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IMPROVED QUALITATIVE METHOD FOR ESTABLISHING FLUNITRAZEPAM ABUSE USING URINE SAMPLES AND COLUMN LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

J.P. WEIJERS-EVERHARD*

Laboratorium Jellinek Centrum, Sarphatistraat 75, 1018 EX Amsterdam (The Netherlands)

and

J. WIJKER, R. VERRIJK, H.H. VAN ROOIJ and W. SOUDIJN

University of Amsterdam, Department of Pharmaceutical Chemistry, Plantage Muidergracht 24, 1018 TV Amsterdam (The Netherlands)

SUMMARY

The abuse of flunitrazepam (Rohypnol®), a potent benzodiazepine-type hypnotic, cannot be established with the widely used immunoassays for urine analysis owing to lack of specificity. A simple method is described which is based on the formation of acridine derivatives and measures the sum of metabolites of flunitrazepam in urine samples using column liquid chromatography with fluorimetric detection.

INTRODUCTION

Flunitrazepam (Rohypnol®), 5-(2-fluorophenyl)-1,3-dihydro-1-methyl-7-nitro-2H-1,4-benzodiazepine-2-one (I in Fig. 1), is used parenterally for premedication and induction of anaesthesia (dose 0.015—0.030 mg/kg) and as a potent oral hypnotic (dose 0.5—2 mg). Abuse of various benzodiazepines by drug addicts is a distinct phenomenon that has been described for diazepam (II in Fig. 1) and oxazepam (III in Fig. 1) [1, 2].

Among the available techniques for the determination of flunitrazepam and its metabolites, gas chromatography [3] and column liquid chromatography (LC) with UV detection [4] or fluorimetric detection [5] have found their
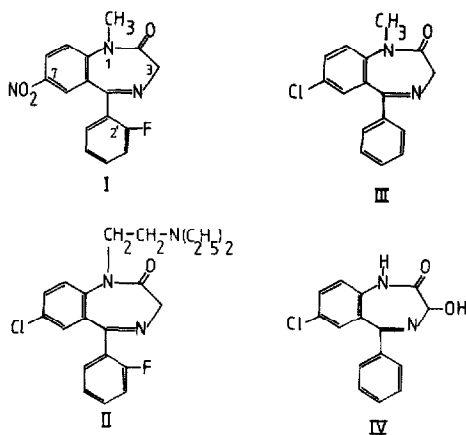
Fig. 1. Structural formulae of flunitrazepam (I), flurazepam (II), diazepam (III) and oxazepam (IV).

application in pharmacokinetic studies using plasma samples. For urine analysis, immunoassays are more suitable, owing to their large sample capacity. However, the available immunoassays for benzodiazepines are unable to discriminate between different benzodiazepines, owing to cross-reactivity of the antibodies used in this type of assay.

Recently, a thin-layer chromatographic method with fluorimetric detection has been described [6], based on the formation of acridine derivatives [7], which can be used for the occasional analysis of flunitrazepam in urine samples. However, for routine analysis of larger amounts of samples, this is definitely not the method of choice.

This paper describes a simple method using LC with fluorimetric detection, also based on the formation of acridine derivatives, which can be used for routine analysis of urine samples from drug addicts and which measures the sum of flunitrazepam metabolites, as flunitrazepam is excreted in the urine in metabolized form [8].

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a high-pressure pump (Varian Type 8500, U.S.A.), a Type Sph125/DDS HPLC autosampler (Kipp & Zonen, Delft, The Netherlands), a column of 316 stainless-steel with dimensions of 100 mm × 4.6 mm I.D., packed with Hypersil ODS (5 μm), and a fluorimeter (Aminco Bowman, Silver Springs, MD, U.S.A.) equipped with a 25-μl through-flow cell (Hellma, The Hague, The Netherlands). The wavelengths were set at 396 nm (excitation) and 445 nm (emission).

Drugs and chemicals

All chemicals were of analytical grade and obtained from Merck (Darmstadt, F.R.G.). Flurazepam and flunitrazepam, as well as their metabolites, their respective benzophenones and chloracridanon (IX in Fig. 1), were kindly donated by Hoffmann-La Roche (Basle, Switzerland).

Procedures

Chromatography. The column was packed with Hypersil ODS (5 µm) using a slurry technique. The mobile phase was methanol—water (55:45) containing 0.050 M acetic acid buffer (pH 4.7) and 0.050 mM tetramethylammonium hydroxide.

Extraction and derivatization. Urine samples were stored at −18°C. Portions (0.5 ml) of thawed urine samples were mixed with 10 M hydrochloric acid (0.5 ml). The solution was heated for 15 min at 100°C. After cooling, the pH was adjusted to 9 with 0.55 ml of 10 M sodium hydroxide and 0.75 ml of 0.05 M borate buffer (pH 9.0). The mixture was extracted twice with 1.2-ml portions of ethyl acetate. The combined organic extracts were evaporated on a water-bath at 50°C under a gentle stream of nitrogen. The residue was dissolved in 0.5 ml of a saturated solution of sodium nitrite in dimethylformamide. The vials were then firmly closed with an aluminium cap and placed in a heating and stirring module (Model 18971, Pierce, Rockford, IL, U.S.A.) for 2 h at 180°C. After cooling, 0.5 ml of water filtered through a Millipore filter was added, and 20-µl aliquots were injected. Peak heights were measured and compared with appropriate standards.

RESULTS AND DISCUSSION

Flunitrazepam is extensively metabolized by reduction of a nitro group to an aromatic amine group, followed by acetylation; by hydroxylation at the 3-position, followed by conjugation with glucuronic acid; and by desmethylation at the N-1 position (Fig. 1) [8]. A total assay of these metabolites is adequate and sufficient to answer the question as to whether flunitrazepam has been ingested. Depending upon the definition of abuse, a cut-off concentration level can be selected.

Hydrolysis of the metabolites to benzophenones [9], followed by cyclization to highly fluorescent acridine derivatives, is a simple method as only two derivatives — compounds V and VI (Fig. 2) [5] — are formed from the many
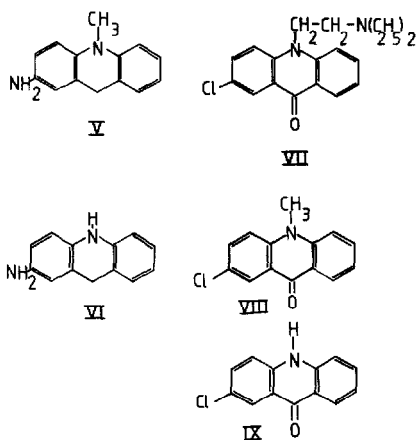

Fig. 2. Structural formulae of acridine derivatives derived from all 7-aminomethyl metabolites of flunitrazepam (V), desmethyl metabolites of flunitrazepam (VI), flurazepam (VII), diazepam (VIII) and oxazepam (IX).

possible metabolites. This simplifies the conclusion — abuse or no abuse — to be drawn from the analytical results.

We found that the formation of highly fluorescent acridine derivatives is easily accomplished with benzodiazepines having a fluoro atom substituted at the 2-position in the phenyl ring. We observed that the fastest rate of formation occurs with flurazepam (II in Fig. 1). Even at temperatures as low as 100°C, the reaction has a high yield. Mass spectrometry showed that compound VII (Fig. 2) is formed.

The cyclization reaction is much slower, however, with the main metabolites of flunitrazepam having an amino group at the 7-position. It was therefore necessary to increase the temperature to 180°C in order to obtain a high yield or sensitivity, as demonstrated in Fig. 3.

When 7-chloro-substituted benzodiazepines were used as starting compounds, a slight formation of acridine derivatives (VIII and IX, Fig. 2) was observed at this temperature. These compounds were formed from benzophenones, obtained after acid hydrolysis of diazepam (III in Fig. 1) and oxazepam (IV in Fig. 1), respectively. No further attempt was made to optimize the reaction conditions under which these compounds could be formed from the chloro-substituted benzodiazepines diazepam and oxazepam.

Fig. 4 shows the chromatographic separation of acridine derivatives V—IX. The mixture dissolved in the derivatization reagent, was diluted with water to obtain a slow-eluting sample liquid. As can be seen from the chromatogram, all derivatives can be separated, which implies that this method can selectively discriminate between the most widely abused benzodiazepines.

The whole procedure was checked for interferences by other drugs frequently encountered, such as morphine, codeine, methadon, barbiturates, cocaine, methaqualon and quinine; no interference was observed. However, carbamazepine (an antiepileptic drug) and its metabolite 10,11-dihydro-10,11-dihydroxycarbamazepine decomposed into two highly fluorescent compounds under these reaction conditions and interfered with the method: this phenomenon probably involves thermal degradation, as has been described
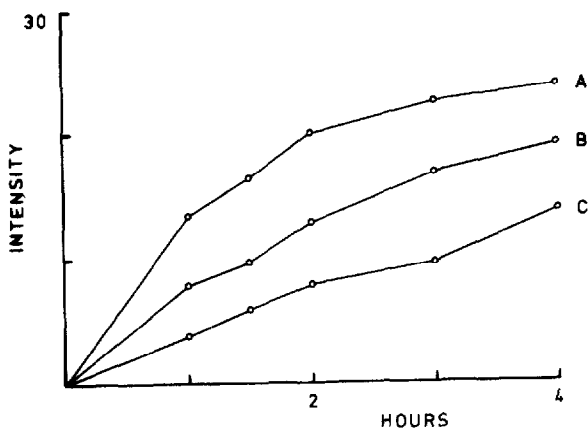

Fig. 3. Yield of the cyclization reaction, expressed as fluorescence intensity, at different temperatures. (A) 180°C; (B) 170°C; (C) 160°C. Starting compound: 2'-fluoro-2,5-diamino-benzophenone.
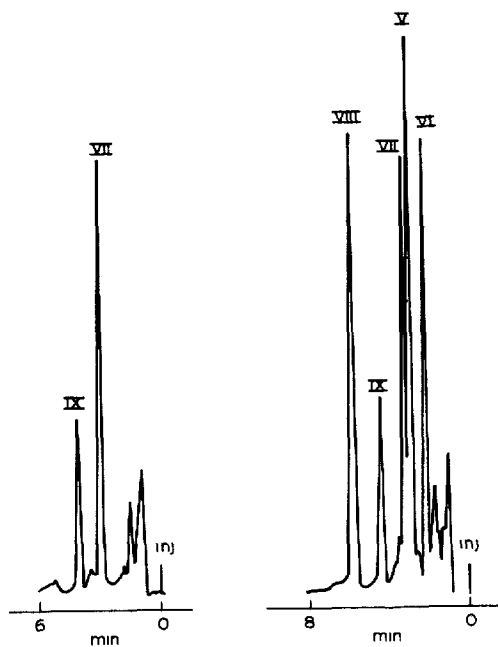
Fig. 4. Chromatogram of a test mixture of acridine derivatives obtained from different benzodiazepines. For meaning of V—IX, see Fig. 2.

when carbamazepine is subjected to gas—liquid chromatographic analysis [10]. Using thin-layer chromatographic systems [6, 7], this interference can be recognized.

Quantitative aspects

Although the method is essentially qualitative, quantitative aspects are important for establishing a cut-off concentration below which abuse cannot be concluded.

Precision and linearity of the chromatographic method were determined by injecting ($n = 5$) 20 μl of solutions of a test compound (IX) and by measuring peak heights. The calibration line was linear over the investigated concentration range (5—2500 ng/ml), with a regression coefficient of 0.9993. The coefficient of variation was found to be 0.99% at 2 ng injected, and 0.37% at 100 ng injected. The precision of the extraction procedure was measured by spiking urine samples ($n = 7$) with 7-aminoflunitrazepam (500 ng/ml) and by measuring peak heights, and was found to be 6.6%. The detection limit, defined as three times the peak-to-peak value of the noise, amounted to 50 pg, calculated as aminoflunitrazepam and aminodesmethylflunitrazepam, respectively. This corresponds to a calculated detection limit of 1.5 ng/ml of urine.

Analysis of urine samples

In the present pilot study, the method was applied to 33 urine samples of drug addicts. A typical chromatogram of a blank urine (a), a positive urine sample (b), and a blank urine to which 7-aminoflunitrazepam has been added (c) is shown in Fig. 5. The concentration of this unknown sample was 2 μg/ml 7-aminoflunitrazepam.
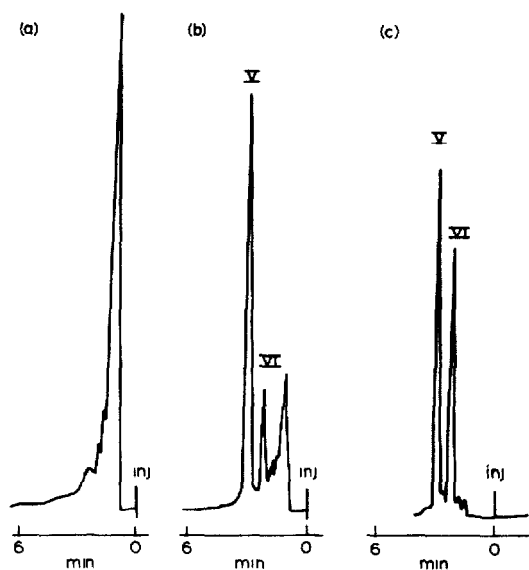
Fig. 5. Typical chromatograms of: (a) a blank urine sample, (b) a positive urine sample, and (c) a standard mixture of acridine derivatives of the amino metabolites of flunitrazepam in urine. For meaning of V and VI, see Fig. 2.

TABLE I

CONCENTRATION OF THE 7-AMINOMETHYL METABOLITES OF FLUNITRAZEPAM IN URINE SAMPLES OF DRUG ADDICTS

Concentration range (ng/ml)	Number of urine samples
0—100	8
101—250	5
251—500	2
501—1000	6
1001—5000	8
>5000	4
Total	33

Table I contains the concentration ranges of the 7-aminomethyl metabolite in these samples. Establishing an appropriate cut-off limit for abuse is a difficult problem, in this case not depending upon the limitations set by the detection limit of the used method.

In different therapeutic settings, e.g. drug-free therapeutic communities or methadone-maintenance programmes, different definitions of abuse prevail. In some cases, the ingestion of even a small dose of the drug has to be established; in other cases, the assessment of the intake of larger doses may be appropriate. Large-scale investigations of urine samples from various populations of drug-addicts, in combination with other clinical data, might solve this problem. This study is now in progress. For the moment we propose an abuse limit of 500 ng/ml, based on the results in Table I.
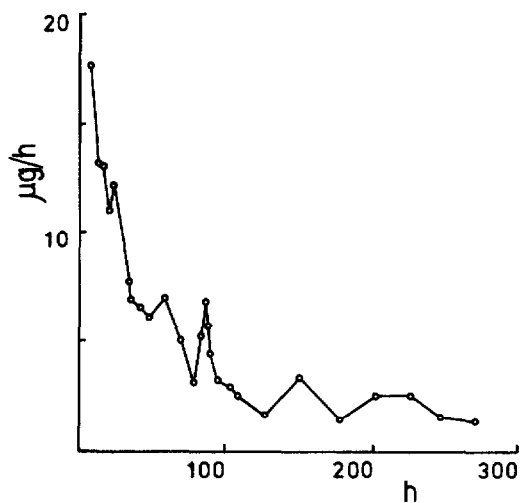
Fig. 6. Urinary excretion rate of the 7-aminomethyl metabolites, expressed as flunitrazepam, as a function of time, after oral administration of 1 mg of flunitrazepam in man.
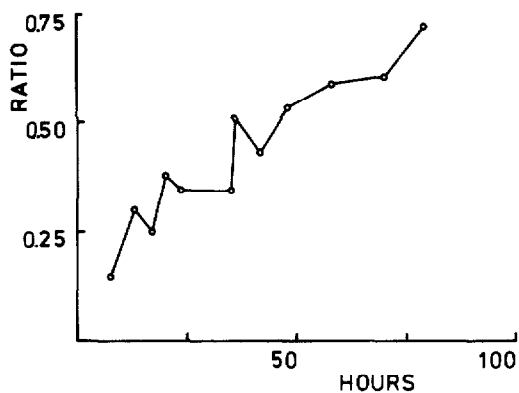
Fig. 7. Ratio of the urinary excretion rates of the 7-aminodesmethyl metabolites and the 7-aminomethyl metabolites of flunitrazepam after oral administration of 1 mg of flunitrazepam in man.
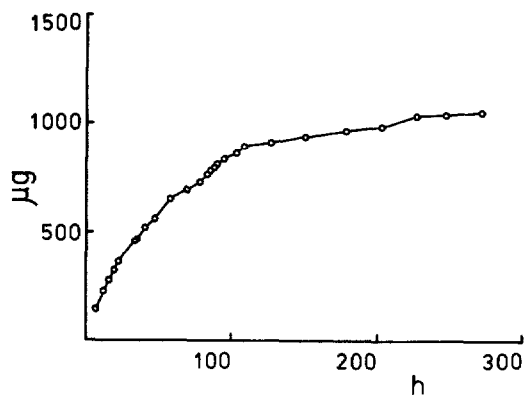
Fig. 8. Cumulative amount of flunitrazepam excreted in urine as amino metabolites in flunitrazepam equivalents after oral administration of 1 mg of flunitrazepam in man.

The results of an investigation of urine samples from a healthy volunteer who received a 1-mg dose of flunitrazepam are shown in Figs. 6—8.

Urine was collected and the urinary excretion rate was calculated from urine flow and urine concentrations of the sum of metabolites containing a methyl group and of desmethylated metabolites. All concentrations are expressed as flunitrazepam equivalents.

Fig. 6 shows the urinary excretion rate of methyl metabolites and Fig. 7 shows the ratio of the urinary excretion rates of desmethyl and methyl metabolites as a function of time. This shows that, in this volunteer, the desmethyl metabolites appear later than the methyl metabolites.

Fig. 8 shows the cumulative amount of flunitrazepam excreted as metabolites, from which it can be concluded that, in this volunteer, flunitrazepam is virtually completely excreted as 7-amino metabolites. Which part of these metabolites is excreted in the form of the acetylamino derivatives was not further investigated.

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