Assay of Adinazolam in Plasma by Liquid Chromatography

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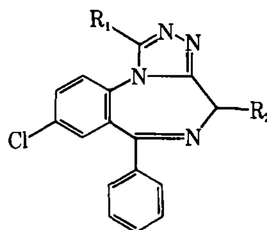
Abstract □ A procedure for the quantitative determination of adinazolam in plasma was developed. The drug, an *N*-demethylated metabolite, and an internal standard were extracted from basified plasma into ethyl acetate. After evaporation, the residue was dissolved in toluene which was washed with sodium hydroxide. The toluene was evaporated and the residue was dissolved in a mixture of acetonitrile, methanol, and water for chromatography. The concentrations of the drug and the metabolite were determined using reverse-phase liquid chromatography with UV detection at 254 nm. The assay methodology showed good peak height ratio-concentration linearity, precision, and accuracy and has been used to analyze plasma samples collected from human subjects after oral administration of adinazolam mesylate in compressed tablets. The low plasma background interferences allowed the quantitative determination of concentrations as low as ~5 ng/mL.

Keyphrases □ Adinazolam and *N*-demethyl metabolite—liquid chromatographic assay in plasma □ Liquid chromatographic assay—adinazolam and *N*-demethyl metabolite in plasma

A number of compounds of the triazolobenzodiazepine class have been found to have important pharmacological properties (1, 2), *i.e.*, the antianxiety activity of alprazolam (3) and the hypnotic activity of triazolam (4) and estazolam (1, 5). More recently, adinazolam mesylate, the methanesulfonate salt of 8-chloro-1-[(dimethylamino)methyl]-6-phenyl-4*H*-*s*-triazolo[4,3-*a*][1,4]benzodiazepine (I), was shown to have both antidepressant and antianxiety properties (6–8).

Adinazolam mesylate is currently undergoing clinical testing for the treatment of depression in humans (9). Since little is known about the pharmacokinetic disposition of adinazolam, such an understanding would facilitate the design and evaluation of clinical efficacy studies. For this purpose, a liquid chromatographic method was developed for the separation and quantitative determination of adinazolam in human plasma samples. 8-Chloro-1-[(methylamino)-methyl]-6-phenyl-4*H*-*s*-triazolo[4,3-*a*][1,4]benzodiazepine (II) (8), an *N*-demethylated metabolite of adinazolam, found in abundant amounts in the plasma samples, were simultaneously analyzed.



I: $R_1 = CH_2N(CH_3)_2$, $R_2 = H$
II: $R_1 = CH_2NHCH_3$, $R_2 = H$
III: $R_1 = CH_2CH_3$, $R_2 = CH_2N(CH_3)_2$

EXPERIMENTAL SECTION

Materials—Adinazolam (I) mesylate, the *N*-demethyl metabolite (II), and the internal standard, 8-chloro-1-ethyl-4[(dimethylamino)methyl]-6-phenyl-4*H*-*s*-triazolo[4,3-*a*][1,4]benzodiazepine (III) (6) were used as received[1]. Acetonitrile and methanol were distilled-in-glass grade[2]. All other solvents and chemicals were reagent grade. Plasma samples were obtained from volunteer human subjects after oral administration of compressed tablets of adinazolam mesylate.

Sample Preparation—Aliquots of 1-mL plasma samples in 15-mL culture tubes equipped with polytef-lined screw caps[3] were supplemented with internal standard, basified with 1 mL of 0.1 M dibasic potassium phosphate[4], and extracted with 5 mL of ethyl acetate[2]. After shaking on a horizontal shaker for ~5 min and centrifuging at 2000 rpm for 5 min, these tubes were placed in a shallow bath of dry ice–acetone mixture to freeze the aqueous phase. The ethyl acetate was transferred to clean tubes and evaporated under a stream of nitrogen[5]. The residues were taken up in toluene[2], (2-mL aliquots) which were in turn washed with 2 mL of 2.0 M NaOH by shaking and centrifugation. After freezing the aqueous layer, the toluene extracts were transferred to clean tubes and evaporated under nitrogen at a temperature <50°C. The residues from the toluene extracts were dissolved in 0.1 mL of acetonitrile–methanol–water (1:1:2, v/v/v), mixed on a vortex mixer[3], and centrifuged prior to chromatography. Calibration standard samples were prepared by supplementing aliquots of blank human plasma with the drug, its metabolite, and the internal standard. These standard samples were similarly extracted and concurrently chromatographed with the plasma samples.

Chromatography—The liquid chromatographic system consisted of a solvent delivery pump[6], a fixed-wavelength UV absorbance detector[7], and a sample injection valve with a 100-µL loop[8]. The prepared plasma samples and the standard samples were chromatographed on a prepacked reverse-phase column[9] using acetonitrile–methanol–phosphate buffer (30:20:85, v/v/v) as the mobile phase. The phosphate buffer contained 0.06 M monobasic ammonium phosphate and 0.02% phosphoric acid. The mobile phase was filtered, deaerated, and pumped through the system at a flow rate of 2 mL/min. The chromatograms monitored by UV absorbance at 254 nm were recorded on a strip chart recorder[10]. The adinazolam–internal standard and *N*-demethyl metabolite–internal standard peak height ratios were measured for quantitative determination. The chromatography was carried out at ambient temperature (20–22°C).

RESULTS AND DISCUSSION

The recovery of adinazolam from the plasma samples by the extraction procedure was investigated by comparison of the chromatograms of the drug standards and the chromatograms of the extracts of plasma samples supplemented with the same quantities of adinazolam mesylate. The internal standard was added to these samples just prior to chromatography, and the peak height ratios measured from the resulting chromatograms were used to estimate the extraction recovery. The solvent extraction recovery of adinazolam from plasma was found to be ~82% in the concentration range of ~10–800 ng/mL of adinazolam mesylate and was essentially independent of the concentration. The extraction of the *N*-demethyl metabolite appeared to be less efficient than the parent drug because of the higher polarity of the metabolite. The extraction procedure was satisfactory for the preparation of the plasma samples for the chromatographic separation of the drug and the metabolite. Ethyl acetate extraction gave very good recovery of the drug and the metabolite from the plasma samples, but the high background interferences rendered the chromatographic separation unsuitable for quantitative determination of these compounds. Toluene extraction gave poor recovery of the metabolite, but the background interference was minimal. The two-step extractions using ethyl acetate and toluene were necessary to remove background interferences and to allow the quantitative determination of the drug and the metabolite at ~5 ng/mL levels.

Figure 1 shows the chromatograms of the extracts of plasma samples collected shortly before (Fig. 1A) and 4 h after (Fig. 1B) the administration of

[1] The Upjohn Company, Kalamazoo, Mich.
[2] Burdick and Jackson Laboratory, Muskegon, Mich.
[3] Fisher Scientific Co., Fair Lawn, N.J.
[4] Mallinckrodt, Inc., St. Louis, Mo.
[5] Multivap; Organomation Associates, Inc., Northborough, Mass.
[6] Model 6000A; Waters Associates, Milford, Mass.
[7] Model 440; Waters Associates, Milford, Mass.
[8] Valco Instruments Co., Inc., Houston, Tex.
[9] µ-Bondapak C_{18}; Waters Associates, Milford, Mass.
[10] Linear Instruments Corp., Irvine, Calif.

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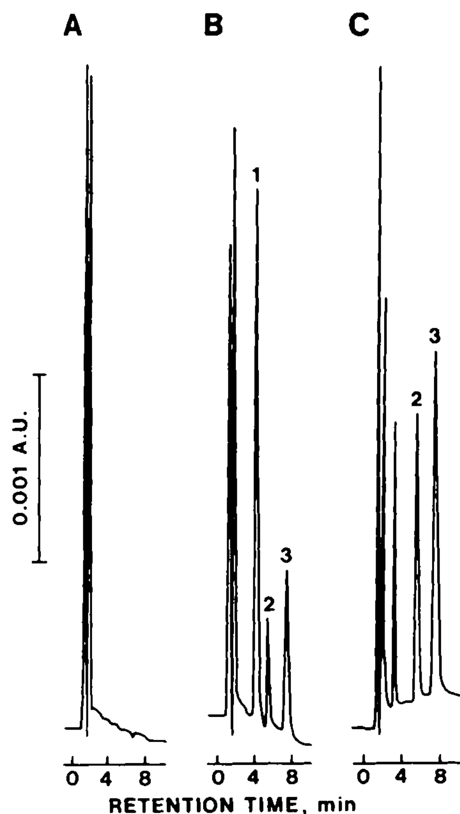

Figure 1—*Chromatograms of the extracts of plasma samples collected from a human subject shortly before (A) and 4 h after (B) the administration of a 20-mg dose of adinazolam mesylate in tablets, and the chromatogram of the extract of a blank plasma sample supplemented with adinazolam mesylate and internal standard (C). Key: (1)* N*-demethyl metabolite; (2) adinazolam; (3) internal standard.*

adinazolam mesylate. Both samples were obtained from the same human subject who received orally a single 20-mg dose of the drug in compressed tablets. Clinical studies showed that ≤30 mg of adinazolam mesylate three times a day for a total daily dose of 90 mg was efficacious in treatment of depression (9). Figure 1 also shows a chromatogram of the extract of a sample

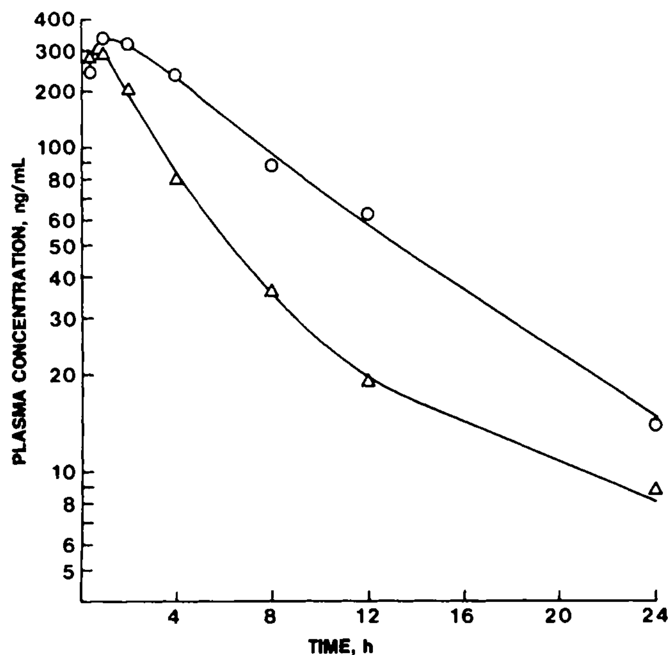

Figure 2—*Plasma concentration-time profiles of adinazolam (△) and the* N*-demethyl metabolite (○) after administration of a 30-mg dose of adinazolam mesylate in tablets to a human subject.*

of blank plasma with added adinazolam mesylate and the internal standard (Fig. 1C). Samples of blank plasma from several sources showed minimal interferences during the chromatographic separation. The chromatographic separation eluted adinazolam and the internal standard with retention volumes of ~11.2 and 16 mL, respectively. In the post-medication samples, an additional drug-related peak with a retention volume of ~8.8 mL was found (Fig. 1C). This peak was later identified as the *N*-demethyl metabolite of the parent drug. No other material could be identified as drug related in the chromatograms under the experimental conditions. The retention volumes of these sample components varied slightly from day-to-day and were especially sensitive to the changes in the proportions of acetonitrile, the concentrations of ammonium phosphate, and the pH of the buffer in the mobile phase. These compounds were more retentive on the column[9] using mobile phase with a lower proportion of organic solvents, a lower concentration of ammonium phosphate in the buffer, or a higher pH of the buffer. This knowledge was used to optimize the chromatographic separation by adjusting the mobile phase compositions. The limitations of these modifications were the excessive peak tailing and the prolongation of the retention times.

Like other benzodiazepines, the triazolobenzodiazepines undergo reversible ring-opening reactions in acidic media involving the hydrolysis of the azomethine linkage. These reversible reactions have been reported for triazolam (10) and estazolam (11). Adinazolam also showed similar ring-opening reactions in acidic solutions. At the pH of the buffer for the chromatographic mobile phase (pH 2.5-4.0), and in the mobile phase containing organic solvents, adinazolam was hydrolyzed to its ring-open form to a large extent. However, chromatographic separation using the acidic mobile phase indicated that essentially no ring-opening reaction took place by eluting adinazolam through the reverse-phase column as monitored by the UV detector. Under the same conditions, when an acidic solution of adinazolam was chromatographed, the intact drug and its ring-open form were separated: the ring-open form was much less retentive on the column than the intact drug. The reason for the discrepancy between the apparent stability of adinazolam during the acidic reverse-phase column separation and its instability in acidic solution was uncertain. It is possible that with the acid-catalyzed hydrolysis of the azomethine linkage, the environment inside the column was different from a simple acidic solution of a mixture of the buffer and organic solvents.

The adinazolam-internal standard and the *N*-demethylated metabolite-internal standard peak-height ratios showed good linear relationships with their corresponding concentrations. The linear regression of the peak height ratio-concentration data of both compounds usually resulted in calibration curves with correlation coefficients >0.998 and with positive or negative intercepts statistically zero, judging from 95% confidence limits. The slopes of the calibration curves were quite reproducible from day-to-day. For example, 12 sets of the calibration curves of adinazolam prepared over a period of three weeks showed a *RSD* of 4.3%, and eight sets of the calibration curves of the metabolite prepared over the same period showed a *RSD* of 9.7%. These data indicated the good interassay reproducibility for these compounds. The intra-assay reproducilibity and the accuracy of this methodology were also very good; *i.e.*, in the analyses of aliquots of blank plasma samples supplemented with 12, 74.5, 165, and 462 ng/mL of adinazolam mesylate, the concentrations found were 11.9 ± 0.38, 73.6 ± 1.0, 164.6 ± 17.24, and 462.5 ± 2.33 ng/mL, respectively. The relative *SD* of these replicate assays (N = 4) were from 0.5 to 10.5%.

The assay methodology has been used to analyze the plasma samples collected from human subjects after oral administration of adinazolam in compressed tablets. The plasma concentration of the parent drug reached its peak usually at ~1 h postdose and then declined following a biexponential pattern. In the same sample, the concentration of the *N*-demethyl metabolite was higher than the parent drug at most sampling times. A representative set of data, demonstrating the utility of the assay methodology, is shown in Fig. 2.

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Potential Antitumor Agents X: Synthesis and Antitumor Activity of Two Nitrogen Mustard Derivatives Related to Ketocaine

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Received July 19, 1982, from the **Istituto di Chimica Farmaceutica e Tossicologica, University of Bologna* and the *‡Istituto di Farmacologia, University of Milan, Italy.* Accepted for publication May 23, 1983.

Abstract □ The synthesis of two nitrogen mustard derivatives (VIII and IX) related to the well-known local anesthetic ketocaine (III) is reported. These compounds were tested in mice implanted with Ehrlich ascites tumor cells, and the antitumor activity was compared with that of two previously synthesized analogues (I and II) lacking the nitro group and with that of doxorubicin. The monofunctional compound IX was inactive, but the bifunctional compound VIII showed potent antitumor activity (%T/C > 254 at 20 mg/kg).

Keyphrases □ Antitumor agents—potential, nitrogen mustard derivatives, Ehrlich ascites carcinoma screen □ Nitrogen mustard derivatives—antitumor agents, potential, Ehrlich ascites carcinoma screen

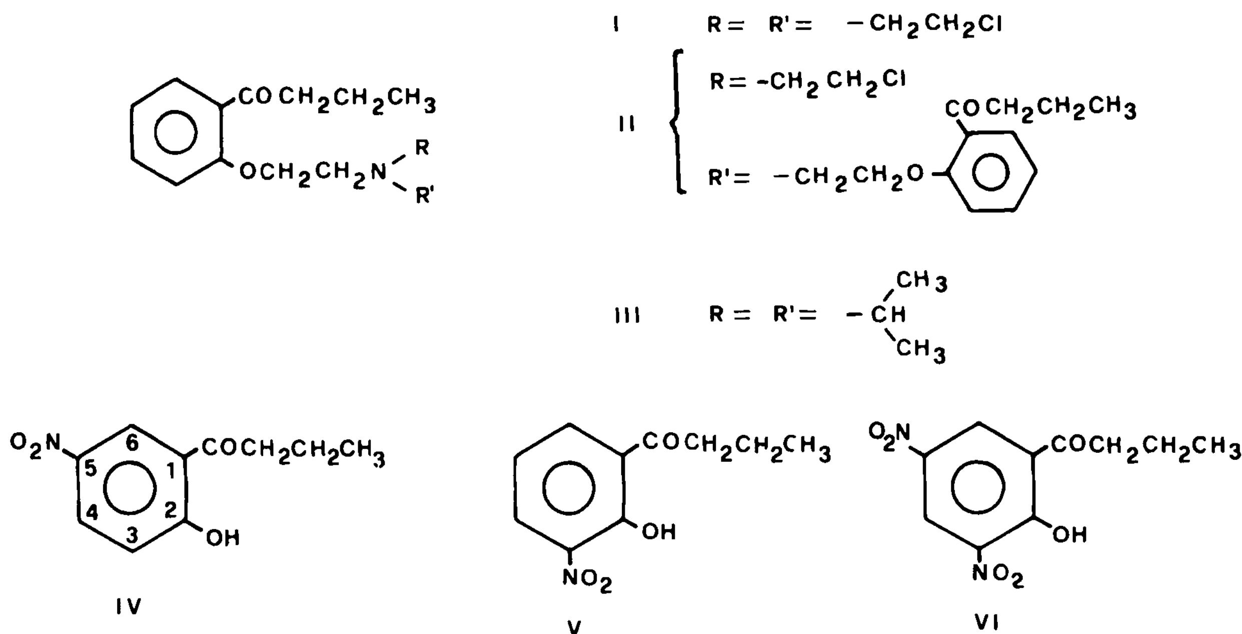
In a previous paper of this series (1), we reported the synthesis of I and II, two analogues of the well-known local anesthetic ketocaine (III). This work was based on the hypothesis that the *in vitro* antimitotic activity of ketocaine is related to its effect on oxygen consumption by tissues with prevailing anaerobic metabolism; this, in turn, demonstrates the ability of ketocaine to pass through the cell membrane (2). While ketocaine does not show antitumor activity, I and II were active (1). On the other hand, a series of butyrophenone derivatives showed that ketocaine and one of its nitro derivatives had analogous behavior to oxygen consumption (3). This study describes the nitration of *o*-hydroxybutyrophenone and the subsequent alkylation of the nitro derivative with tris(2-chloroethyl)amine hydrochloride, as previously described (1). The antitumor activity and the effect on oxygen consumption of these substances were compared with those of the previously described I and II.

RESULTS AND DISCUSSION

The nitration of *o*-hydroxybutyrophenone always gave a mixture of IV–VI. These materials were separated by column chromatography and identified by means of spectrometric data (see *Experimental Section*). The reaction between the nitro derivative (IV) and tris(2-chloroethyl)amine hydrochloride (VII) gave VIII and IX, as well as X (Scheme I). Compounds VIII and IX were tested, as the hydrochlorides, for antitumor activity.

The bifunctional compound VIII inhibited tumor growth and prolonged the life span of mice bearing Ehrlich ascites carcinoma beyond that of the untreated animals. The %T/C at 4 mg/kg ip was 144, and it was >254 at 20 mg/kg. The monofunctional compound IX was inactive. Table I reports the antitumor activity of VIII and IX compared with that of the previously reported I and II (1) and doxorubicin.

Compounds I, II, VIII, and IX also affected the oxygen consumption of tumor cells. Figure 1 shows a difference in the respiratory inhibition that may be related to the different lipophilicity of the four compounds: in fact respiratory inhibition is a linear function of lipophilicity in a series of 4-hydroxyquinoline-3-carboxylic acids (4). The potent antitumor activity of VIII under the experimental conditions employed is evident. From the results so far available it appears that VIII is also active against other experimental tumors.



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