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Quantification of flunitrazepam's oxidative metabolites, 3-hydroxyflunitrazepam and desmethylflunitrazepam, in hepatic microsomal incubations by high-performance liquid chromatography

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Abstract

A high-performance liquid chromatographic assay for the quantification of the oxidative metabolites of flunitrazepam, 3-hydroxyflunitrazepam and desmethylflunitrazepam, in human liver microsomal incubations was developed. Both metabolites were quantifiable in a single assay following a solvent extraction and reversed-phase high-performance liquid chromatography with UV detection. Standard curve concentrations for both metabolites ranged from 0.2 to 10 μ*M*. Assay performance was determined using quality control samples and the intra- and inter-day accuracy and precision as determined by the coefficient of variations which were less than 15% (0.5–6 μ*M*) for both metabolites. This method provides good precision and accuracy for use in kinetic studies of the oxidative metabolism of flunitrazepam in human liver microsomes.

Keywords: Flunitrazepam; 3-Hydroxyflunitrazepam; Desmethylflunitrazepam

1. Introduction

Flunitrazepam (Rohypnol) is a 7-nitrobenzodiazepine that has been used as a hypnotic and for the induction of anaesthesia [1] for over 20 years. It is also a widely abused benzodiazepine [2]. It undergoes oxidative metabolism to 3-hydroxyflunitrazepam and desmethylflunitrazepam (Fig. 1), mediated by the cytochrome P450 (CYP450) mixed-function oxidase system. However it is not known which CYP450 isoforms catalyse these reactions. It also undergoes reductive metabolism to 7-aminoflunitrazepam.

Several methods for the analysis of flunitrazepam and its metabolites have been developed for pharmacokinetic investigations, therapeutic drug monitoring and forensic testing. Cirimele and co-workers developed gas chromatographic methods for the detection of flunitrazepam and its 7-amino metabolite in human hair [3,4]. Although these mass spectrometry assays are sensitive (quantification limit of ~17 pg/mg flunitrazepam and 3 pg/mg 7-aminoflunitrazepam) and selective, they do not detect the oxidative metabolites of flunitrazepam. ElSohly et al. also developed a gas chromatography–mass spectrometry (GC–MS) method for quantification in urine [5]. The disadvantage with this method is that the quantification of individual metabolites is not possible, as the acid hydrolysis of the urine converts related metabolites to a common benzophenone

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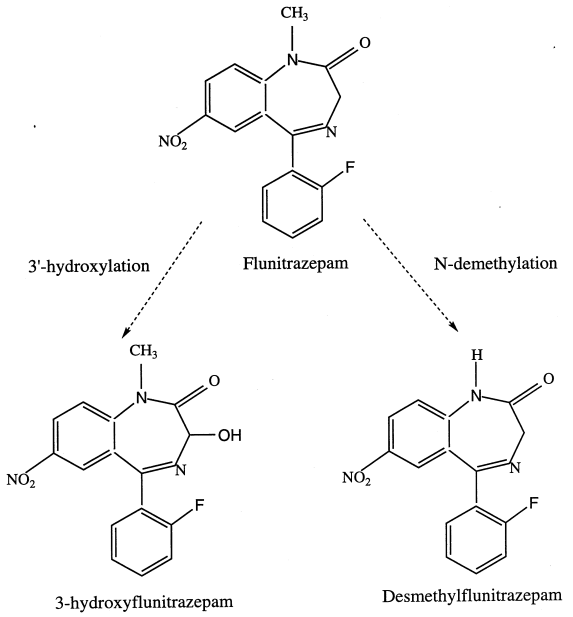

Fig. 1. Oxidative metabolic pathway of flunitrazepam.

which is then quantified by GC–MS. Four individual benzophenones were identified by these authors from: (a) flunitrazepam, 3-hydroxyflunitrazepam and norflunitrazepam; (b) 3-hydroxynorflunitrazepam; (c) 7-aminoflunitrazepam and 7-amino-3-hydroxyflunitrazepam; (d) 7-aminonorflunitrazepam and 7-amino-3-hydroxynorflunitrazepam, respectively. Thus the method can only be used to confirm the presence of flunitrazepam, its metabolites and their 3-hydroxy derivatives collectively.

Immunoassays have also been developed to detect the presence of flunitrazepam and its metabolites in urine [2]. The OnLine and OnTrak immunoassay are used as kits and can detect concentrations as low as 26 ng/ml, however the metabolites are confirmed collectively, preventing the quantification of each metabolite alone.

Robertson and Drummer [6] used reversed-phase high-performance liquid chromatography (HPLC) to quantify flunitrazepam and its 7-amino metabolite in blood, with a limit of quantification of 10 ng/ml. Berthault et al. used a HPLC method which was the first to quantify flunitrazepam and all its known metabolites [7]. The disadvantages of this method were the low extraction efficiency for some metabolites (30%) and the high intra-assay variability (17–27%).

No method has yet been reported for quantification of flunitrazepam and its metabolites in hepatic microsomal incubations. The considerable time and expense associated with GC, and poor reproducibility with previous HPLC methods have led to the development of a method that involves solvent extraction followed by reversed-phase HPLC with UV detection for quantification of the oxidative metabolites of flunitrazepam. The method is suitable for investigation of the CYP450 isoforms involved in flunitrazepam metabolism in human liver microsomes.

2. Experimental

2.1. Chemicals

Flunitrazepam, 3-hydroxyflunitrazepam, desmethylflunitrazepam, and dextromethorphan were supplied by Roche Products (Dee Why, Australia). Lorazepam was a kind donation from Wyeth Labs. (Sydney, Australia). Furafylline was a kind donation from Professor Wolfgang Pfleiderer (Chemogen, University of Konstanz, Germany). Other materials were obtained from the following sources: *S*(+)-mephenytoin from Ultrafine Chemicals (Manchester, UK); omeprazole from Astra Pharmaceuticals (Sydney, Australia); DL-isocitric acid (trisodium salt), diethyldithiocarbamate, isocitrate dehydrogenase (NADP, type IV), quinidine sulfate, sulphaphenazole, and troleandomycin from Sigma (St. Louis, MO, USA); nicotinamide adenine dinucleotide phosphate disodium salt (NADP-Na_2) from Merck (Darmstadt, Germany); diethyl ether, dimethylformamide, sodium dihydrogenorthophosphate monohydrate, and sodium carbonate from Merck (Kilsyth, Australia); acetonitrile and hexane from BDH (Poole, UK). All chemicals and solvents were of analytical or HPLC grade.

Due to the lack of aqueous solubility of flunitrazepam, stock solutions of 100 m*M* (~31 mg/ml) were prepared in 100% dimethylformamide. Lorazepam, 3-hydroxyflunitrazepam and desmethylflunitrazepam were dissolved in 100% methanol,

and 100% dimethylformamide, respectively, for stock solutions.

2.2. Instrumentation

The reversed-phase HPLC system consisted of a pump (Model LC-6A, Shimadzu, Kyoto, Japan), a WISP autoinjector (Model 710B, Waters, Milford, MA, USA), a variable-wavelength UV absorbance detector (Model 875-UV, Jasco, Japan), and a C-R6A chromatopac integrator (Shimadzu). Compounds were detected at a wavelength of 210 nm following separation using a Pellicular ODS C_{18} pre-column (1 cm) and a stainless steel column (15 cm×4.6 mm) packed with C_{18} 5 μm ODS-2 packing material (Spherisorb; Phase Separations, Queensferry, UK). The mobile phase consisted of 26% acetonitrile and 50 m*M* NaH_2PO_4 in water, adjusted to pH 4 with orthophosphoric acid, and was pumped through the system at 1.5 ml/min.

2.3. Microsomal incubation

Microsomal fractions were prepared by differential centrifugation of human liver homogenates and stored at −80°C until used [8]. Ethics approval was obtained from the Royal Adelaide Hospital Research Ethics Committee, and consent for hepatic tissue to be removed given by patients undergoing partial hepatectomy. The oxidative metabolism of flunitrazepam in vitro was investigated via incubation of the substrate (flunitrazepam, 10–600 μ*M*) with microsomal protein (0.2 mg), a NADPH regenerating system and 0.1 *M* sodium phosphate buffer (pH 7.4) in a final incubation volume of 250 μl in 10-ml glass tubes. All incubations including controls contained equivalent amounts of dimethylformamide (1%), the solvent for flunitrazepam. Incubations were carried out in a water bath at 37°C for 30 min and reactions stopped by the addition of 250 μl of 1 *M* sodium carbonate.

2.4. Sample preparation

Fifty microliters of internal standard (lorazepam, 10 μ*M*) and 3 ml of extraction solvent (hexane–diethyl ether, 50:50, v/v) were added to glass tubes containing the incubates and sodium carbonate. Each was mixed on a mechanical vortex for 10 min and then centrifuged for 10 min (1500 *g*). The organic phase was removed using glass pasteur pipettes into clean 5-ml glass tubes and evaporated to dryness at 55°C under vacuum. The residue was resuspended in 250 μl mobile phase and 25 μl injected onto the HPLC system.

2.5. Calibration, precision, accuracy and extraction efficiency

Calibration curves were constructed for 3-hydroxyflunitrazepam and desmethylflunitrazepam, with eight final concentrations ranging from 0.2 to 10 μ*M*. Low, medium and high quality control (QC) samples were also prepared, with final concentrations of 0.5, 2.5 and 6 μ*M* for both compounds. Standards and QC samples containing both metabolites were prepared identically to the microsomal incubations (excluding the addition of flunitrazepam) in 10-ml glass tubes and placed in a water bath at 37°C for 30 min. Following this, 250 μl of 1 *M* sodium carbonate was added and sample preparation as described above was performed.

The robustness of the method was assessed by assaying 15 QC samples (five each of low, medium and high concentrations) and five of the lowest standard (0.2 μ*M*) on a single assay day to determine the intra-day accuracy and precision. Inter-day accuracy and precision were determined by analysis of six QC samples (two each of low, medium and high concentrations) and the lowest standard (0.2 μ*M*) on seven different assay days.

Extraction efficiency was analysed for both metabolites at each QC concentration and for the internal standard (lorazepam). The peak heights of 3-hydroxyflunitrazepam, desmethylflunitrazepam and lorazepam extracted from the QC samples were compared to those obtained by direct injections of solution of these compounds.

Peak heights of each metabolite were converted into peak height ratios using the peak height of the internal standard and, linear regression analysis of peak height ratios against nominal concentrations provided an estimate of slope, intercept and coefficient of determination (r^2).

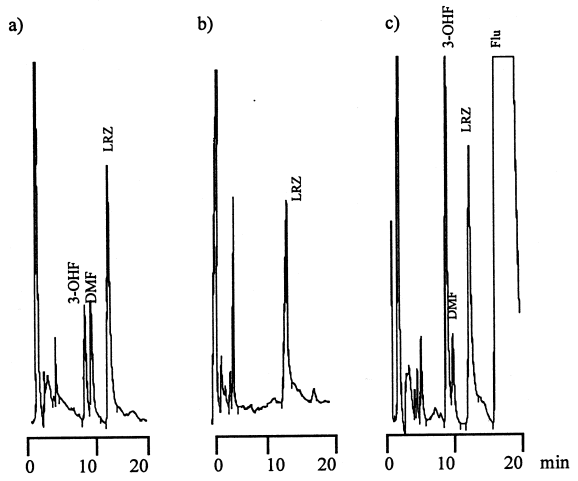

Fig. 2. Representative chromatograms from aqueous extraction of 500 n*M* 3-hydroxyflunitrazepam and desmethylflunitrazepam, and 1.6 μ*M* lorazepam (a); blank microsomal extraction with 1.6 μ*M* lorazepam (b); incubated microsomal extraction with 500 μ*M* flunitrazepam and 1.6 μ*M* lorazepam (c). 3-OHF=3-Hydroxyflunitrazepam, DMF=desmethylflunitrazepam, LRZ=lorazepam, Flu=flunitrazepam.

2.6. Selectivity

The selectivity of the method was assessed based on potential chromatographic interference by other chemicals used to inhibit the oxidative metabolism of flunitrazepam having similar retention times.

3. Results and discussion

Chromatograms resulting from the extraction of a substrate-free microsomal mixture (blank), an incubated low QC sample (0.5 μ*M* 3-hydroxyflunitrazepam, desmethylflunitrazepam and lorazepam), and a microsomal incubation of 500 μ*M* flunitrazepam are shown in Fig. 2. The microsomal incubation resulted in two peaks with identical retention times to 3-hydroxyflunitrazepam and desmethylflunitrazepam in the QC sample. Under the HPLC conditions described, the retention times for 3-hydroxyflunitrazepam, desmethylflunitrazepam,

Table 1
Intra- and inter-day accuracy and precision of the lowest standard and QC samples for 3-hydroxyflunitrazepam and desmethylflunitrazepam

	Actual concentration (n*M*)	Mean calculated concentration (n*M*)	Precision (%)[a]	Accuracy (%)[b]
Intra-day (*n*=5)				
3-OHF	200	180.6	3.3	−9.7
	500	542.1	9.5	+8.4
	2500	2597.9	13.5	+3.9
	6000	6920.7	13.8	+15
DMF	200	199.5	5.9	−0.2
	500	502.6	3.7	+0.5
	2500	2419.9	4.4	−3.2
	6000	5606.4	13.1	−6.5
Inter-day (*n*=7)				
3-OHF	200	205.9	16.4	+2.9
	500	521.8	8.1	+4.3
	2500	2497.2	6.8	−0.1
	6000	6361.7	9.4	+6.0
DMF	200	194.6	2.5	−2.7
	500	508.6	8.3	+1.7
	2500	2541.9	5.6	+1.7
	6000	6262.8	6.2	+4.4

[a] Precision=(S.D./mean)×100.
[b] Accuracy=[(Mean calculated concentration−Actual concentration)/Actual concentration]×100.

lorazepam and flunitrazepam were 8, 9, 13 and 18 min, respectively, with a total run-time of 30 min.

After extraction of the unincubated microsomal mixture spiked with flunitrazepam, a small peak corresponding to the retention time of desmethylflunitrazepam was observed. The identity of this peak has not yet been determined, but has been found to be substrate-concentration dependent and is likely to be a small contaminant of desmethylflunitrazepam in the pure flunitrazepam sample rather than a degradation product of flunitrazepam.

Calibration curves were linear over the concentration range used for 3-hydroxyflunitrazepam and desmethylflunitrazepam with mean r^2 values of 0.994 and 0.996, respectively ($n=6$). The mean ($\pm$S.D.) y-intercepts for 3-hydroxyflunitrazepam and desmethylflunitrazepam were 0.003 (0.0024) and 0.0033 (0.0277), respectively, and the mean ($\pm$S.D.) slopes of the calibration curves over seven independent assay days for 3-hydroxyflunitrazepam and desmethylflunitrazepam were 0.0071 (0.00029) and 0.00089 (0.00029), respectively. The accuracy and precision of QC samples are shown in Table 1. The limit of quantification for both metabolites was 200 n*M* (~66 ng/ml 3-hydroxyflunitrazepam, ~99 ng/ml desmethylflunitrazepam).

Extraction efficiency was analysed at low, medium and high QC concentrations and at the limit of quantification for 3-hydroxyflunitrazepam and desmethylflunitrazepam, and for lorazepam. The percentage extraction efficiencies (mean$\pm$S.D.) for 3-hydroxyflunitrazepam at concentrations of 200 n*M*, 500 n*M*, 2.5 μM, and 6 μM were 79$\pm$2 ($n=3$), 82$\pm$3 ($n=2$), 78$\pm$15 ($n=4$) and 79$\pm$3 ($n=3$), respectively. For desmethylflunitrazepam at identical concentrations the values were 76$\pm$3 ($n=4$), 73$\pm$15 ($n=3$), 72$\pm$4 ($n=4$) and 70$\pm$2 ($n=3$), respectively, and for lorazepam at 1.6 μM percentage extraction efficiency was 96$\pm$2 ($n=14$). These extraction efficiencies are better than those reported by Berthault

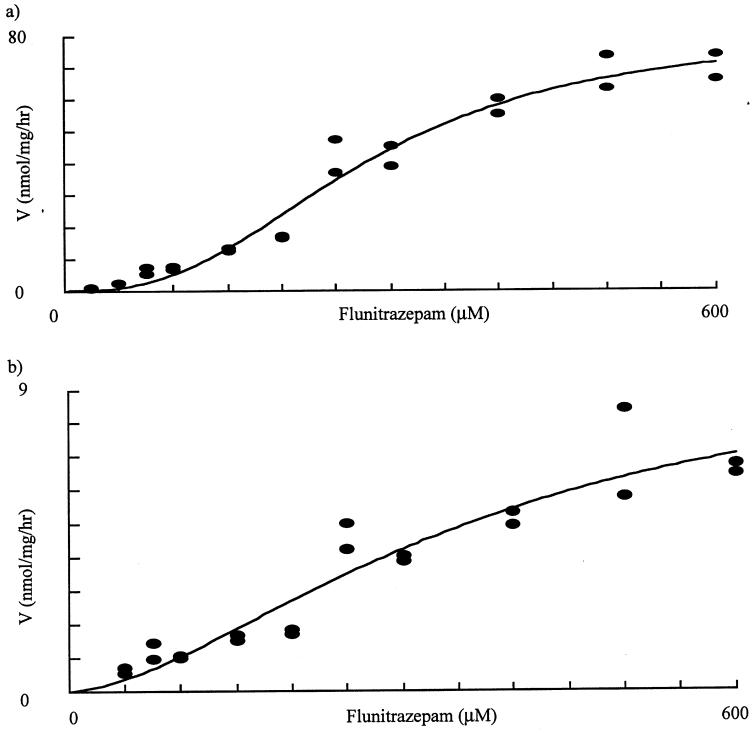

Fig. 3. Rate of formation [V (nmol/mg/h)] of 3-hydroxyflunitrazepam (a) and desmethylflunitrazepam (b) versus substrate (flunitrazepam) concentration in duplicate in human liver microsomes (line represents modelled data derived from the Hill equation).

et al. [7], 57% for desmethylflunitrazepam, who used the extraction solvent of diethyl ether–chloroform (80:20) in their HPLC method.

The applicability of the assay method was used to study the oxidative metabolism of flunitrazepam in a pilot study of one liver. Metabolite formation rate versus substrate concentration curves for 3-hydroxyflunitrazepam and desmethylflunitrazepam are shown in Fig. 3. Kinetic parameters were best predicted with the Hill equation due to non-linear Eadie–Hofstee plots. Additionally it was observed that the following chemicals routinely used to determine the involvement of various CYP450 isoforms did not interfere with the chromatography; diethyldithiocarbamate, furafylline, sulphaphenazole, ketoconazole, troleandomycin, omeprazole, *S*-mephenytoin and dextromethorphan. Quinidine was observed to have a retention time between the two oxidative metabolites and hence interfered with their chromatography.

In summary, we report a reversed-phase HPLC method for the quantification of the two oxidative metabolites of flunitrazepam, 3-hydroxyflunitrazepam and desmethylflunitrazepam. The assay is simple, inexpensive, precise and accurate allowing investigations into identifying the specific cytochrome P450 isoforms involved in the oxidative metabolism of flunitrazepam in human liver microsomes.

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