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DETERMINATION OF 3-HYDROXY-GUANFACINE IN BIOLOGICAL FLUIDS BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

M. GUERRET, C. JULIEN-LAROSE, J.R. KIECHEL* and D. LAVÈNE

Laboratoires Sandoz, Centre de Recherches Pharmacocinétiques, 14, Boulevard Richelieu, 92506 Rueil-Malmaison (France)

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SUMMARY

An electron-capture gas—liquid chromatographic method was developed for measuring 3-hydroxy-guanfacine, the main metabolite of guanfacine in human plasma and urine. After extraction, the metabolite was derivatized by condensing the amidino group with hexafluoroacetylacetone and by methylating the NH and OH groups with methyl iodide. The obtained derivative possessed good bioanalytical gas chromatographic properties, using a capillary column. The O-glucuronide was measured after enzymatic hydrolysis. Unchanged guanfacine could be determined in urine together with its 3-hydroxy metabolite by this method.

INTRODUCTION

Guanfacine (I) is a new derivative of guanidine which possesses antihypertensive activity and a central site of action [1]. Biotransformation has been studied in animals and man [2]. Metabolism occurs on the aromatic moiety of the molecule; the main route of biotransformation yields 3-hydroxy-guanfacine (II) which is then conjugated to the O-glucuronide (III) or O-sulphate (IV) (Fig. 1).

The present study describes an analytical procedure for the determination of 3-hydroxy-guanfacine in biological fluids (plasma and urine). After extraction, the metabolite is derivatized by condensing the amidino group with hexafluoroacetylacetone and by methylating the NH and OH groups with methyl iodide. The obtained derivative possesses good bioanalytical properties, allowing the quantitation of the OH metabolite in plasma and urine; the O-glucuronide can be determined after enzymatic hydrolysis.

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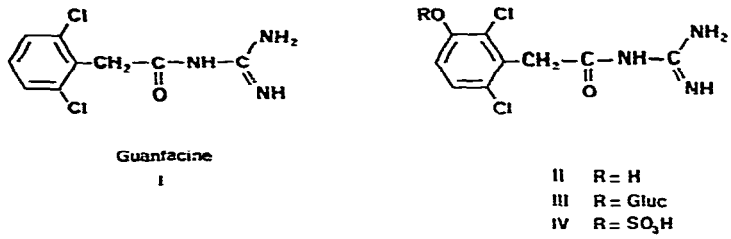


Fig. 1. Chemical formulae of guanfacine and its main metabolites.

EXPERIMENTAL

Reagents

Dichloromethane and methyl isobutyl ketone (MIBK) were obtained from Baker (Deventer, The Netherlands) and glass distilled under nitrogen prior to use. Toluene and hexane RS for pesticide analysis were obtained from Carlo-Erba (Milan, Italy) and were used without prior distillation. Tetrabutylammonium hydroxide was purchased from O.S.I. (Paris, France) and the final concentration used was 0.15 *M* in water. The 10% dilution in methanol of hexafluoroacetylacetone (HFAA), obtained from Fluka (Buchs, Switzerland), was prepared just before use.

The solution of 0.5 *M* methyl iodide was obtained by dissolution of 3.12 ml of CH_3I in 100 ml of toluene just before use.

Extraction solvent was prepared by mixing 50 volumes of MIBK with 50 volumes of dichloromethane.

β-Glucuronidase was obtained from Sigma (St. Louis, MO, U.S.A.). It was diluted to 40,000 UF/ml in pH 4.6 acetate buffer. Ethyl- and butylanthraquinone were purchased from Aldrich (Milwaukee, WI, U.S.A.).

Gas chromatography

A Hewlett-Packard 5710 A gas chromatograph equipped with a 15 mCi ^{63}Ni electron-capture detector was used for analysis.

Packed column. The glass column (2 m × 2 mm I.D.) was packed with 3% OV-225 on 100—120 mesh Chromosorb W (Erba-Sciences, Paris, France). The injector port, detector and oven were maintained at 250°C, 300°C and 210°C, respectively. Flow-rate was 30 ml/min for the argon—methane (90:10) carrier gas.

Ethylanthraquinone was used as internal standard. The retention times were 4 min and 5.5 min for the guanfacine metabolite and the internal standard, respectively.

Capillary column. Separation was performed on a glass capillary column (25 m × 0.25 mm I.D.) coated with CP Sil 5 from Chrompack (Middelburg, The Netherlands). The chromatograph was equipped with a glass solid injector (moving needle). The column was operated at 150°C for 2 min, then the oven temperature was increased at 8°C/min to 210°C. Flow-rates were 3 ml/min for the hydrogen carrier gas and 30 ml/min for the argon—methane (90:10) auxiliary gas.

The internal standard was butylanthraquinone. Retention times were 8 min

30 sec and 9 min 45 sec for the guanfacine metabolite and the internal standard, respectively.

Gas chromatography—mass spectrometry

Analyses by gas chromatography—mass spectrometry (GC—MS) were carried out on a Nermag R 10-10 Sidar 11A. A Girdel gas chromatograph was connected to the mass spectrometer and the system was completed by a PDP/8 computer system (Nermag, Rueil-Malmaison, France).

The chromatograph was equipped with a glass solid injector (moving needle) and a glass capillary column (30 m × 0.3 mm I.D.) coated with SE-52 from Chrompack. Helium at a pressure of 1.9 bars was the carrier gas. The oven temperature was programmed from 150°C to 210°C at 8°C/min. Injector and interface temperatures were 300°C. The source temperature was 200°C, the ionisation voltage was 70 eV for the electron-impact mode and 100 eV for the chemical-ionisation mode; the emission current was 250 μA. The chemical-ionisation (CI) spectra were obtained with methane as reactant gas.

Glassware

All glassware was washed with a 1% diluted cleaning solution of Liquinox from Alconox (New York, NY, U.S.A.) and rinsed thoroughly with water, distilled water and methanol. Then it was silanized with 10% hexamethyldisilazane in hexane.

Standard stock solutions

Guanfacine and its 3-hydroxy derivative were synthesized in the Sandoz Laboratories in Basle (Switzerland).

The standard stock solutions of pure butylanthraquinone, ethylanthraquinone and 3-hydroxy-guanfacine were prepared by dissolving 5 mg in 50 ml of methanol. They were stable for one month when stored at 4°C. The dilutions of internal standard, butylanthraquinone (capillary column) or ethylanthraquinone (packed column), were prepared every day. They were first diluted 1 to 20 with methanol and diluted again 1 to 10 with hexane to give a solution containing 1 ng per 2 μl.

Analytical procedure

To 2 ml of plasma or urine were added 1.4 g of sodium chloride, and 5 ml of solvent for extraction. The tube was sealed with a glass cap and shaken for 15 min. After centrifugation (5 min, 2400 *g*), the upper organic phase was transferred to a reaction tube and then evaporated to dryness under a nitrogen stream at 35°C. The dry extract was redissolved in 200 μl of 10% HFAA in methanol. The tube, stoppered with Polytef capsules, was heated in an aluminium heating block at 100°C for 1 h and then evaporated to dryness under a nitrogen stream at 50°C. The resulting dry residue was then taken up in 2.5 ml of methyl iodide solution and 200 μl of tetrabutylammonium hydroxide to which 2 ml of 1 *N* sodium hydroxide were added. The tube was shaken for 20 min. After a brief centrifugation, the organic phase was transferred to a small glass-stoppered tube and evaporated to dryness under a stream of nitrogen at 40°C. The dry residue was dissolved in 400 μl of internal stan-

dard solution, then 1 ml of 1 *N* sodium hydroxide was added to eliminate the excess reagent. The sample was vortexed, centrifuged and 2 μl of the hexane phase were injected into the chromatograph.

In the first collections of urine, the concentration was high, so the samples were first diluted 1 to 4 with distilled water.

Enzymatic hydrolysis

Urine or plasma (2 ml) was buffered at pH 4.6 with acetate buffer and incubated with 0.5 ml of β-glucuronidase solution at 37°C. After 48 h, 1.4 g of sodium chloride were added and the extraction was carried out as for the unchanged drug.

RESULTS AND DISCUSSION

In order to determine the optimal conditions of derivatization and extraction of II, some preliminary experiments were performed.

(1) The derivative obtained by condensing the amidino group of the metabolite with HFAA, as reported previously for guanfacine [3], possesses unsatisfactory GC properties, probably due to the high polarity of the hydroxyl group. After methylation of this group, the response of the resulting O-CH_3 derivative analyzed by GC with an electron-capture detector was more satisfactory.

(2) The extraction conditions used for I do not allow the extraction of the metabolite and therefore different solvents and pH conditions have been tested.

(3) III was essentially recovered in human urine as the O-glucuronide. So, the enzymatic hydrolysis was worked out.

(4) Finally, in order to increase the sensitivity amd specificity, the method has been adapted to a capillary column.

Derivative identification

The structure of the ultimate derivative obtained after the two chemical reactions on I and its metabolite II was determined by GC—MS.

(A) Methylation of the pyrimidino derivative of guanfacine. After condensation of I with HFAA (derivative A), methylation with methyl iodide led to V (Fig. 2), the structure of which was elucidated by GC—MS. A comparison of the spectra of V and of the pyrimidino guanfacine derivative A is shown in Fig. 3.

The following observations are of relevance. (1) For the two compounds, in the electron-impact mode, the molecular ions are very small, If the spectrum is taken in the chemical-ionisation mode (Fig. 4), the quasi-molecular ion MH^+ confirms the structure for V. (2) The characteristic ions of the aromatic moiety were seen in both spectra of V and A. (3) All the ions containing the nitrogen atoms had a mass increase of 14 units for derivative V. The methylation of derivative A occurs on the nitrogen atom in the α-position to the carbonyl group.

The fragmentation pattern of V is displayed in Fig. 5.

(B) Methylation of the pyrimidino derivative of 3-hydroxy-guanfacine. The

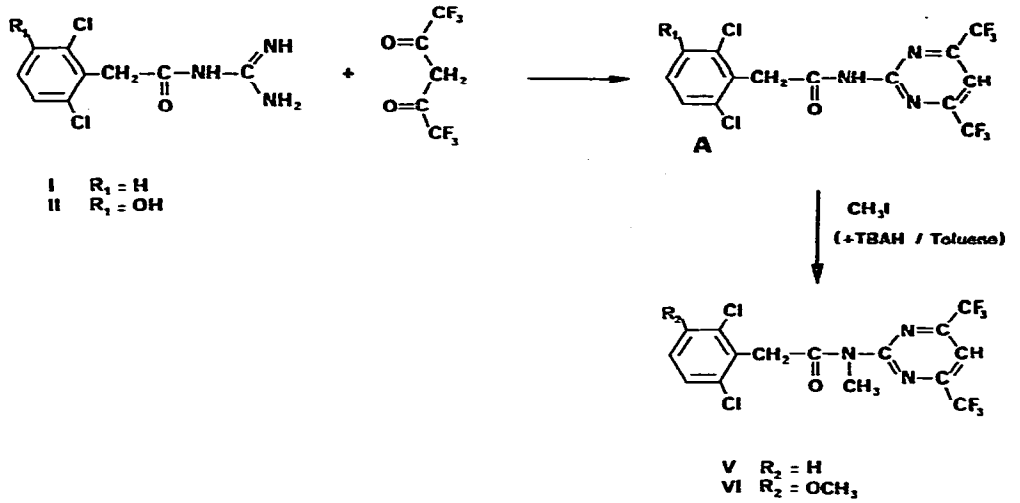
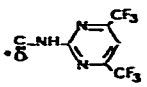
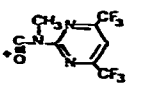
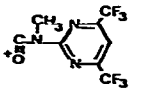
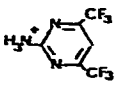
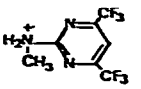
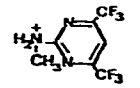
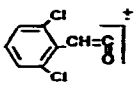
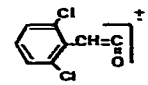
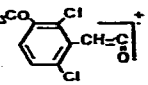
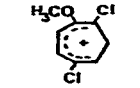


Fig. 2. Formation of guanfacine derivatives by condensation with HFAA and extractive alkylation.

Derivative A			Derivative V			Derivative VI		
m/e	Characteristic ions	A*%	m/e	Characteristic ions	A*%	m/e	Characteristic ions	A*%
417–419	$M^{\pm}$	10	431–433	$M^{\pm}$	3	461–463	$M^{\pm}$	1
382–384	$M^{\pm}-35=M^{\pm}-Cl$	30	396–398	$M^{\pm}-35=M^{\pm}-Cl$	18	426–428	$M^{\pm}-35=M^{\pm}-Cl$	75
258		10	272		25	272		20
232		20	245		20	246		15
186–188		98	186–188		45	216–218		60
159–161		75	159–161		45	189–191		50

*:A % = ABUNDANCE PEAK in % of BASIC PEAK

Fig. 3. Main fragment ions obtained in electron-impact mass spectra of guanfacine derivatives.

structural similarity of I and II allows us to presuppose that methylation on the nitrogen atom in the α-position of the carbonyl group also occurred for the metabolite. However, for the hydroxyl group on the benzene ring, methylation is also likely in addition. This was confirmed by GC—MS analysis.

In the chemical-ionisation mode (Fig. 6) the quasi-molecular ion MH^+ obtained is 30 units higher than that of the guanfacine derivative V, showing that one hydrogen was substituted by the O-CH_3 group. The assumed struc-

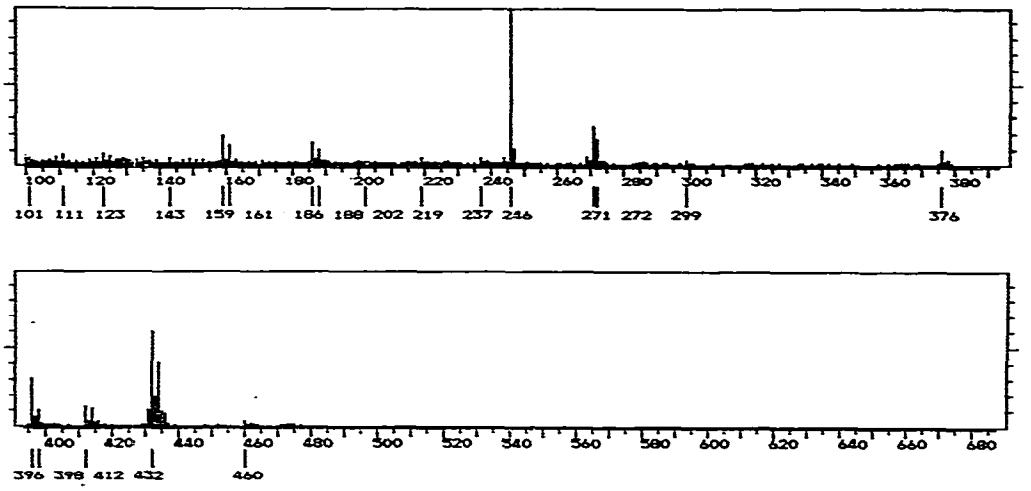


Fig. 4. Chemical-ionisation mass spectrum of guanfacine derivative V.

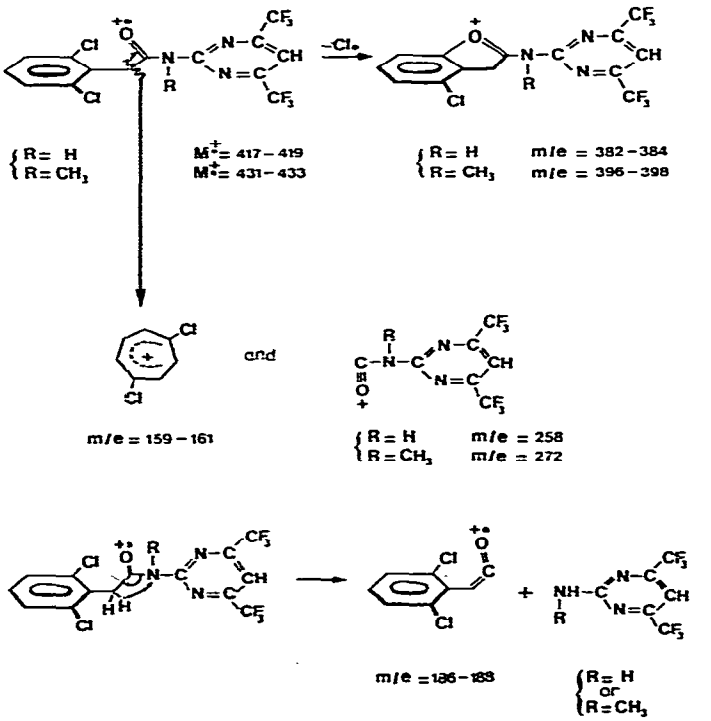


Fig. 5. Fragmentation pattern of guanfacine derivatives.

ture for this compound (VI, Fig. 2) is corroborated by examination of the main fragment ion, displayed in Fig. 3.

It is possible to conclude that the hydroxyl group of the metabolite is a position of methylation. This is confirmed by the presence of two fragments at *m/e* 216 and *m/e* 189, respectively, homologous to ions *m/e* 186 and *m/e*

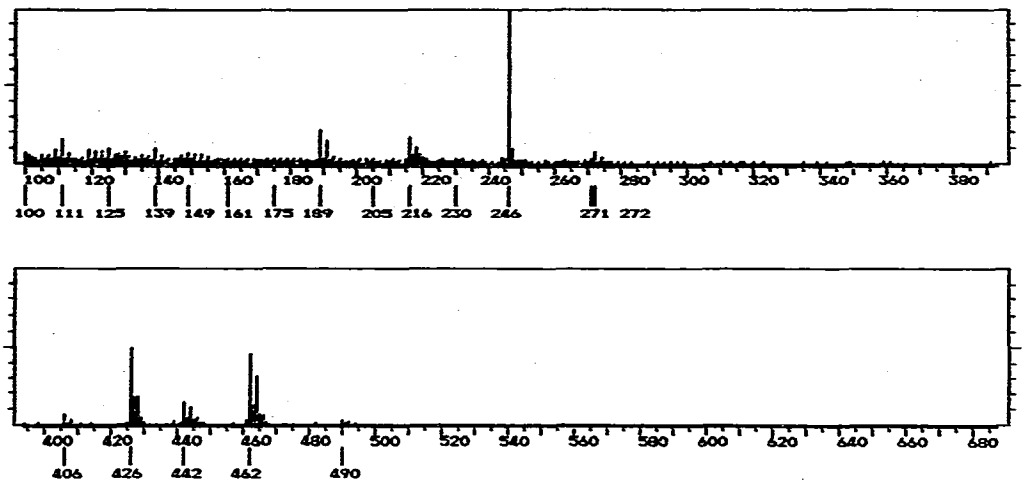


Fig. 6. Chemical-ionisation mass spectrum of 3-hydroxy-guanfacine derivative VI.

159 seen in the spectra of the two guanfacine derivatives A and V in the electron-impact mode. As for guanfacine, the second position of methylation is the nitrogen atom in the α-position to the carbonyl group. This is confirmed by the presence of fragments at *m/e* 272 and *m/e* 246, which are also seen in the spectrum of V. Furthermore, the pattern of fragmentation for the derivative of II is the same as that of the guanfacine derivatives A and V, as shown in Fig. 5.

Derivative synthesis

The formation of the pyrimidino derivative by condensation of the amidino group of II with HFAA was carried out as reported previously for I [3]. It has been verified by thin-layer chromatography that the reaction was complete. The methylation was performed as for the extractive alkylation [4] described previously for a diuretic, Brinaldix [5].

Solvents of the methylation reaction. Among the different solvents used for the extractive methylation, chloroform, dichloromethane, benzene and toluene gave good yields, but toluene was chosen on account of some advantages: indeed, the organic phase is the top layer in contrast to chloroform and dichloromethane, and the toxicity of toluene is lower than of benzene. Extraction by chloroform or dichloromethane leads to a larger, unfavorable residue. Extraction and methylation are as complete with 2.5 ml as with 5 ml of solvent. So, 2.5 ml are used to accelerate the evaporation step.

Temperature and heating time. Assays with different temperatures (ambient, 45°C and 60°C) and heating times (15 to 45 min) were performed. At 60°C the reaction was nearly complete after 20 min and the reproducibility was better than at 45°C..

Under these conditions, I derivatized by condensation with HFAA is completely methylated without degradation.

Sample extraction

Various parameters of the extraction procedure were analyzed. Different solvents (chloroform, dichloromethane, benzene, diethyl ether or MIBK) resulted in less than 10% yield at different pH values (1.4, 5.4, 7, 9, 13).

Since metabolite II is water-soluble, saturation with sodium chloride was necessary. Instead of MIBK which gave the best yield but took a long time to be evaporated, a mixture of MIBK and dichloromethane was preferred.

Recovery

The recovery of II was determined by adding various known amounts of II to human urine (Table I) and analyzing each sample in triplicate according to the described procedure. Compared to a similar series of unextracted reference standard of II, the recoveries varied from 81.5 to 101.5% with an average of 87.7 ± 3.0%, and were independent of the concentration within the tested range. In plasma the recovery of II was 80.1 ± 5.2%.

TABLE I

RECOVERY OF 3-HYDROXY-GUANFACINE ADDED TO HUMAN URINE SAMPLES

Assays were performed on 2 ml of urine.

3-Hydroxy-guanfacine added (ng/ml)	Recovery (mean* ± S.E.M.)
25	101.5 ± 3.6
50	83.4 ± 2.5
100	93.4 ± 0.9
200	83.0 ± 0.1
400	81.5 ± 2.3
600	83.5 ± 2.0

*Average of three determinations.

Selectivity

Evidence of selectivity of the method was furnished by characteristic GC retention times of the reference compounds and the lack of interfering peaks in plasma or urine extracts from subjects who had not received guanfacine. Figs. 7 and 8 show chromatograms obtained after determination on a packed column or on a capillary column. Some drugs, usually prescribed with guanfacine, which might interfere in the assay were tested, e.g. diuretics (furosemide or clopamid) and β-blocking agents. No interfering peak was observed under the described conditions.

Reproducibility and accuracy

Reproducibility and accuracy of the determination were estimated by preparing urine standards of several concentrations for triplicate analysis. The results presented in Table II for the packed column and the capillary column show that the overall accuracy of the procedure was good.

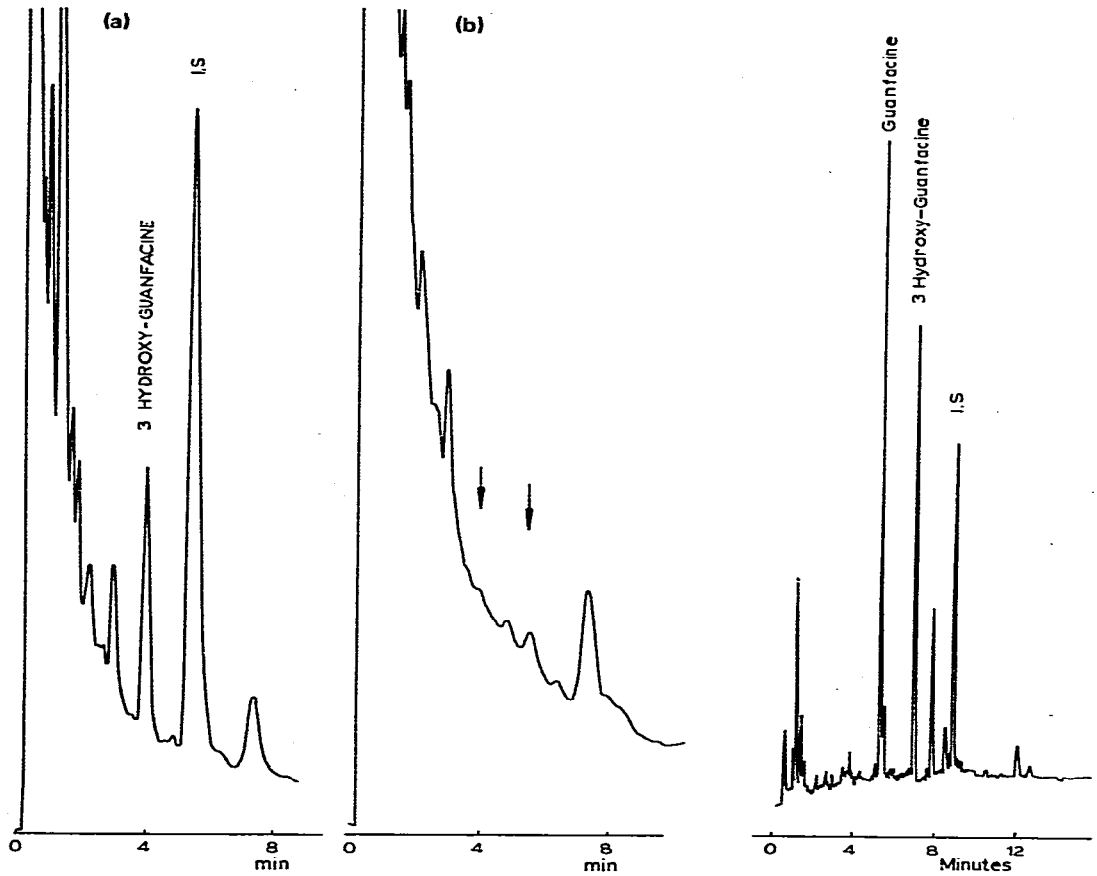


Fig. 7. Gas chromatogram of a 0—24-h urine extract from a subject given 2 mg of guanfacine orally, on a packed column (a). Blank urine (b).

Fig. 8. Gas chromatogram of a 0—24-h urine extract from a subject given 2 mg of guanfacine orally on a capillary column.

The relative variations of the determination are better using capillary columns (range 0.2—6.3) than packed columns (range 5.1—9.6).

The accuracy of the method expressed by the mean percentage deviation of all concentrations from the theoretical value, ranged from 3.6 to 10.1% (mean 6.8%) for the packed column and from 1.6 to 9.9% (mean 4.8%) for the capillary column.

Linearity and sensitivity

The linearity of the method was demonstrated by calculation of the linear correlation coefficients of peak area ratio versus metabolite II concentration.

On the packed column the coefficients were $Y = 0.0080X - 0.0063$ ($r = 0.9951$).

On the capillary column the obtained curve was split into two straight lines. For concentrations below 25 ng/ml the coefficients were $Y = 0.0091X$

TABLE II

REPRODUCIBILITY AND ACCURACY OF DETERMINATION OF 3-HYDROXY-GUANFACINE ADDED TO HUMAN URINE

Urine samples were 2 ml.

Amount added (ng/ml)	Amount recovered (ng/ml) (mean ± S.E.M.)	Coefficient of variation (%)	Accuracy (mean ± S.E.M.)
Packed column			
10	10.62 ± 0.51	8.2	6.21 ± 1.80
20	20.17 ± 0.70	6.0	4.87 ± 0.80
50	44.93 ± 2.50	9.6	10.13 ± 6.01
100	81.70 ± 5.25	9.1	6.57 ± 2.13
120	123.02 ± 6.32	8.9	9.25 ± 1.53
200	201.27 ± 7.25	5.1	3.63 ± 0.64
Capillary column			
5	5.22 ± 0.16	5.31	9.01 ± 2.36
25	26.81 ± 0.98	6.34	7.84 ± 2.23
50	44.80 ± 1.50	4.73	6.42 ± 2.80
100	108.07 ± 1.15	1.51	8.07 ± 1.15
200	196.82 ± 0.23	0.17	1.59 ± 0.12
400	392.54 ± 10.47	4.62	4.14 ± 0.86
600	607.17 ± 14.98	3.49	2.50 ± 1.19

(r = 0.9986). For concentrations between 25 and 600 ng/ml the coefficients were $Y = 0.00434X + 0.11021$ (r = 0.9994).

The sensitivity, defined as a signal-to-noise ratio greater than 3, was 10 ng/ml from 2 ml of urine with packed columns and 2 ng/ml from 1 ml of urine with capillary columns. In the latter case, the reproducibility was about 10%. In plasma, from a 1-ml sample, the sensitivity is about 5 ng/ml though lower levels can be detected with a less good precision.

In biological fluids, the 3-hydroxy metabolite (II) is also found as an O-glucuronide conjugate and its determination can be performed after enzymatic hydrolysis. The optimal conditions were determined on a 0—24-h urine sample from a subject who received 2 mg guanfacine orally. The optimal pH was determined previously and was found to be 4.6 (acetate buffer).

Incubations with β-glucuronidase using various reaction times showed that after two days the hydrolysis appears to be complete. The same assays with 30,000 UF instead of 20,000 UF of β-glucuronidase enzyme led to comparable results.

Application

The described procedure can be used for the determination of II in biological fluids. The use of a wall-coated capillary column with a high efficiency allows both high specificity and very good sensitivity. In addition, guanfacine itself, after the derivatization steps of this procedure, elutes on packed column at the beginning of the chromatogram together with interfering sub-

stances from the reaction mixture. In contrast, on the capillary column, the peak of guanfacine occurs on the chromatogram at a retention time of about 6 min (Fig. 4), and is well separated from peaks of interfering substances.

Although the sensitivity for I is not as good as that of the direct method described previously [3], the determination of I is possible together with its metabolite II in urine by using capillary columns since the levels of guanfacine in urine are sufficiently high. This procedure has the advantage of reducing the analysis time. In Table III are displayed the precision and the accuracy for the assay of the parent drug in urine.

TABLE III

REPRODUCIBILITY AND ACCURACY OF DETERMINATION OF GUANFACINE ADDED TO HUMAN URINE ASSAYED AS DESCRIBED FOR 3-HYDROXY-GUANFACINE (GC WITH CAPILLARY COLUMN)

Amount added (ng/ml)	Amount recovered (ng/ml, mean ± S.E.M.)	Coefficient of variation (%)	Accuracy (mean ± S.E.M.)
25	26.24 ± 1.65	10.91	9.91 ± 7.89
50	48.03 ± 0.71	2.55	3.93 ± 1.41
100	99.03 ± 3.74	6.55	5.04 ± 1.34
200	201.18 ± 7.67	6.60	6.76 ± 1.45
400	400.15 ± 15.86	6.87	5.19 ± 1.50
600	602.50 ± 9.96	2.87	2.12 ± 0.72

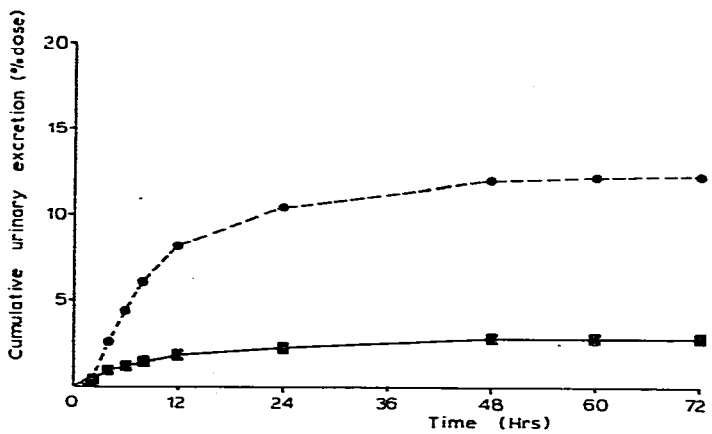


Fig. 9. Mean urinary cumulative excretion curve (n = 6) as a function of time for 3-hydroxy-guanfacine (——) and its glucuronide (·····).

Nevertheless, for the determination of I in plasma, the previous method [3] should preferably be used since the therapeutic levels are lower.

The described method was used for the determination of both II and III in the urine of six subjects who received 2 mg of guanfacine orally. The mean urinary cumulative excretion (percentage of administered dose) and the mean urinary excretion rate (expressed in μg/h) are shown in Figs. 9 and 10, re-

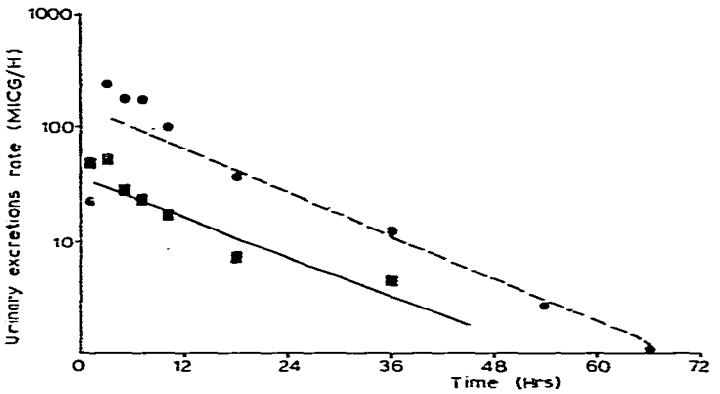


Fig. 10. Mean urinary excretion rate curve (n = 6) as a function of time for 3-hydroxyguanfacine (——) and its glucuronide (·····).

spectively. Fig. 9 shows that the hydroxy metabolite is excreted in urine essentially as the O-glucuronide. The apparent half-life of III (about 13 h) is not significantly different from that of metabolite II.

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