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RAPID, SENSITIVE AND SPECIFIC ELECTRON CAPTURE—GAS CHROMATOGRAPHIC METHOD FOR THE QUANTITATION OF 6-CHLORO-2-(1-PIPERAZINYL)PYRAZINE IN BIOLOGICAL FLUIDS

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SUMMARY

A highly specific and sensitive gas chromatographic method for the determination of 6-chloro-2-(1-piperazinyl)pyrazine (MK-212), a central serotonin-like agent, in biological fluids is described. MK-212 and a related internal standard are extracted into benzene from an alkaline solution, back-extracted into acid and then re-extracted into benzene at an alkaline pH. The amines are converted to the trifluoroacetyl derivatives (characterized by gas—liquid chromatography—mass spectrometry), chromatographed and detected with a ^{63}Ni electron capture detector. The sensitivity of the method is such that 10 ng of drug can be measured per aliquot of biological fluid. The precision and accuracy of the method are well within acceptable limits. Specificity of analysis was established by gas—liquid chromatography—mass spectrometry techniques.

INTRODUCTION

Recently, MK-212 was shown to exhibit central serotonin-like activity. The compound, when administered systemically, was shown to elicit four distinct responses characteristic of serotonin-receptor activation in the nervous system [1]. Complete abolition of these responses to MK-212 was achieved by pretreatment of the animals with a centrally acting indoleamine antagonist, whereas a peripherally acting antagonist was ineffective [1]. The anorexigenic and ancillary actions of MK-212 have also been described [2]. The synthesis of a series of 2-(1-piperazinyl)pyrazines, including MK-212, has recently been reported [3]. These authors also discussed the use of molecular orbital calculations and computer graphics to gain insight into the structural features of MK-212 which might interact with serotonic receptors.

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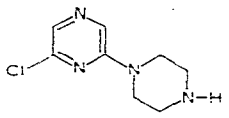
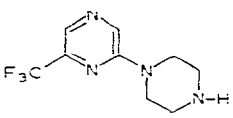
This report describes a highly specific and sensitive gas—liquid chromatographic (GLC) method for the determination of MK-212 using the internal standard, 6-trifluoromethyl-2-(1-piperazinyl)pyrazine (I), derivatization with trifluoroacetic anhydride, and detection using a ^{63}Ni electron capture detector.



MK-212



I

Analysis of biological specimens from rats, dogs and monkeys were utilized to demonstrate the applicability of the assay procedure following pharmacological doses of MK-212.

EXPERIMENTAL

Reagents and Chemicals

The reagents and chemicals used were: 6-chloro-2-(1-piperazinyl)pyrazine (MK-212), the internal standard 6-trifluoromethyl-2-(1-piperazinyl)pyrazine (I), pesticide grade benzene, 2.5% solution of trifluoroacetic anhydride (Aldrich, Milwaukee, Wisc., U.S.A.) in benzene, 0.2 *M* phosphate—NaOH buffer (pH 9) and 5% aqueous ammonium hydroxide. The amines were supplied as the hydrochloride salts; however, all concentrations are expressed in terms of the free bases. Standard solutions of MK-212 were prepared in water and were diluted to final concentrations of 0.125—2 μg/ml. The internal standard solution was diluted to a final concentration of 5 μg/ml.

Instrumentation

GLC. Analyses during the past year were performed on either a Packard gas chromatograph (Model 7400) equipped with a ^{63}Ni electron capture detector (ECD) and a glass column (1.2 m × 4 mm I.D.) packed with 3% OV-210 on Gas-Chrom Q (100—120 mesh) or a Hewlett-Packard gas chromatograph (Model 5830A) with a ^{63}Ni detector and a similar column. The instruments were operated isothermally with a carrier gas (helium, Packard; argon—methane, Hewlett-Packard) flow-rate of 50 ml/min. The injection port and detector temperatures were 20—30° higher than the column temperature (190—205°).

GLC—mass spectrometry (MS). All mass spectra were obtained on an LKB-9000S mass spectrometer using a GLC inlet system. A 1.2 m × 3 mm I.D. glass column packed with 3% OV-210 was used. The gas chromatograph was operated isothermally at 200° with a helium flow-rate of 30 ml/min. The mass spectrometer ionizing and accelerating potentials were 70 eV and 3.5 KV, respectively. The source, separator and injection port temperatures were 270°, 250° and 245°, respectively.

Measurement of MK-212 in biological samples

The concentration of MK-212 in biological fluids is determined as follows. To an appropriate aliquot of plasma, urine or brain homogenate in a 50-ml glass-stoppered centrifuge tube are added 100 μl of I (500 ng), 1 ml of 0.2 *M*

buffer (pH 9) and 15 ml of benzene. The tube is shaken for 10 min, centrifuged and the organic phase transferred to a clean 25-ml glass-stoppered tube containing 1 ml of trifluoroacetic anhydride (TFAA) reagent. The contents of the tube are heated for 30 min at 65° in a water bath. The tube is cooled and the contents are shaken with 1 ml of water (vortex, 0.1 min) and 1 ml of 5% NH_4-OH. Following centrifugation, the benzene phase is transferred to a clean test tube and the contents evaporated under a gentle stream of nitrogen to about 1 ml. An appropriate aliquot (usually 2 or 5 μl) is injected into the gas chromatograph. The retention times of MK-212 and I as the trifluoroacetyl derivatives were 4.4 and 2.4 min, respectively.

Plasma, urine or brain homogenates spiked with known quantities of MK-212 (12.5—2000 ng) and I (500 ng) are analyzed concurrently with each set of unknown samples. A standard curve is prepared for each series of analyses by plotting the peak height ratio (MK-212/I) vs. the weight ratios of MK-212 to I. Concentrations of MK-212 in the unknown samples are obtained by reference of the particular peak height ratio obtained to the standard curve.

Biological studies

Three rhesus monkeys (3.0—4.4 kg) were administered MK-212 intravenously at a dose of 2.5 mg/kg, and three additional rhesus monkeys (4.0—5.2 kg) were dosed orally (2.5 mg/kg). Male Sprague-Dawley rats received the drug (2.5 mg/kg) either orally (21 rats) or intravenously (24 rats). Six beagle dogs received the drug at the same dose. The animals were fasted overnight prior to MK-212 administration. Blood specimens were collected in heparinized tubes, plasma was separated by centrifugation and appropriate aliquots removed for analysis. Urine specimens were immediately frozen upon collection and remained frozen until analyzed.

RESULTS AND DISCUSSION

During the development of MK-212 for potential use as a serotonin-like agent, a method was needed to determine blood and urinary levels of the compound for absorption, excretion and metabolism studies. Preliminary data in rats using radiolabeled material indicated that low levels (ng range) of MK-212 would be encountered following a pharmacological dose. Since electron-capture techniques have been utilized by a number of investigators [4—8] to enhance the sensitivity of detection, this approach was explored for the analysis of MK-212. The use of trifluoroacetic anhydride as the derivatizing agent provided the required sensitivity. The derivatization procedure is similar to that reported previously [4]. A number of related 2-(1-piperazinyl)pyrazines were examined for possible use as an internal standard. The choice of I was based upon its chromatographic behavior (as the trifluoroacetyl derivative) relative to the MK-212 derivative. The trifluoroacetyl derivatives provided good chromatographic properties on the OV-210 column for these compounds. The formation of each trifluoroacetyl derivative was confirmed by combined GLC—MS.

Fig. 1 presents gas chromatograms of the following samples: (a) control dog plasma, (b) control dog plasma to which 500 ng of MK-212 and 500 ng of I were added, and (c) dog plasma (0.5 ml) obtained 30 min after oral dosing (I was added as described in method). Fig. 2 presents gas chromatograms of: (a)

control dog urine to which 500 ng of MK-212 and I was added and carried through the method, and (b) 0—24 hr dog urine to which 500 ng of I was added. All samples were carried through the described procedure. As stated previously, the trifluoroacetyl derivatives of MK-212 and I exhibited retention times of 4.4 and 2.4 min, respectively. No interfering peaks were obtained with control plasma or urine.

A summary of the recovery results obtained following analysis of various added amounts of MK-212 to control plasma and urine from rat, dog or monkey is presented in Table I. All analyses were performed using the aforementioned instruments with electron-capture detectors over 1 year. In the 12.5—2000 ng range, the mean recovery of MK-212 from control plasma was 99.6%; the recovery from urine was 99.6%. The standard deviations within each set of analysis are listed in Table I. As is evident, the GLC method for MK-212 in

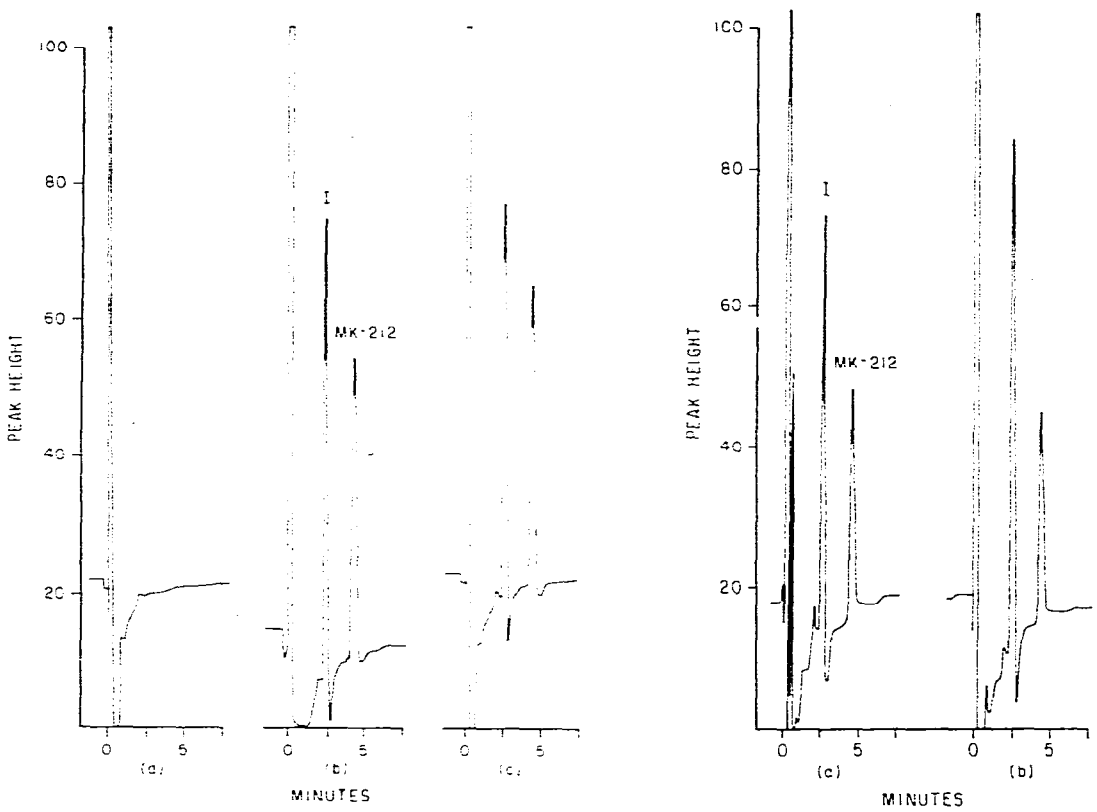


Fig. 1. Gas chromatograms of: (a) control dog plasma carried through extraction procedure, (b) 500 ng each of MK-212 and I added to control dog plasma and carried through procedure, and (c) material obtained from dog plasma following administration of MK-212.

Fig. 2. Gas chromatograms of: (a) control dog urine ti which 500 ng of MK-212 and I was added prior to extraction procedure and (b) material isolated from 0-24 h dog urine following administration of MK-212 (500 ng of I added to urine prior to analysis).

biological fluids is extremely sensitive and the accuracy and precision of the method are well within acceptable limits. Typical levels of MK-212 obtained following the administration of MK-212 to rats and monkeys are presented in Tables II and III, respectively.

TABLE I

RECOVERY OF MK-212 FROM PLASMA AND URINE USING ELECTRON-CAPTURE DETECTION

MK-212 added, (ng)		Amount Recovered* Plasma		Urine	
		n	Mean ± S.D.	*n*	Mean ± S.D.
2000	A	16	1983 ± 76.8	7	1997 ± 268.1
	B		99 ± 3.8		100 ± 13.4
1000	A	20	982 ± 71.6	15	982 ± 110.4
	B		98 ± 7.2		98 ± 10.6
500	A	24	487 ± 43.6	16	487 ± 65.9
	B		97 ± 8.7		97 ± 13.2
250	A	20	246 ± 14.7	16	240 ± 39.0
	B		99 ± 5.9		98 ± 10.7
100	A	21	98 ± 12.1	13	105 ± 13.3
	B		98 ± 12.1		105 ± 13.3
50	A	19	51 ± 9.2	8	52 ± 7.4
	B		103 ± 18.4		104 ± 14.8
25	A	16	26 ± 5.6	5	27 ± 7.6
	B		104 ± 22.4		107 ± 30.6
12.5	A	12	13 ± 3.1	0	
	B		101 ± 24.8		
12.5—2000	B	148	99.6± 13.6	80	99.6 ± 13.1

* Values in A rows represent nanograms recovered; values in B rows represent percent recovery.

TABLE II

MK-212 PLASMA LEVELS FOLLOWING ADMINISTRATION TO RATS (2.5 mg/kg)

Three rats (A, B, C) were sacrificed at each time period. A, B and C do not represent the same rats for all time periods.

Time (h)	MK-212 (ng/ml) I.V.				P.O.			
	A	B	C	Average	A	B	C	Average
0.08	664	591	591	615	*	*	*	—
0.5	306	327	425	353	123	73	160	119
1.0	275	237	344	285	122	93	144	120
2.0	294	231	208	244	45	73	58	59
4.0	368	150	180	233	5	4	*	3

*Less than 5 ng/ml.

Confirmation of specificity of analysis was obtained when representative, unknown, biological specimens from dogs were analyzed by combined GLC—MS. Fig. 3 presents a comparison of the mass spectrum of authentic MK-212 carried through the analytical method with the material isolated from urine and plasma. As observed, the mass spectra obtained from the analysis of the GLC peak

TABLE III

MK-212 PLASMA LEVELS FOLLOWING ADMINISTRATION OF MK-212 TO MONKEYS (2.5 mg/kg)

Time (h)	MK-212 (ng/ml) I.V.				P.O.			
	1	2	3	Average	4	5	6	Average
0.033	1725	1430	1780	1645	—	—	—	—
0.17	1065	1510	1255	1276	*	5	*	2
0.5	1045	1070	995	1036	5	21	*	9
1	710	790	505	668	10	242	6	86
2	500	373	322	398	22	123	21	55
4	328	315	88	243	20	37	36	31
6	196	165	26	129	15	18	20	18
24	8	6	*	5	*	*	*	0

*Less than 5 ng/ml.

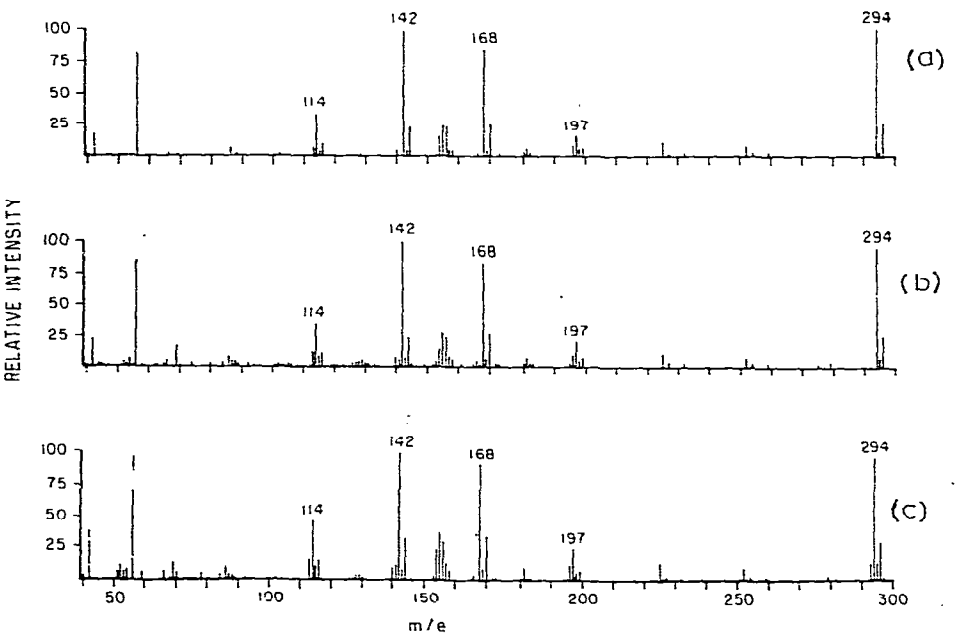


Fig. 3. Mass spectra of: (a) authentic MK-212 following addition (20 μg) to control dog urine, (b) material isolated from the urine of a dog treated with MK-212 and (c) material isolated from the plasma of a dog treated with MK-212.

corresponding to MK-212 (as the trifluoroacetyl derivative) were identical to that obtained when authentic MK-212 was carried through the entire procedure.

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