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GAS CHROMATOGRAPHIC DETERMINATION OF GUANADREL IN PLASMA AND URINE

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SUMMARY

To evaluate the pharmacokinetics and drug availability from various dosage formulations, a method for the determination of guanadrel, (1,4-dioxaspiro[4,5]dec-2-ylmethyl)guanidine, in plasma and urine was required. A gas chromatographic procedure, based on formation of a hexafluoroacetylacetone derivative in a two-phase system of water and toluene, was developed. The limit of determination of the method is 5 ng/ml guanadrel in plasma and 15 ng/ml guanadrel in urine. Statistical analyses indicate average recoveries of 98.1 ± 18.0 and 104.4 ± 15.6% from plasma and urine, respectively. Mass spectrometric analyses, in conjunction with gas chromatography, confirmed the specificity of the method for intact drug. The procedure was applied successfully to drug absorption studies in humans.

INTRODUCTION

Guanadrel sulfate [(1,4-dioxaspiro[4,5]dec-2-ylmethyl)guanidine sulfate; Hylorel®*] (I, Fig. 1) is an orally active guanidine antihypertensive agent in animals and humans [1–9]. To study the absorption and excretion of this agent in humans at relatively low doses (i.e., 50 mg), a sensitive and specific method for drug analysis in plasma and urine was needed.

The utility of hexafluoroacetylacetone to form volatile pyrimidines from guanidino compounds has been demonstrated [8–11]. Quantification of the resulting pyrimidines was achieved using electron-capture detection (ECD) or mass spectrometric (MS) detection. Since it was anticipated that submicrogram sensitivity was needed for measurement of intact I in plasma, a method utilizing gas chromatography (GC) in conjunction with ECD was developed.

*Hylorel®, registered trademark of Upjohn, Kalamazoo, MI, U.S.A. for guanadrel sulfate.

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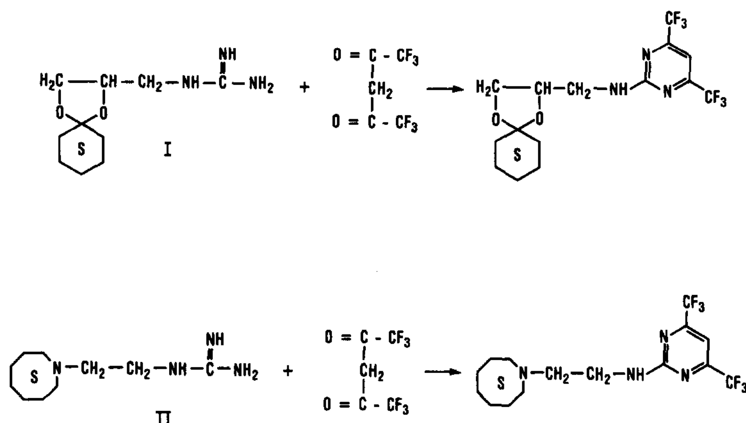

Fig. 1. Reaction scheme for the formation of hexafluoroacetylacetone derivatives of substituted guanidine bases: I = guanadrel free base; II = guanethidine free base.

EXPERIMENTAL

Reagents and materials

The guanadrel used in this study was synthesized within the Research Division (Upjohn, Kalamazoo, MI, U.S.A.) and used without further purification. Guanethidine sulfate (II, Fig. 1), selected as internal standard, was kindly supplied by Ciba-Geigy (Summit, NJ, U.S.A.). Toluene, chloroform, ethyl acetate and methanol were used as supplied (Burdick and Jackson Labs; Muskegon, MI, U.S.A.). Hexafluoroacetylacetone was obtained from Pierce (Rockford, IL, U.S.A.). Stock solutions of aqueous sodium bicarbonate (1 *M*) and aqueous sodium hydroxide (10 and 3 *M*) were stored in glass containers. Separate stock solutions of I and II (100 μg free base equivalents per ml) were prepared by dissolving the sulfate salts in water. All additional dilutions were prepared in methanol. Phenyl methyl silicone fluid (OV-17) on 60–80 mesh Gas Chrom Q (Applied Science Labs., State College, PA, U.S.A. and thin-layer chromatography (TLC) plates coated with a 250-μm layer of silica gel F-254 (EM Labs., Elmsford, NY, U.S.A.) were used as supplied.

Instrumentation

A two-speed reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) was used for sample extraction in the horizontal position. GC measurements were made with a Tracor Model MT-222 chromatograph equipped with a ^{63}Ni electron-capture detector (Tracor, Austin, TX, U.S.A.), a free standing Tracor Model 114556B electron-capture linearizer (Tracor) and a Varian Model A-25 1-mV single-pen recorder (Varian Aerograph, Walnut Creek, CA, U.S.A.). All cylinders of gases used for chromatography [i.e., methane (5%, v/v) in argon] were fitted with filters containing 4-Å molecular sieves. Hexafluoroacetylacetone derivatives of I and II were characterized with an LKB 9000 mass spectrometer (LKB Produkter, Stockholm, Bromma, Sweden) and FTS-14 infrared spectrometer (Digilab, Cambridge, MA, U.S.A.).

Thin-layer chromatography

All TLC was conducted on silica gel F-254; the plates were developed ascendingly in ethyl acetate. The separated materials were visualized by irradiation of the plates with a short-wavelength (254 nm) UV lamp. Under these conditions, the hexafluoroacetylacetone derivatives of I and II had R_F values of 0.90 and 0.65, respectively.

GC conditions

All chromatography was conducted using U-shaped glass columns (1.83 m×3 mm I.D.) packed with 3% (w/w) OV-17 on 60–80 mesh Gas Chrom Q. All newly prepared columns were preconditioned at 250°C for 1 h without carrier gas flow and for 16 h with a carrier gas flow-rate of 10 ml/min. During analysis, the column, injection port and electron-capture detector were maintained isothermally at 125, 225, and 260°C, respectively. Flow-rate of the methane (5%, v/v) in argon, used as carrier gas, was maintained at 60 ml/min. Under these conditions, the hexafluoroacetylacetone derivatives of I and II have retention times of 10.2 and 6.3 min, respectively (Figs. 2 and 3).

Synthesis of standard materials

Place 250 mg of I in a 50-ml screw-cap centrifuge tube and add 5 ml of 3 *M* sodium hydroxide and extract twice with 20 ml chloroform. Pool the extracts and evaporate with nitrogen gas. Add 20 ml toluene and 2 ml hexafluoroacetylacetone and reflux at 100°C for 2 h. Cool the tube to room temperature and extract twice with 10 ml of 3 *M* sodium hydroxide. Transfer the toluene to a clean tube and evaporate with nitrogen gas to a yellow oil. Reconstitute the oil in 5 ml ethyl acetate and chromatograph on a silica gel column using ethyl acetate as mobile phase. Monitor the effluent using TLC (vide infra), pool the fractions with R_F values of 0.90 and evaporate the solvent with nitrogen gas.

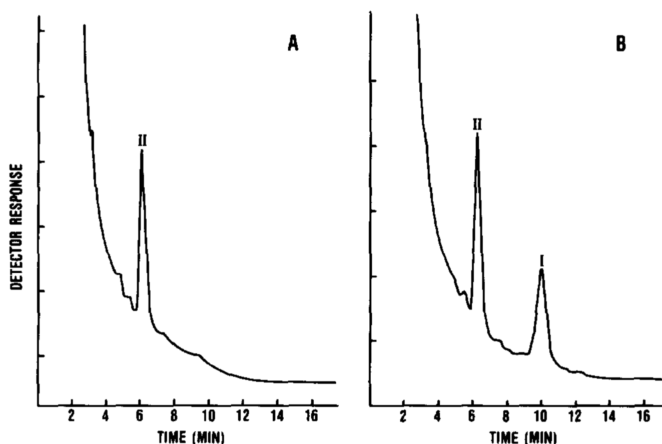

Fig. 2. Gas chromatograms of extracts of (A) blank human plasma containing the hexafluoroacetylacetone derivative of guanethidine (II) and (B) human plasma 3 h after a 50-mg oral dose of guanadrel sulfate, containing the hexafluoroacetylacetone derivatives of guanadrel (I) and guanethidine (II).

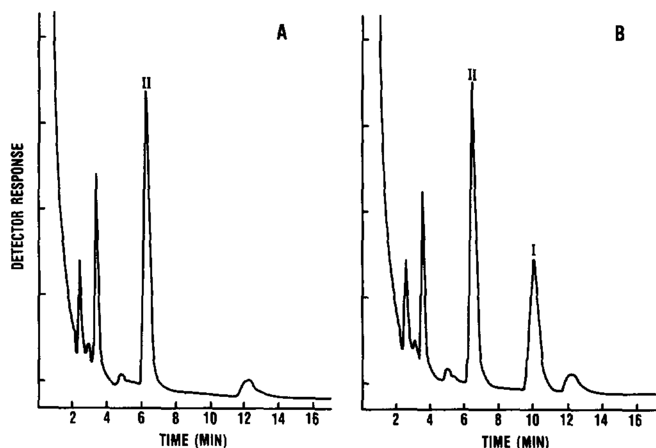

Fig. 3. Gas chromatograms of extracts of (A) blank human urine containing the hexafluoroacetylacetone derivative of guanethidine (II) and (B) human urine during 0–4 h time interval after a 50-mg oral dose of guanadrel sulfate, containing the hexafluoroacetylacetone derivative of guanadrel (I) and guanethidine (II).

Synthesis of the II hexafluoroacetylacetone derivatives uses the same general procedure as described above.

Assay procedure

Preparation of plasma standards. Pipet aliquots of the I methanol stock solution, equivalent to 10, 20, 40, 60, 80 and 100 ng, into screw-capped (PTFE-lined) centrifuge tubes (Bellco Glass, Vineland, NJ, U.S.A.). Evaporate to dryness with a gentle stream of nitrogen gas. Add 1 ml of control plasma to each centrifuge tube and mix well. Prepare an appropriate blank. Extract and react all standards with hexafluoroacetylacetone in the same manner as described for the plasma specimens.

Preparation of plasma specimens. Pipet aliquots (1 ml) of the plasma specimens into screw-capped (PTFE-lined) centrifuge tubes. Add 1 ml of 10 *M* sodium hydroxide and 12 ml of chloroform. Shake in a horizontal position for 20 min. Centrifuge for 10 min at 1000 *g*. Remove the aqueous phase by aspiration. Use a thin glass rod or pipette tip to push aside the protein plug and insert a 10-ml pipette into the chloroform phase. Transfer a 10-ml aliquot of the chloroform layer to a fresh centrifuge tube. Evaporate to dryness with a gentle stream of nitrogen gas. Rinse down the sides of the centrifuge tubes with 1 ml chloroform and evaporate to dryness. Add an aliquot of the internal standard stock solution (i.e., equivalent to 45 ng of guanethidine free base) and evaporate to dryness. Add 150 μl of 1 *M* aqueous sodium bicarbonate solution, 0.5 ml of toluene, 50 μl of hexafluoroacetylacetone and two glass boiling chips (BDH, Poole, U.K.). Screw centrifuge caps tightly and heat for 2 h at 100°C. Cool to room temperature. Add 5 ml of 3 *M* sodium hydroxide and shake in a horizontal position for 10 min. Centrifuge for 10 min at 1000 *g*. Inject a 1–5 μl aliquot of the toluene layer into the chromatograph for analysis.

Preparation of urine standards. Pipet aliquots of the I methanol stock solution, equivalent to 50, 100, 200, 300, 400, 600, 800 and 1000 ng, into screw-capped (PTFE-lined) centrifuge tubes. Add an aliquot of the internal standard stock solution (i.e., equivalent to 1000 ng of guanethidine free base) and evaporate to dryness with a gentle stream of nitrogen gas. Add 0.1 ml of control urine and mix well. Prepare an appropriate blank. React all standards with hexafluoroacetylacetone in the same manner as described for the urine specimens.

Preparation of urine specimens. Pipet aliquots of the II methanol stock solution, equivalent to 1000 ng, into a series of centrifuge tubes and evaporate to dryness with a gentle stream of nitrogen gas. Add 0.1 ml of the urine specimens, two glass boiling chips and mix well. Add 50 μl of 1 *M* aqueous sodium bicarbonate, 0.5 ml of toluene and 50 μl of hexafluoroacetylacetone. Screw centrifuge caps tightly and heat for 2 h at 100°C. Cool to room temperature. Add 5 ml of 3 *M* aqueous sodium hydroxide and shake in a horizontal position for 10 min. Centrifuge for 10 min at 1000 *g*. Inject a 1–5 μl aliquot of the toluene layer into the chromatograph for analysis.

Calculations

Calibration curves for known concentrations of I in plasma or urine are prepared by plotting peak height ratios (I/II) versus free base concentrations, expressed as ng/ml of plasma or urine. Values for unknown concentrations of I in plasma or urine specimens, obtained in the same manner, are then read directly from the graphs or calculated from the slopes of the standard curves.

Drug administration to humans

Informed written consent was obtained from each of six normal human male volunteers prior to participation in this study. All subjects were between the ages of 25 and 33 years and ranged in body weight from 67.1 to 79.4 kg and in height from 1.664 to 1.791 m. All were fasted for 12 h prior to drug administration and for 4 h afterwards.

Each volunteer received 50 mg of I as two 25-mg compressed tablets along with 240 ml water. Blood specimens (10 ml) were withdrawn in heparinized syringes at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0 and 8.0 h post-drug administration. All urine was collected quantitatively during the following time intervals: 0–4, 4–8, 8–12, 12–24, 24–36, 36–48, 48–60 and 60–72 h. All plasma and urine specimens were frozen immediately and stored at −18°C.

RESULTS AND DISCUSSION

Synthesis and characterization of hexafluoroacetylacetone derivatives of guanadrel and guanethidine

Utility of hexafluoroacetylacetone derivatives, for measuring submicrogram amounts of selected guanidino compounds in biological matrices, has been described [8–11]. The reaction scheme for the derivatization of I and II is shown in Fig. 1. Optimal reaction temperatures and times for derivative formation were studied using known amounts of I and II. In studies with I, known amounts of

preformed II-hexafluoroacetylacetone derivative were used as internal standard. Similarly, in studies with II, known amounts of preformed I-hexafluoroacetylacetone derivative were added as internal standard. The results indicated that derivative formation for both compounds was completed at 100 °C (Fig. 4) within 2 h (Fig. 5). Addition of glass boiling chips to the reaction mixture circumvented problems with occasional bumping at the 100 °C reaction temperature.

Varying the amount of hexafluoroacetylacetone in the reaction mixture from 25 to 150 μl had little effect on formation of the II derivative, whereas the optimum amount for the formation of the I derivative was between 25 and 50 μl (Fig. 6). Formation of the II derivative, but not the I derivative, was observed when cyclohexane or acetonitrile was substituted for toluene as the organic solvent. The chromatographic background was superior using 3 *M* as compared to 0.1, 0.3 or 1 *M* aqueous sodium hydroxide.

Synthesis of standard material showed that the hexafluoroacetylacetone deriv-

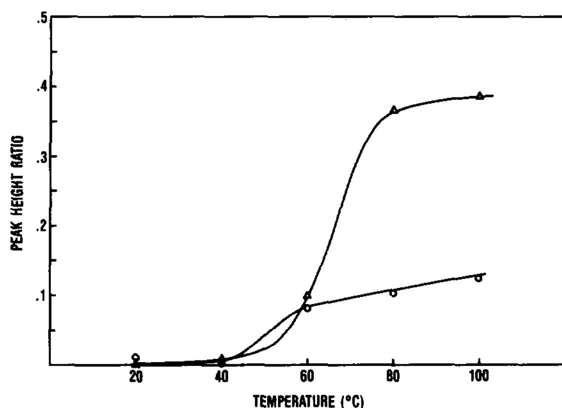

Fig. 4. Effect of reaction temperature on formation of hexafluoroaetylacetone derivatives of guanadrel (△) and guanethidine (○). Reaction time, 2 h.

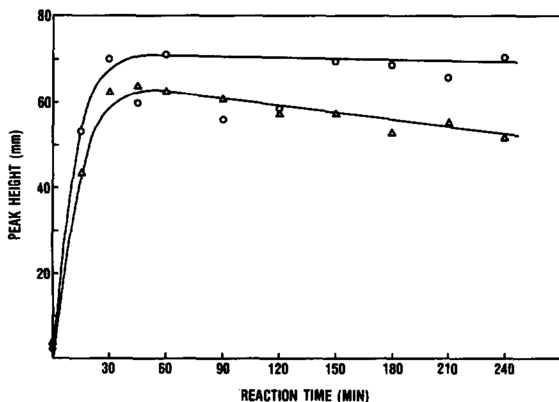

Fig. 5. Effect of reaction time on formation of hexafluoroacetylacetone derivatives of guanadrel (△) and guanethidine (○). Reaction temperature, 100 °C.

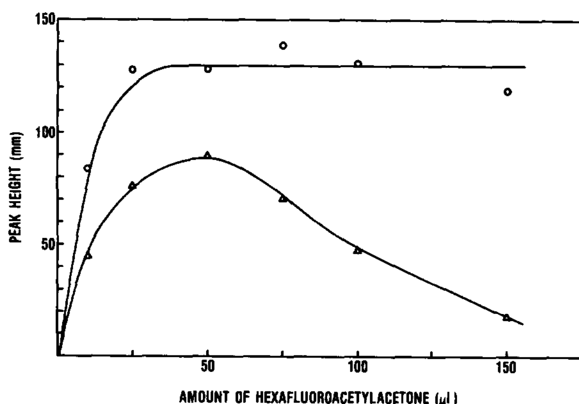

Fig. 6. Effect of hexafluoroacetylacetone volume on derivative formation of guanadrel (△, 50 ng) and guanethidine (○, 75 ng). Reaction temperature, 100 °C, time, 2 h.

ative of I was a pale yellow, almost colorless, oil. TLC and GC analyses showed that the material was a single component. Infrared (IR) and MS analyses supported the proposed structure. The hexafluoroacetylacetone derivative of II was isolated as a pale yellow oil. TLC and GC analyses showed that the material was a single component. IR and MS analyses supported the proposed structure.

MS analyses, before and after GC, confirmed that the hexafluoroacetylacetone derivatives of I and II chromatographed as the intact molecules.

Limit of determination

At an attenuator setting of 4 on the electron-capture linearizer, 0.03 ng of I as its hexafluoroacetylacetone derivative produced a full scale response. However, under the assay conditions, the lower limit of detection sensitivity for I in plasma is 5 ng/ml of the original sample aliquot. This value is based on a sample signal greater than three times the signal-to-noise ratio. Under the assay conditions described, a linear relationship between detector response and concentration is obtained for I over the range of zero to at least 100 ng/ml. For the analysis of guanadrel in human urine, the limit of determination is 15 ng/ml.

Assay recovery and reproducibility

Known amounts of I and II were evaporated in centrifuge tubes and urine or plasma added. The samples were thoroughly mixed and reacted with hexafluoroacetylacetone. The recovery of guanadrel in plasma was measured from 0 to 100 ng/ml (seven steps) and the recovery from urine was measured from 0 to 1000 ng/ml (nine steps), both on each of six days. The overall recoveries (mean ± S.D.) for plasma ($n=33$) and urine ($n=48$) were 98.1 ± 18.0 and 104.4 ± 15.6%, respectively.

Plasma levels of guanadrel in humans

Results from the measurements of plasma I concentrations in six normal human male volunteers after single-dose oral administration of 50 mg I as compressed tablets demonstrated the utility of the analytical methodology (Table I).

TABLE I

PLASMA CONCENTRATIONS AND URINARY ELIMINATION OF GUANADREL FREE BASE IN NORMAL MALE HUMAN VOLUNTEERS FOLLOWING ORAL ADMINISTRATION OF 50 mg GUANADREL SULFATE AS COMPRESSED TABLETS

Volunteer No.	1	2	3	4	5	6	Mean ± S.D.
Body mass (kg)	71.82	75.00	79.55	71.82	68.18	67.27	72.27 ± 4.53
Guanadrel sulfate dose (mg/kg)	0.696	0.667	0.629	0.696	0.733	0.743	0.694 ± 0.042
Time (h)	Urine elimination (mg)*						
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Mean ± S.D.
0–4	4.851	3.033	3.728	8.964	7.814	11.878	6.711 ± 3.43
4–8	2.253	6.078	4.844	2.108	0.323	1.117	2.787 ± 2.22
8–12	2.588	1.807	1.942	0.700	1.108	0.870	1.503 ± 0.73
12–24	2.478	0.935	0.818	0.433	1.343	1.001	1.168 ± 0.71
24–36	1.571	1.298	0.576	0.735	0.681	0.335	0.866 ± 0.47
36–48	0.760	0.650	0.433	0.297	0.266	0.539	0.557 ± 0.17
48–60	0.441	0.334	0.258	0.199	0.490	0.063	0.298 ± 0.16
60–72	0.064	0.168	0.112	0.052	0.178	0.116	0.115 ± 0.05
Total	15.013	14.303	12.711	13.488	12.203	15.919	13.94 ± 1.41
% of dose	30.0	28.6	25.4	27.0	24.4	31.8	27.9 ± 2.8
Time (h)	Plasma concentration (ng/ml)*						
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	Mean ± S.D.
0	0	0	0	0	0	0	–
0.25	0	0	0	0	0	0	–
0.50	0	2.7	4.2	8.8	71.0	38.8	20.9 ± 28.4
0.75	4.3	13.6	14.2	50.2	132.9	35.6	41.8 ± 47.7
1.0	15.3	51.4	55.2	66.6	80.7	101.5	61.8 ± 29.2
1.5	92.9	123.4	56.2	66.6	81.4	117.0	89.6 ± 26.9
2.0	105.3	35.9	49.2	76.7	59.8	171.8	83.1 ± 49.6
2.5	27.4	51.8	46.2	69.7	63.0	112.0	61.7 ± 28.7
3.0	98.3	52.0	30.4	57.5	65.1	124.9	71.4 ± 34.3
4.0	79.2	59.1	36.6	44.2	43.7	88.7	58.6 ± 21.2
6.0	52.7	38.1	16.5	23.7	57.4	48.8	39.5 ± 16.5
8.0	32.2	34.4	6.7	25.5	31.6	40.1	28.4 ± 11.6
$AUC_{0\to 8\,h}$**	449.7	367.2	218.5	328.6	444.1	621.5	404.9 ± 135.9

*All values expressed as free base equivalents.
**Area under the plasma level curve calculated using the trapezoidal rule.

Peak plasma I concentrations, ranging from 56.2 to 171.8 ng/ml, were observed between 0.75 to 2.0 h after drug administration, indicating rapid drug absorption from the gut. At 8 h, plasma I concentrations ranged from 6.7 to 40.1 ng/ml. With the limited data available, between 2 and 8 h post-drug administration, the plasma half-life was estimated graphically to range from 2.08 to 4.90 h.

Urinary excretion of guanadrel in humans

Results from the measurement of intact drug eliminated in urine of normal human male volunteers after single-dose oral administration of 50 mg I demonstrated the utility of the analytical methodology (Table I). During the 0–72 h time interval post-drug administration, 27.88 ± 2.82% of the dose administered was excreted in the urine as intact I. Of this amount, greater than 90% was eliminated during the first 36 h. The combined results from these investigations showed that the GC method could be used for (a) evaluating the pharmacokinetics, (b) evaluating drug availability from various dosage formulations and (c) selecting an optimum dosage regimen for guanadrel sulfate administration to humans.

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