Radioimmunoassay of Flurazepam in Human Plasma

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Abstract □ A radioimmunoassay for the determination of the hypnotic flurazepam in plasma was developed. Plasma levels of intact flurazepam were measured following oral administration of therapeutic doses to humans. The procedure employs an antiserum obtained from a rabbit immunized with 3-hemisuccinyloxyflurazepam covalently coupled to bovine serum albumin and tritium-labeled flurazepam as the radioligand. Assay specificity was achieved by chromatographic isolation of flurazepam from plasma extracts on Sephadex LH-20 prior to analysis. The method has a sensitivity limit of 0.1 ng of flurazepam/ml using a 1-ml plasma sample, and the intra- and interassay coefficients of variation did not exceed 9% over a range of 0.1–5 ng/ml. In eight subjects who received a single 30-mg dose of flurazepam, peak plasma concentrations of 0.5–3.0 ng of intact drug/ml were reached after 0.5–1 hr, except in one subject where the peak occurred at 4 hr. The plasma concentrations of flurazepam declined with a harmonic mean apparent half-life of 2.3 hr.

Keyphrases □ Flurazepam—radioimmunoassay, plasma levels in humans □ Radioimmunoassay—flurazepam, plasma levels in humans □ Hypnotic agents—flurazepam, radioimmunoassay, human plasma

Flurazepam hydrochloride[1] (Table I) was synthesized by Sternbach *et al.* (1) and now is the most widely prescribed hypnotic agent for the treatment of insomnia in the United States (2). Studies on the biotransformation of flurazepam in humans and dogs have shown that the drug is metabolized extensively (3). The major metabolite in human plasma is *N*-1-desalkylflurazepam (Table I). Intravenous studies (3) of the elimination of flurazepam in dogs showed that the blood levels of the intact drug disappeared rapidly (half-life of 1.4 hr).

BACKGROUND

Kaplan *et al.* (4) used a fluorometric assay to study the blood level profile of the major flurazepam metabolites in humans following chronic dosage. This procedure requires a two-stage derivatization process involving acid hydrolysis of flurazepam and its metabolites to their respective benzophenones followed by cyclization to the 9-acridinone derivatives. The derivatives then are separated by TLC and are analyzed by a spectrofluorometric procedure. However, the levels of the intact drug were below the assay sensitivity and could not be measured.

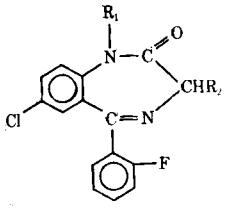
More recently, an electron-capture GLC assay was developed by de Silva *et al.* (5) with sufficient sensitivity to measure the intact *N*-1-desalkyl metabolite. While this method is useful for toxicological work and measurement of the major metabolite present in plasma, it lacks sufficient sensitivity to measure the intact drug after administration of therapeutic doses. Thus, the plasma level profile and the apparent half-life of flurazepam in humans have not been established.

The present report describes the development of a radioimmunoassay to determine the plasma levels of intact flurazepam in humans following a single 30-mg oral dose.

EXPERIMENTAL

Preparation of Immunogen—3-Hydroxyflurazepam (Table I) was refluxed with succinic anhydride in dichloromethane containing triethylamine to yield the desired hapten, 3-hemisuccinyloxyflurazepam (Table I), which was coupled covalently to bovine serum albumin by the mixed anhydride procedure of Erlanger *et al.* (6). The resulting conjugate was dialyzed against 0.05 *M* $NaHCO_3$ followed by dialysis against distilled water and isolated by lyophilization. UV analysis of the immunogen (6) indicated that ~18 moles of the hapten was covalently linked to 1 mole of albumin.

Table I—Flurazepam, the Hapten 3-Hemisuccinyloxyflurazepam, and the Cross-Reactivity of Metabolites Present in Plasma



Compound	R_1	R_2	Cross-Reactivity, %
Flurazepam	$(CH_2)_2N(C_2H_5)_2$	H	100
3-Hydroxyflurazepam	$(CH_2)_2N(C_2H_5)_2$	OH	—
3-Hemisuccinyloxyflurazepam	$(CH_2)_2N(C_2H_5)_2$	$OCO(CH_2)_2COOH$	—
Monodesethylflurazepam	$(CH_2)_2NHC_2H_5$	H	17.0
Didesethylflurazepam	$(CH_2)_2NH_2$	H	3.5
N-1-Hydroxyethylflurazepam	$(CH_2)_2OH$	H	<1.0
N-1-Desalkylflurazepam	H	H	<1.0

Immunization and Antibody Production—A female New Zealand White rabbit was immunized intradermally and boosted intravenously as described previously (7). Serum was collected at monthly intervals and stored at −20°. The antiserum obtained following the second booster immunization was used throughout this study. The antiserum was diluted 1:2000 with assay buffer[2] prior to use.

Extraction of Flurazepam from Plasma—Plasma (1 ml) was buffered to pH 9 with 1 ml of 1 *M* borate buffer and extracted twice with 5 ml of hexane[3] on a reciprocating shaker for 15 min. The combined hexane extracts were evaporated to dryness in a 15-ml conical tube at 40° under nitrogen, and the inside wall of each tube was rinsed down with 1 ml of hexane. Duplicate control plasma samples and internal standards containing 0.125, 0.5, and 1 ng of flurazepam/ml were processed along with the unknowns.

Column Chromatography of Plasma Extracts—Prepacked columns containing 1 g of Sephadex LH-20[4] were washed first with methanol and then equilibrated with hexane–benzene–methanol (95:5:4).

Each plasma extract was dissolved in 0.2 ml of the solvent system, applied to the top of column, and allowed to percolate into the bed. The tube was rinsed with another 0.5-ml portion of the solvent and transferred to the column. The column was developed using the solvent system, and the first 3.5 ml of eluate (which previously was shown to be devoid of flurazepam) was discarded. The next 5 ml of eluate containing the flurazepam was collected in a 20-ml glass scintillation vial and evaporated to dryness under nitrogen at 40°. The residue was dissolved in 0.5 ml of the assay buffer, and the vial was capped and incubated for 1 hr at 37° to obtain maximum dissolution of flurazepam prior to radioimmunoassay.

[1] Flurazepam hydrochloride is the active drug substance in Dalmane. Its chemical structure is 7-chloro-1-[2-(diethylamino)ethyl]-5-(*o*-fluorophenyl)-1,3-dihydro-2*H*-1,4-benzodiazepin-2-one dihydrochloride.

[2] Assay buffer is Dulbecco phosphate-buffered saline (GIBCO, Grand Island, NY 14072).

[3] Hexane is UV grade, Burdick & Jackson Laboratories, Muskegan, Mich.

[4] Isolab, Akron, OH 44321.

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Table II—Plasma Concentrations[a] (Nanograms per Milliliter) of Flurazepam in Eight Human Subjects following a 30-mg Oral Dose

Hour	Subject 1	2	3	4	5	6	7	8
0.0	NM	NM	NM	NM	NM	NM	NM	NM
0.5	1.00	0.30	0.15	1.00	0.14	1.80	0.24	NM
1.0	0.80	1.10	0.80	1.40	0.75	3.00	0.50	NM
							0.30[b]	
2.0	0.70	0.50	0.40	0.75	0.40	0.60	0.24	NM
3.0	0.50	0.40	0.30	0.45	0.30	0.80	0.20	0.35
4.0	0.30	0.25	0.20	0.40	0.20	0.30	0.19	0.61
							0.12[c]	0.27[c]
6.0	0.17	NM	0.10	0.28	0.17	0.20	0.12	0.27
								0.14[d]
8.0	NM	NM	NM	0.16	NM	0.16	NM	NM

[a] The reported concentrations are based on duplicate analyses of each plasma sample. NM = not measurable. [b] At 1.5 hr. [c] At 5 hr. [d] At 7 hr.

Radioimmunoassay—A calibration curve was generated by adding [^{3}H]flurazepam[5] (15,000 dpm) in 0.1 ml of buffer to duplicate 12 × 75-mm disposable glass culture tubes containing 0.03–2 ng of flurazepam in 0.2 ml of buffer. Following preparation of the standards, duplicate 0.2-ml aliquots of the reconstituted unknown flurazepam fractions were added to tubes containing 0.1 ml of the [^{3}H]flurazepam. Then the diluted antiserum (0.2 ml) was added to all tubes, except the nonspecific-binding control specimen to which was added 0.4 ml of buffer.

Each tube was mixed gently on a vortex mixer and allowed to stand at room temperature for 1 hr. Following the incubation, the antibody-bound radioligand was separated from the unbound fraction by precipitation with saturated ammonium sulfate (7). After the pellet was dissolved in 0.4 ml of water and 3 ml of scintillator[6] to give a clear solution, radioactivity in each tube was quantified in a modified liquid scintillation counter as described previously (8).

The calculation of the unknown flurazepam levels was performed by interpolation from a logit–log standard curve.

Determination of Assay Blank—One-milliliter aliquots of pooled normal human control plasma were extracted and chromatographed as described previously on eight separate columns. A separate control column was developed in the absence of any plasma extract. The fraction containing flurazepam eluate from each column was reconstituted with 0.5 ml of buffer and then analyzed by the radioimmunoassay.

Recovery of Flurazepam from Human Plasma—Various concentrations (0.125–5 ng) of unlabeled flurazepam were added to the control human plasma, incubated for 30 min at 37° and then stored at −20° until they were assayed. Then 1 ml of each plasma standard was extracted and chromatographed as described previously and analyzed by the radioimmunoassay.

RESULTS AND DISCUSSION

Radioimmunoassay Characteristics—The antiserum bound 50% of the radioligand at a final dilution of 1:5000 in a 0.5-ml incubation volume. A logit–log plot of B/B_0 (percent) *versus* concentration of unlabeled flurazepam gave a linear response between 0.03 and 2 ng, where B and B_0 are the amounts of antibody-bound radioligand in the presence and absence of the unlabeled drug, respectively, following correction for nonspecific binding. A typical calibration curve had a slope of −0.98 with a correlation coefficient of 0.99. Repeated analyses of the 0.03-ng standard gave a B/B_0 value of 87%; the working sensitivity of the procedure was ~0.1 ng of flurazepam/ml using a 1-ml plasma sample.

Blank values for eight 1-ml aliquots of control plasma following the extraction and chromatographic procedures were well below (mean B/B_0 = 95 ± 2%) the detection limit while the recovery of internal standards over the range of 0.1–5 ng of flurazepam/ml averaged 82.5 ± 3%.

The intra- and interassay coefficients of variation were determined by repeated analysis of six randomly selected plasma samples from subjects who had received a single 30-mg oral dose of flurazepam over a 6-week period. The coefficients of variation did not exceed 9% over a range of 0.1–1.6 ng of flurazepam/ml.

[5] [^{3}H]Flurazepam, with a specific activity of 27 Ci/mmole, was stored as the free base in toluene at −20°.

[6] Aquasol, New England Nuclear, Boston, MA 02118.

Radioimmunoassay Specificity—The specificity of the antiserum initially was evaluated by cross-reactivity studies involving all of the flurazepam metabolites known to be present in plasma (3). The mono- and didesethyl metabolites exhibited a cross-reactivity of 17 and 3.7%, respectively, while the other possible competitors cross-reacted less than 1% (Table I). As the large substituent at the N-1 position of flurazepam was progressively metabolized to the N-desalkyl metabolite, cross-reactivity decreased, indicating that the substituent at N-1 on the hapten is a major antigenic determinant.

In view of the cross-reactivity of both the mono- and didesethyl metabolites, a specific assay for flurazepam in plasma could not be developed without first separating it from its metabolites. Therefore, column chromatography on Sephadex LH-20 was employed. The elution profile of flurazepam was determined as described previously. Under these conditions, the plasma metabolites are not eluted from the column, thus providing a rapid and reproducible procedure for separating flurazepam from its interfering metabolites.

Plasma Flurazepam Levels in Humans—The method was applied to the analysis of plasma samples from eight subjects who received single 30-mg doses of the drug. Peak plasma concentrations of intact flurazepam were reached after 0.5–1 hr in seven subjects and at 4 hr in one subject (Table II). Although the data could not be used for detailed pharmacokinetic analysis, the intact drug had a harmonic mean apparent half-life in plasma of 2.3 hr.

This study demonstrates that, although orally administered flurazepam undergoes extensive first-pass metabolism to yield N-1-desalkylflurazepam as its major metabolite in plasma, some intact flurazepam does reach the peripheral circulation. The extent to which intact flurazepam contributes to the hypnotic activity of the drug is unknown.

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