block at 37 °C for 20 minutes, then cooled at room temperature; to all the tubes 20 µl of internal standard solution were added (Table 1) and homogenized, then the samples were diluted with 1 ml of pure water, homogenized and transferred to autosampler vials for automatic sample injection. 50 µl were injected into the HPLC system (Fig. 3, for the scheme of sample preparation).

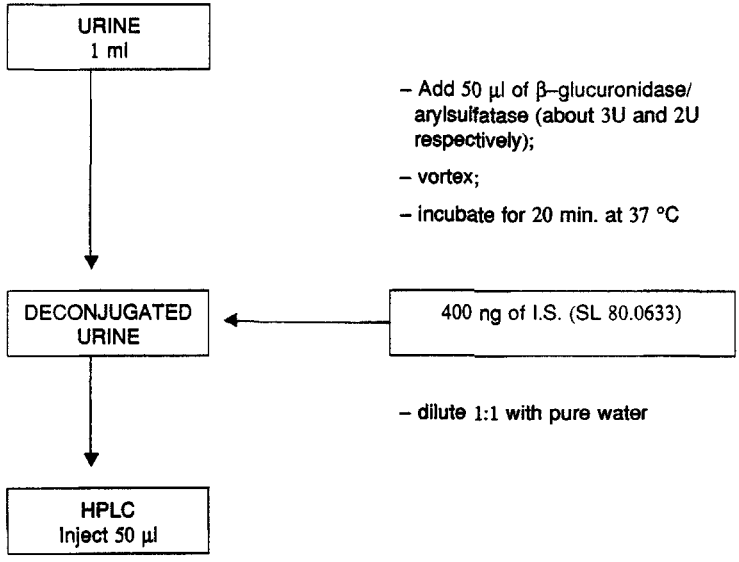

FIGURE 3: Scheme of the sample preparation for the analysis of metabolite 2 (SL 83.0912) in urine.

Quantitative determination

Peak height ratios of metabolite 2/internal standard, obtained from urinary standards, plotted versus the nominal concentration of the metabolite 2, were used to generate the linear least square regression line (calibration equation). The concentration of metabolite 2 in the unknown specimens were obtained by interpolation from the calibration equation using peak height ratios of metabolite 2/internal standard, obtained from unknown specimens. All the operations concerning quantitation and calibrations were automatically performed on an integrator SP 4290.

RESULTS AND DISCUSSION

Stability

The metabolite 2 and internal standard were stable in methanol, at least one month, if maintained at 0–5 °C conditions (stock solutions) in comparison with freshly prepared solutions. Both the compounds were stable in deconjugated and diluted human urine for at least 12 hours at room temperature (pre–injection conditions). The metabolite 2, in urine, resulted stable if submitted to 2 freezing–thawing cycles (–20 to 37 °C).

Selectivity

Several pre–dose urinary samples from different subjects were tested for the absence of interfering compounds; in no case was any chromatographic interference found at the retention time of metabolite 2 and internal standard (Fig. 4a). The possibility of interference by some commonly prescribed drugs (anxiolytics, hypnotics,

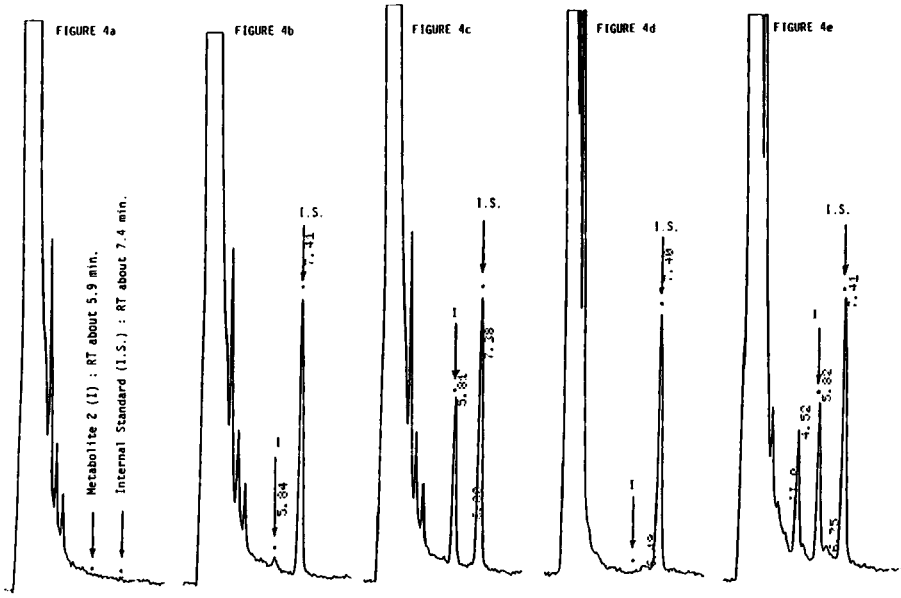

FIGURE 4a: chromatogram of pre–dose human urine after deconjugation; metabolite 2 = I, internal standard = I.S.

FIGURE 4b: chromatogram showing the limit of determination of the method in human urine deconjugated : 20 $ng.ml^{-1}$

FIGURE 4c: chromatogram of a quality control sample (QC) recovered from pre–dose human urine deconjugated at nominal concentration of 300 $ng.ml^{-1}$ of I and 400 $ng.ml^{-1}$ of I.S.

FIGURE 4d: chromatogram of a urine sample taken from a subject treated orally with 50 mg of alpidem b.i.d.; non–deconjugated sample

FIGURE 4e: chromatogram of the same sample reported in Figure 4d after being submitted to deconjugation process. The value found for metabolite 2 was 292.7 $ng.ml^{-1}$ (referred to 0–10 hours urinary collected fraction)

antidepressants, β–blockers, anti–H_2) was also checked; diazepam, nordiazepam, nitrazepam, flunitrazepam, oxazepam, lorazepam, triazolam, amitriptyline, fluoxetine, propanolol, betaxolol and ranitidine were not detectable in the described conditions. Trazodone and zolpidem and its metabolites, which possess fluorescence properties, were detectable, however these were eluted at shorter retention times than metabolite 2 and well separated from it.

Linearity

A linear correlation between peak height ratio of metabolite 2 and the internal standard versus the concentration of metabolite 2 was found in the range 20–1000 ng.ml^{-1} of human urine (as shown in Fig. 5).

Limit of quantitation

The limit of quantitation was 20 ng.ml^{-1} of metabolite 2 in human urine (Fig. 4b). The coefficient of variation (C.V.) of the limit of quantitation was ± 7% (n=4).

Precision and accuracy

The described method was validated by two analysts working with the same chromatographic apparatus on different days. Each analyst, after the daily calibration (performed in quadruplicate), analysed two quality control samples (low and medium concentration, performed in quintuplicate) respectively at about 300 and 60 ng.ml^{-1} of metabolite 2 in human urine, over a two day period. The results of this experiment, reported in Table 2, showed good precision and accuracy. A relevant chromatogram of the experience is shown in Fig. 4c.

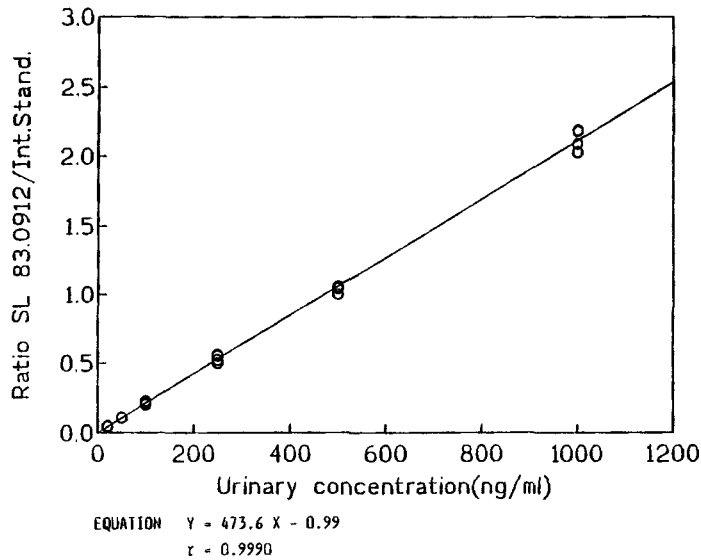

FIGURE 5: Linearity for alpidem metabolite 2(SL 83.0912) in human urine (n=24).

Application of the method to "real" urinary specimens

The method described was applied to the determination of metabolite 2 in urinary samples of volunteers administered orally with alpidem at 50 mg dose b.i.d. (therapeutic regimen); the urinary excretion values are reported in Table 3; Fig. 4d shows a chromatogram of the non-deconjugated urinary sample (fraction 0–10 hours after drug treatment) without the presence of metabolite 2, on the contrary Fig. 4e, referring to the same urinary sample submitted to deconjugation, shows the presence of metabolite 2 at concentration of 292.7 $ng.ml^{-1}$ of urine.

Conclusions

The metabolite 2 of alpidem, can be detected in human urine of subjects treated orally with alpidem only after deconjugation of the urinary samples, as a consequence, the analysis of urine samples

TABLE 2

Precision and accuracy of the method of determination of metabolite 2 (Alpidem) in human urine evaluated by analysing quality control specimens at 59.1 ng.ml^{-1} and 292.5 ng.ml^{-1} by two analysts over 4 day period.

	59.1 ng.ml^{-1}	292.5 ng.ml^{-1}
Precision C.V. intra (%) @	5.3	3.4
C.V. inter (%) #	0.0	4.3
C.V. total (%)	5.3	5.5
LS*	7.7	10.4
Accuracy **	93.2 ± 1.7	94.0 ± 4.3
n	20	20

@ C.V. intra = coefficient of variation within days
# C.V. inter = coefficient of variation between days
* 95% upper confidence limit for the relative standard deviation
** it is the recovery (expressed as per cent of the nominal concentration) with 95% as confidence limit

before and after deconjugation can be considered as an identification criterion of the intake of the anxiolytic drug. The chromatography of the deconjugated urine also showed the presence of other possible metabolites eluted in the early part of the chromatogram, not well separated from the solvent front and from other endogenous peaks. The sample preparation of the proposed method is extremely simple since it requires no extraction or clean–up steps, only incubation with

TABLE 3

Urinary excretion of metabolite 2 in two healthy volunteers administered with alpidem orally at 50 mg dose b.i.d.

Fraction	Subject B.M.		Subject V.A.	
	Collection time (hours)	Metabolite 2 (μg)	Collection time (hours)	Metabolite 2 (μg)
1	0–7	202.7	0–10	117.8
2	7–15	80.5	10–14	92.8
3	15–23	139.1	14–23	266.9
4	23–32	114.0	23–34	109.5
Cumulative excretion	0–32 (hours)	536.3 (μg)	0–34 (hours)	587.0 (μg)

the deconjugation enzyme, dilution of the sample with water and direct injection onto the chromatographic column. The selectivity of the method in regard to endogenous compounds and other eventually co-administered drugs (benzodiazepine anxiolytics, hypnotics, antidepressants, β-blockers, anti-H_2) is quite satisfactory; no interference was found. The method has to be tested on urine samples collected from subjects overdosed with alpidem, but the results on samples obtained under therapeutic treatment allow us to think that the method should be suitable in the case of overdosing. Moreover in such case the urinary levels of metabolite 2 should be higher than those found in normal dose. In conclusion the described method is proposed for the rapid identification and quantitation of the metabolite 2 of alpidem in toxicological and emergency laboratory in

case of a suspected overdose (voluntary or accidental) of alpidem; the method could also be useful for assessing drug compliance during clinical investigations (phase 3 and 4).

REFERENCES

1. M.Dimsdale, J.C.Friedman, P.L.Morselli, B.Zivkovic, Drugs of the Future, 13, 106–109 (1988)

2. B.Zivkovic, E.Morel, D.Yoly, G.H.Perrault, D.J.Sanger, K.G.Lloyd, Pharmacopsychiatry, 23, 108–113 (1990)

3. S.Z.Langer, S.Arbilla, Pharmacol.Biochem. Behav., 29, 763–766 (1988)

4. G.Bianchetti, F.Thiercelin, J.H.G.Jonkman, V.Ascalone, P.Guinebault, J.P.Thenot, European Congress of Biopharmaceutics and Pharmacokinetics, Abstract 339, April 21–24, 1987, Freiburg (Germany)

5. V.Ascalone, P.Catalani, L.Dal Bo', J. of Chromatogr., 414, 101–108 (1987)

6. G.Bianchetti, C.Dubruc, J.P.Thenot, V.Ascalone, Abstracts from International Symposium on "Imidazopyridines in anxiety disorders", September 26–28, 1991,Paris (France)

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