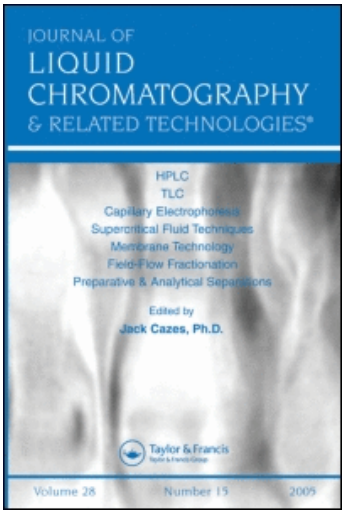

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Simultaneous Determination of Zopiclone and Its Two Major Metabolites (N-Oxide and N-Desmethyl) in Human Biological Fluids by Column Liquid Chromatography After Solid-Phase Extraction

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SIMULTANEOUS DETERMINATION OF ZOPICLONE AND ITS TWO MAJOR METABOLITES (N-OXIDE AND N-DESMETHYL) IN HUMAN BIOLOGICAL FLUIDS BY COLUMN LIQUID CHROMATOGRAPHY AFTER SOLID-PHASE EXTRACTION

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ABSTRACT

A reverse phase liquid chromatographic procedure with fluorescence detection for the simultaneous determination of zopiclone and its main metabolites, N-desmethylzopiclone and zopiclone-N-oxide, in serum, blood and urine is described. An aliquot (0.5 - 1 mL) of the sample after the addition of 0.25 mL of 250 ng/mL solution of harmane in 0.2 M NaH_2PO_4 as the internal standard is passed through a 1-mL BondElut C_{18} silica extraction column. The column is selectively washed with water and acetonitrile to remove polar, neutral and acidic compounds. The desired compounds are eluted with a 0.25 mL aliquot of a mixture of methanol + 35% perchloric acid (100:1 v/v). A 10 - 25 μL aliquot of the eluate is injected onto a 150 X 4.6 mm I.D. column packed with 5-μm C_{18} silica particles which is eluted at ambient temperature with a mobile phase of acetonitrile - 0.1% tetramethylammonium perchlorate (17:83 v/v) adjusted to pH 3.8

with 10% perchloric acid at a flow rate of 1.8 mL/min. The peaks are detected with a fluorescence detector (ex = 320 nm, em = 520 nm). The extraction recovery of all the compounds is in the range of 90 - 95%. The chromatogram is clean and the desired peaks are well separated from each other and from extraneous peaks.

INTRODUCTION

Zopiclone (Imovane®) belongs to a novel chemical class (cyclopyrrolone) of hypnotics-sedatives which is structurally unrelated to benzodiazepines or barbiturates. However, the pharmacological profile of zopiclone is similar to that of benzodiazepines.[1] Like other sedatives, zopiclone also has a considerable potential of being ingested in overdose. Severe overdose with zopiclone produces somnolence, confusion and coma with reduced or absent reflexes. Though the treatment of zopiclone overdose like that of benzodiazepines overdose is supportive in response to clinical signs and symptoms, it is desirable to include zopiclone in hypnotic-sedative screen in clinical and forensic laboratories.

Mannaert and Daenens[2] have recently described a radioimmunoassay for the determination of N-desmethylzopiclone, the most persistant metabolite of zopiclone. However, immunoassay reagents for the detection or determination of zopiclone or its metabolites are not yet commercially available. Antibodies for barbiturates or banzodiazepines do not show any cross reactivity with this drug. Relatively low serum peak drug concentration of 20 - 60 ng/mL observed after a therapeutic dose of 7.5 mg of zopiclone does not allow the use of spectrophotometric or spectrofluorometric procedures for the determination of zopiclone. Gas chromatgraphy is not particularly suitable as zopiclone is thermally labile and produces multiple peaks.[3,4]

At present, column liquid chromatgraphy (LC) appears to be the most suitable technique for the identification and quantification of zopiclone in biological fluids. A number of LC procedures using fluorescence[5-8] or UV absorbance detection[3,9,10] have been described for the determination of zopiclone in serum/blood. However, only Liboux et al.[7] describe the simultaneous determination of zopiclone and its metabolites in serum and urine. Analysis of urine is required for forensic detection of zopiclone use because of the short half-life (3.5 h) of zopiclone. A high performance thin layer chromatographic procedure for the determination of zopiclone has also been described.[12]

In all the LC procedures described so far, the sample has been extracted by liquid/liquid extraction and the extract evaporated prior to chromatography. Gaillard et al.[4] have described a solid-phase extraction (SPE) procedure requiring evaporation of the extract. In this procedure, zopiclone is isolated as its decomposition product for its determination by gas chromatography. Now I describe a rapid SPE procedure which allows the direct injection of the extract without its prior evaporation for the simultaneous determination of intact zopiclone and its main metabolites.

EXPERIMENTAL

Materials

Stock solutions of zopiclone, N-desmethylzopiclone and zopiclone-N-oxide (all from Rhone-Poulenc Rorer, Canada) of 1 mg/mL each were prepared in acetonitrile. The solutions were stored at -20°C.

A serum standard of only zopiclone of 2 μg/mL and a urine standard of zopiclone and its two metabolites of 2 μg/mL each were prepared in blood bank outdated plasma and drug free pooled urine. Serum standard was serially diluted to prepare 8 standards and the urine standard was serially diluted to prepare 6 standards.

Stock internal standard (IS) solution of 1 mg/mL of harmane hydrochloride (Sigma Chemical Co. St.Louis, MO) was prepared in methanol and stored at -20°C. Working IS solution was prepared by diluting 5 μL of the stock solution with 20 mL of 0.2 M NaH_2PO_4 This solution was stored at 4°C for one week.

Procedures:

Extraction

The required number of 1-mL BondElut C_{18} extraction columns (Varian, Harbor City, CA, USA) was placed on a VacElut system. The columns were washed once (one column volume) with 1 M HCl , twice with methanol and once with water, each time aspirating the liquid completely with suction. A

0.25 mL aliquot of the working IS solution was placed in each column, then 1 mL of serum sample or supernatant of blood hemolysate (prepared by diluting 1.0 mL of blood with 0.5 mL of water and centrifugation) or 0.5 mL of urine sample was applied. The liquid was allowed to pass through the columns at a slow rate of about 1 mL/min.. using mild suction. The columns were washed twice with water and twice with 0.5 mL aliquots of acetonitrile making sure that each column was drained completely after every wash. The tips of each column was wiped with tissue and placed on 16 X 100 mm glass tubes containing correspondingly labelled 1.5 mL plastic sample cups. An aliquot of 0.25 mL of methanol containing 1 mL/100 mL of 35% perchloric acid was applied to each column. The liquid was allowed to pass through the column bed by gravity and finally drained completely by centrifugation for 20 s. The cups were covered with aluminium foil and loaded in the autosampler. A 25-µl aliquot of serum extract or a 10-µL aliquot of urine extract was injected onto the chromatographic system.

Chromatography

A modular chromatographic system comprising of a Model LC-6A pump, a Model SPD-10A absorbance detector, a Model RF-535 fluorescence detector and a Model CR-501 integrator plotter (all from Shimadzu Scientific Co., Columbia MD, USA) was used. A 150 X 4.6 mm I.D. Ultrasphere ODS reverse phase column packed with 5-µm C_{18} bonded silica particles (Beckman Instruments, San Ramon, CA, USA) protected by a 15 X 3.2 mm I.D. RP-18 guard cartridge packed with 7-µm silica particles (Applied Biosystems, San Jose, CA, USA) was used as the analytical column. A mobile phase consisting of acetonitrile - 0.1% tetramethylammonium perchlorate (17:83 v/v) adjusted to pH 3.8 with 10% perchloric acid was pumped at a flow rate of 1.8 mL/min. resulting in an operating pressure of 12 Mpa. Chromatography was performed at ambient temperature. The fluorescence was monitored at 520 nm (excitation at 320 nm).

RESULTS AND DISCUSSION

Detection

Zopiclone has been determined by both UV absorbance[3,9,10] and fluorescence[5-8] detection. The two modes of detection were compared by connecting the exit of the absorbance detector to the inlet of the fluorescence

detector. There was virtually no distortion in the fluorescence peaks. Both detectors working optimally were set for maximum possible sensitivity. The fluorescence response of zopiclone and its two metabolites (ex = 310 nm; em = 500 nm) was about 4 times that of absorbance detection (305 nm) with a stable base line for both the detectors. Extracts of serum samples showed clean chromatograms by both detection modes. However, extracts of some urine samples showed additional extraneous peaks than observed by fluorescence detection. Therefore, fluorescence detection was selected for quantification of zopiclone and its metabolites. Simultaneous UV/fluorescence detection is used to confirm the identification of zopiclone and its metabolites by comparing the ratios of peak areas of absorbance/fluorescence of the unknown peak to that of the zopiclone standard.

Internal Standard

Tracqui et al.[10] did not use any internal standard as this publication describes only a screening procedure for the detection of zopiclone and other analogous sedatives. Royer-Morrot et al.[9] used dihydroquinidine as the internal standard for the determination of zopiclone in plasma. This compound is present in the blood of patients receiving quinidine therapy and elutes close to quinidine. Foster et al.[11] have used chlordiazepoxide as the IS for stereospecific assay of zopiclone. However, this compound does not appear to be a suitable compound as an IS for toxicological and forensic determinatioon of zopiclone as chlordiazepoxide is a commonly prescribed sedative drug.

In all other procedures,[3,5-8] a quinolyl analogue of zopiclone (RP 29481) has been used as the internal standard. It is an appropriate compound to be used as an IS for the determination of zopiclone. However, the chromatographic run time in the procedure of Liboux et al.[7] was more than 35 min. and there is an interval of about 20 min. between the last peak of the IS and the preceeding peak of zopiclone. In an attempt to reduce the chromatographic run time, a number of commercially available fluorescent non-drug compounds were screened for use as an IS for the simultaneous determination of zopiclone and its metabolites. Harmane proved to be the most suitable compound. It elutes as the first peak in the selected chromatographic system. The chromatographic run time of 20 min. in the present system is still quite long. However, this long time is due to the requirement of baseline separation of zopiclone from its metabolite N-desmethylzopiclone. Chromatographic time can be reduced to about 12 min. by increasing the acetonitrile content to about 25%, when the presence of only zopiclone is expected in a given sample. Harmane is highly fluorescent at the optimal

conditions of fluorescence of zopiclone (ex = 310 nm; em = 500 nm). Fluorescence response of harmane is reduced to one-half at ex = 320 nm and em = 520 nm with only a 10% reduction in the fluorescence of zopiclone. These settings were selected as a compromise so that harmane could be monitored by both fluorescence and UV absorbance detection. It is not possible to do so if harmane fluorescence peak is reduced by decreasing the concentration of the IS solution. These settings also reduce the fluorescence of procainamide, quinidine and quinine and other indole compounds.

Extraction

Liboux et al.[7] have used a mixture of dichloromethane and 2-propanol to extract biological samples for the isolation of zopiclone and metabolites. It seems that the extraction recovery of the various compounds has not been described in this publication. The SPE procedure described in this report is environmentally friendly as water immiscible solvents, particularly halogenated hydrocarbons, are not used. The extraction recovery determined by comparing the peak areas of extracts of serum and urine of 0.1 and 1 μg/mL of each compound with those of unextracted standards of corresponding concentrations showed extraction recovery of each compound in the range of 90 - 95%. The internal standard, harmane, behaves similarly to zopiclone during extraction as there is no change in the ratios of peak areas of analyte/IS after extraction by the described SPE.

Gaillard et al.[4] have used a SPE for the isolation of zopiclone only and give the impression that zopiclone is completely converted to a decomposition product during extraction. Some compounds do undergo decomposition during SPE because compounds are exposed to large volumes of air during wash and elution steps. However, there is no indication of any change in the structure of zopiclone or that of zopiclone metabolites as a result of SPE. The retention times of these compounds in the methanolic extract are identical to those observed when an unextracted acetonitrile solution of these compounds is chromatographed.

It is now well established that zopiclone is unstable in nucleophilic solvents such as methanol or ethanol[3] even when stored at 4°C or even at -20C. However, this decomposition is quite slow. Zopiclone concentration decreased by about 20% over a period of 4 weeks when 1 mg/mL methanolic solution of zopiclone was stored at 4°C. The methanolic eluate did not show any decrease in the peak area of any of the three compounds or show any change in the ratio of peak area of analyte/IS when the extract was stored at room temperature for

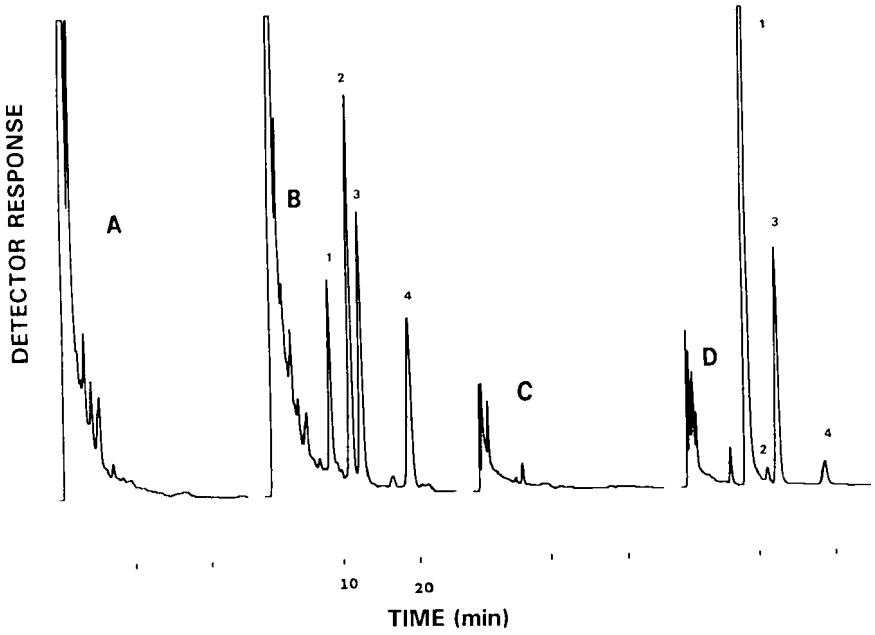

Figure 1. Chromatograms of extracts obtained from (A) drug free urine; urine of a volunteer collected 15 h after the ingestion of a 15-mg dose of zopiclone; (C) drug free serum; (D) serum 1.5 h after zopiclone dose. Peaks: 1 = Harmane (8.3 min.), 2 = N-desmethylzopiclone (10.8 min.), 3 = zopiclone (12.2 min.), 4 = zopiclone-N-oxide (18.1 min.). Detector: signal output 1 V, sensitivity = high, response time = slow. Integrator: attenuation = 2, chart speed 2 mm/min.

5 h or at 4°C for 24 h. There was a 20% decrease in the peak areas of each of three compounds when the extract was kept at 60°C for 1h in a stoppered tube to avoid evaporation. The extraction recovery of zopiclone is reduced to about 70% when the extraction column is eluted with a 0.25 mL aliquot of acetonitrile containing 1% of 35% perchloric acid.

Chromatograms of extracts of drug free urine (Fig. 1A) and of drug free serum (Fig. 1C) show the absence of extraneous peaks from the biological matrices or materials of extraction columns after the solvent peaks.

Method Validation

The relationship between the ratios of peak areas of zopiclone/IS (y) and

Table 1

Precision and Accuracy of the Method

	Zopiclone			N-Desmethylzopiclone			Zopiclpone-N-oxide		
	Mean ng/mL	%CV	%Dev[a]	Mean ng/mL	%CV	%DEV	Mean ng/mL	%CV	%DEV
Within Batch (n=8)									
u-low	120	2.6	-4.0	238	2.9	-4.9	251	2.8	+0.4
u-high	1263	2.2	+1.0	2410	2.2	-3.6	2510	1.2	+0.4
s-low	53	3.3	+6.0		---			---	
s-high	1000	1.4	0.0		---			---	
Between Batch (n=8)									
u-low	117	10.4	-6.4	242	5	-3.2	248	5.3	-0.8
u-high	1168	3.8	-6.6	2285	2.2	-9.4	2439	3.4	-2.4
s-low	51	11.1	+2.0		---			---	
s-high	1016	5.3	+1.6		---			---	

[a]Bias from the spiked value

the serum zopiclone concentration (x) is linear and the curve passes through the origin ($y = -0.01 + 0.883x$, $r^2 = 1.000$). The relationship between the peak area ratios of analyte/IS and urine zopiclone and its metabolite concentrations are also linear and the curves pass through the origin ($y = -0.03 + 0.048x$, $r^2 = 0.998$ for zopiclone; $y = 0.04 + 0.03x$, $r^2 = 0.995$ for N-desmethylzopiclone and $y = -0.031 + 0.054x$, $r^2 = 0.997$ for zopiclone-N-oxide).

Sensitive fluorescence detection and high extraction recovery allow quite low limits of quantitation. Zopiclone and its metabolites can be quantitated down to 2 ng/mL in serum and 10 ng/mL in urine. The sensitivity of detection can be further improved by injecting a larger volume of the extract. There is no distortion of peaks when up to 40 µl of the extract are injected. Analysis of serum spiked with zopiclone and of urine spiked with zopiclone and its metabolites showed acceptable precision (Table 1).

Fig. 1B shows a chromatogram of an extract of a random urine sample

obtained from a volunteer who had ingested a 15-mg dose of zopiclone, 15 hours prior to sample collection. This 64 yr old male volunteer has been on chronic therapy of 20 mg/day of glyburide, 1 g/day of metformin and 325 mg/day of aspirin. The concentration of zopiclone corresponds to 1.2 μg/mL; of N-desmethylzopiclone to 1.3 μg/mL and of zopiclone-N-oxide to 0.84 μg/mL. Fig. 1D shows a chromatogram of an extract of serum obtained from blood collected 1.5 h after the ingestion of zopiclone dose. The concentration of zopiclone corresponds to 57 ng/mL. It is generally believed that zopiclone metabolites are non-detectable in serum after therapeutic or mild overdoses (6). It is interesting that in this case both N-desmethylzopiclone (4 ng/mL) and zopiclone-N-oxide (8 ng/mL) can be observed in serum after only a high therapeutic dose of zopiclone. Renal and liver function tests are normal in this individual.

Specificity

The described procedure has a high specificity due to selective extraction and fluorescence detection in the visible range. Acidic and neutral compounds including barbiturates, salicylates and acetaminophen are removed during wash steps with acetonitrile. However, basic drugs including benzodiazepines, if present are co-extracted. Only a few drugs show fluorescence at the selected excitation and emission wavelengths.

In a number of LC procedures, some basic drugs e.g. morphine, codeine, beta blockers (atenolol, metoprolol, nadolol etc.) and antidepressants (paroxetine, fluoxetine, impramine etc.) have been determined by monitoring their native fluorescence under optimal conditions for the detection of these drugs. However, under the present conditions these drugs show poor response. Foster et al.[11] have used chlordiaepoxide as the IS for the determination of zopiclone enantiomers by LC with fluorescence detection. However, in the present procedure, chlordiazepoxide and other benzodiazepines show very poor fluorescence response. Further, benzodiazepines and antidepressants which are commonly ingested in overdose, elute after zopiclone-N-oxide and do not interfere with the assay of zopiclone and its metabolites.

CONCLUSION

This report describes a simple procedure which is quite suitable for use in routine clinical laboratories for sensitive screening and quantification of

zopiclone and its major metabolites, N-desmethylzopiclone and zopiclone-N-oxide.

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