Biotransformation and Excretion of Nitromethaqualone in Rats and Humans

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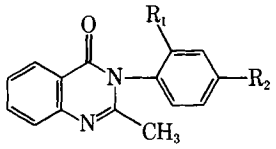
Abstract □ The metabolic disposition of ^{14}C-labeled nitromethaqualone was investigated in rats. Unlabeled nitromethaqualone was used for studies on humans. Nitromethaqualone was eliminated from the body after most of it had undergone biotransformation. Both humans and rats reduced the nitro group of nitromethaqualone to the corresponding amino derivative, which was partially transformed to the corresponding acetylated form. Cleavage of the quinazolinone nucleus resulting in 2-methoxy-4-nitroaniline was also observed in humans. In rats additional major metabolites arose from the oxidation of the 2-methyl group into hydroxymethyl resulting in 2-hydroxymethyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone and concomitant *in vivo* reduction of the latter resulting in 2-hydroxymethyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone. Both metabolites were also excreted as glucuronides. In rats fecal excretion accounted for 55–60% of the administered dose, while 24–27% was excreted in the urine. Protracted excretion in both humans and rats indicated an extensive enterohepatic circulation.

Keyphrases □ Biotransformation—excretion, nitromethaqualone in rats and humans, metabolism □ Nitromethaqualone—biotransformation and excretion in rats and humans, metabolism □ Metabolism—biotransformation and excretion of nitromethaqualone in rats and humans

Nitromethaqualone[1] (I) or 2-methyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone has been available in Europe since 1967 as a nonbarbituric hypnotic of the quinazolinone series, which includes methaqualone [2-methyl-3-*o*-tolyl-4(3*H*)-quinazolinone (II)] and mecloqualone [2-methyl-3-(2′-chlorophenyl)-4(3*H*)-quinazolinone (III)].



$R_1 = OCH_3$, $R_2 = NO_2$ (Nitromethaqualone, I)
$R_1 = CH_3$, $R_2 = H$ (methaqualone, II)
$R_1 = Cl$, $R_2 = H$ (mecloqualone, III)

The abuse of methaqualone (1–3) and mecloqualone (4) has been reported previously. Both drugs are metabolized extremely well in humans and animals (5–12), with detection of the unchanged drug in the urine being difficult when therapeutic amounts have been ingested. Detection in urine samples is usually done by means of the metabolites (13, 14).

Neither the metabolism of nitromethaqualone nor its analysis in urine samples has been established previously.

The present report deals with the metabolism of nitromethaqualone in humans and rats, along with the detection of the drug in urine samples for clinical and medicolegal practice. The excretion in rats was studied with [2-^{14}C]2-methyl -3- (2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone.

[1] Parnox, Sopar, Brussels, Belgium.

EXPERIMENTAL

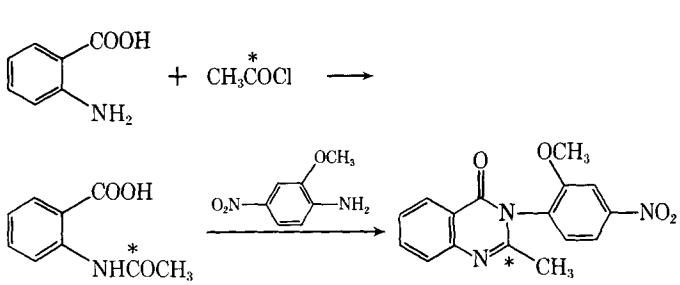
Synthesis of [2-^{14}C]2-Methyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*) - quinazolinone—[2-^{14}C]2-Methyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone was synthesized as shown in Scheme I by condensation of labeled *N*-acetylanthranilic acid with 2-methoxy-4-nitroaniline in toluene in the presence of phosphorus trichloride according to a procedure described previously (15). Labeled *N*-acetylanthranilic acid was prepared by refluxing 1 mmole of anthranilic acid in toluene with 1 mmole of [1-^{14}C]acetyl chloride (specific activity, 5.0 mCi/mmole) until no more hydrochloric acid was liberated. The reaction mixture was cooled to 70°. 2-Methoxy-4-nitroaniline (1 mmole) was added followed by the addition of phosphorus trichloride in toluene over a 10-min period (Scheme I). The mixture then was mixed thoroughly and refluxed for 2 hr in an oil bath. Toluene was evaporated under vacuum and the yellow residue was shaken with 10% aqueous sodium carbonate and extracted twice with 50 ml of chloroform. After evaporation of the solvent, the residue was taken up in benzene and purified on a 10-g column[2]. The column was eluted with 1 liter of benzene. Benzene was removed under vacuum and the residue recrystallized from methanol–water.

The specific activity determined by liquid scintillation counting was 5.0 mCi/mmole. The purity of the prepared compound was tested by TLC using two different solvents: chloroform–acetone–ammonia (50:50:1) and cyclohexane–chloroform–diethylamine (70:20:10). In both solvent systems only one spot could be detected accounting for the complete radioactivity with an R_f value identical to a standard of unlabeled nitromethaqualone. The radioactive spots were localized with a thin-layer scanner[3].

Animal Experiments—A single 2-mg dose of [^{14}C]nitromethaqualone with an activity of 5 μCi/mg suspended in medicinal oil was introduced directly into the stomach of two male Wistar rats, 260 and 312 g, respectively. The rats were housed in metabolic cages; urine and feces were collected separately at 12-hr intervals over 6 days. Urine samples were counted without any treatment; feces samples were counted as described under quantification of radioactivity. Two other rats were each given 30 mg of unlabeled nitromethaqualone. Urine was collected during 6 days and examined for metabolites.

Clinical Study—Two male volunteers were administered single 25-mg nitromethaqualone tablets[1]. Urine was collected at 2–4-hr intervals on the first day and 12-hr specimens were sampled over the next 6 days.

Quantification of Radioactivity—The isotope disintegration rates of all specimens were determined by liquid scintillation spectrometry[4]. Counting mixtures were prepared as follows: 2-ml urine samples were taken up in 15 ml of scintillation liquid[5]; 20- to 40-mg feces samples were rehydrated for 2 hr with 200 μl of water in plastic counting vials and 1 ml of tissue solubilizer[6] was added. The mixtures were incubated for 5 hr



Scheme I—Synthesis of [^{14}C]nitromethaqualone.

[2] Florisil column.
[3] Dünnschicht Scanner LB 2723, Berthold Instruments.
[4] Liquid scintillation counter, model BF 5000/300, Berthold Instruments.
[5] Insta-Gel, Packard.
[6] Soluene-350, Packard.

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Table I—TLC and GLC of Nitromethaqualone and Metabolites (R_f and R_t Values)

	TLC Solvent System[a] A	B	C	GLC (R_t, min)[b]
2-Methyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone (nitromethaqualone)	0.79	0.90	0.60	10.6
2-Methyl-3-(4′-nitrophenyl)-4(3*H*)-quinazolinone (internal standard for GLC)	—	—	—	9.0
2-Hydroxymethyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone (Metabolite I)	0.77	0.84	0.30	Decomposition
2-Methyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone (Metabolite II)	0.52	0.70	0.00	12.8
2-Hydroxymethyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone (Metabolite III)	0.45	0.61	0.00	Decomposition
2-Methyl-3-(2′-methoxy-4′-acetylaminophenyl)-4(3*H*)-quinazolinone (Metabolite IV)	0.52	0.61	0.00	38.4
4-Nitro-2-methoxyaniline (Metabolite V)	0.75	0.92	0.08	1.0
2-Methyl-3-(2′-hydroxy-4′-aminophenyl)-4(3*H*)-quinazolinone (Metabolite VI)	0.25	0.16	0.00	Not eluted
2-Methyl-3-(2′-hydroxy-4′-nitrophenyl)-4(3*H*)-quinazolinone	0.18	0.05	0.00	Not eluted
Trimethylsilyl derivative of Metabolite I	—	—	—	13.4
Trimethylsilyl derivative of Metabolite III	—	—	—	16.6

[a] Solvent A: benzene–*n*-propanol–ammonia (80:20:1); Solvent B: chloroform–acetone–ammonia (50:50:1); Solvent C: cyclohexane–chloroform–diethylamine (70:20:10). [b] OV-17/QF, (1.5%); 260°.

at 60°. After cooling to room temperature, 0.5 ml of isopropanol was added followed by 0.2 ml of hydrogen peroxide (30%) for bleaching. The mixtures were kept at room temperature for 4 hr until transparent.

Finally, 15 ml of a mixture of 9 parts scintillation liquid[5] and 1 part 0.5 *N* HCl was added and the sample counted. Counting efficiency was determined by the internal standard method with ^{14}C-labeled toluene. Efficiency of the different urine samples examined varied between 85 and 99% and of the feces between 65 and 90%. Silica gel zones scraped from thin-layer chromatograms were suspended in 15 ml of scintillation fluid. Solvent extracts were measured following evaporation of the solvent and dissolution of the residue in 15 ml of scintillation liquid[7].

Extraction and Fractionation of Drug Components from Urine Samples—Urine samples were extracted on prepacked kieselguhr columns[8]. Twenty-milliliter samples were poured onto the columns, the aqueous solution was distributed as stationary phase on the chemically inert support, and then eluted with 100 ml of extraction solvent consisting of a mixture of dichloromethane–*n*-propanol (85:15). In this way the lipophilic substances were extracted from the aqueous phase into the organic phase, the aqueous phase remaining on the support (16, 17). The solvent was evaporated under vacuum and the residue taken up in 100 μl of methanol for TLC, GC, and GC–MS examination.

For the study of glucuronides, urine samples were extracted with chloroform at pH 3.0 followed by an extraction with ethyl acetate at pH 9.0. The remaining aqueous phases were submitted to an enzymatic hydrolysis. Therefore, 5 ml of the previously extracted samples were adjusted to pH 5.2 and incubated for 24 hr at 37° with 0.1 ml of β-glucuronidase[9]. The treated samples were handled on columns as described for the direct extraction. TLC examination of radioactive extracts was performed on precoated silica gel plates[10].

The presence of intact [^{14}C]nitromethaqualone was determined by one-dimensional TLC of extracts[8] using System C (Table I). For metabolites, Systems A and B (Table I) were used. Measurements of the isotope disintegration rates of the silica gel areas were carried out as described. For nonradioactive extracts, different metabolites were located by spraying the plates with different reagents specific for definite chemical functions. Aromatic amino functions were detected by the Bratton–Marshall reaction. The plates were sprayed first with a 1:1 mixture of 1% sodium nitrite in water and 1 *N* HCl followed by a solution of 200 mg of *N*-(1-naphthyl)ethylenediamine dihydrochloride in methanol.

Aromatic acetyl amino functions were detected in the same way as described for the aromatic amino functions following acid hydrolysis on the TLC plate to the free amino function. Plates were first sprayed with 2 *N* H_2SO_4 and put in an oven at 130° for 15 min. After cooling, the plates were sprayed as described earlier. Aromatic nitro functions were first reduced to an aromatic amino function by spraying the plates with a 1% titanium trichloride solution. After drying with warm air, the aromatic amino function was detected as described. Phenols were detected by the Folin–Ciocalteu reagent.

The metabolites present in the extracts from human urine samples were isolated by preparative TLC. Silica gel plates (20 × 20 cm, 250-μm thick[11]) were used. The extracts were applied as a band across the plate. Following development with Solvent System B, the separated zones were scraped off and eluted in minicolumns with 10 ml of methanol. The solvent was evaporated under a stream of nitrogen. The residues were then analyzed by GC and GC–MS.

Metabolite Characterization—Metabolites were identified by comparing their mass spectra and chromatographic data (TLC and GLC) with those of synthesized metabolites. The structure of the synthesized nitromethaqualone was confirmed by comparing the obtained physicochemical data with those of the literature (18). The structure of the synthesized metabolites was proved by NMR and mass spectrometry.

The mass spectra were recorded on a single-focusing apparatus[12] operated at 8-kV accelerating voltage, 100-μA trap current, and 70-eV ionization energy. The spectra were recorded either by the direct inlet method for isolated metabolites or synthesized products or by coupling the gas chromatograph to the mass spectrometer.

Optimal GC conditions were as follows: mixed phase, 1.5% OV-17-1.5% QF_1 on 80–100 mesh Gaschrom Q (180 cm × 2-mm i.d.) at 260° or with temperature programming (4°/min from 100 to 260°). The carrier gas was either helium at 60 ml/min (GC–MS) or nitrogen at 30 ml/min (GC).

Products were detected with a nitrogen-phosphorus detector for conventional GC. Identity of the proposed structures from the metabolites were confirmed by synthesis, and NMR spectra[13] were recorded in deuterochloroform as a solvent and tetramethylsilane as an internal standard.

Synthesis of Metabolites—*2-Methyl-3-(2′-hydroxy-4′-nitrophenyl)-4(3H)-quinazolinone*—Acetylanthranilic acid was condensed with 2-hydroxy-4-nitroaniline (15). White crystals with mp 225° were obtained.

2-Methyl-3-(2′-methoxy-4′-nitrophenyl)-4(3H)-quinazolinone (Nitromethaqualone)—The previous product was dissolved in methanol and a solution of diazomethane in ether added in excess. After 30 min the solvents were evaporated and the residue was crystallized. Light yellow crystals with mp 193° were obtained. The structure was identical with the product isolated from commercial nitromethaqualone tablets and confirmed by mass spectrometry and NMR (18). Nitromethaqualone was also prepared by direct condensation of acetylanthranilic acid with 2-methoxy-4-nitroaniline, yield 70%.

2-Methyl-3-(2′-methoxy-4′-aminophenyl)-4(3H)-quinazolinone—Nitromethaqualone (0.5 g) was dissolved in 200 ml of warm 0.2 *N* NaOH; 1 g of sodium dithionite was added and the mixture kept at 80° for 10 min, then it was cooled and extracted twice with a mixture of chloroform–1-propanol (85:15). The solvent was evaporated under vacuum and the yellow residue recrystallized from ethanol. White crystals (mp 205–206°) were obtained, yield 80%. The NMR spectrum of the synthesized compound showed a broad singlet integrating for two protons at $\delta = 4.9$ proving the presence of an amino group. The latter gave a positive reaction with the Bratton–Marshall reagent proving its aromatic nature. The mass spectrum (Table II) confirmed the proposed structure.

Anal.—Calc. for $C_{16}H_{15}N_3O_2$: C, 68.31; H, 5.37; N, 14.94. Found: C, 68.26; H, 5.41; N, 14.82.

2-Methyl-3-(2′-methoxy-4′-acetylaminophenyl)-4(3H)-quinazolinone—2-Methyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone (250 mg) was heated in 200 ml of dry toluene, an equivalent quantity

[7] LipoLuma, Lumac Systems AG, Basel, Switzerland.
[8] Extrelut, Diagnostica Merck, Darmstadt, West Germany.
[9] β-glucuronidase *Escherichia coli*, 100 U/ml, Boehringer, Mannheim, West Germany.
[10] Sil G-25UV$_{254+366}$, Machery-Nagel, Düren, Germany.
[11] DC Fertig Platten Kieselgel 60, F 254, Merck, Darmstadt, West Germany.
[12] A Kratos AEI MS-12 apparatus was used.
[13] NMR spectra were obtained with a Hitachi Perkin-Elmer R 24, 60-MHz apparatus.

Table II—Mass Spectra of Nitromethaqualone and Synthesized Metabolites

		Molecular Ion	Major Peaks m/z (Relative Intensity)				
2-Methyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone		311 (85)	296 (32)	280 (100)	250 (38)	143 (27)	
2-Hydroxymethyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone	A	327 (52)	296 (100)		250 (19)		
Corresponding trimethylsilyl derivative	B	384(M-15) (30)	368 (100)	324 (32)	264 (40)		
2-Methyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone	C	281 (100)		264 (27)	250 (80)	143 (35)	
Corresponding trimethylsilyl derivative	D	369 (53)	354 (100)	266 (42)	248 (53)		
2-Hydroxymethyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone	E	297 (41)	266 (21)	123 (100)			
2-Methyl-3-(2′-methoxy-4′-acetylaminophenyl)-4(3*H*)-quinazolinone	F	323 (75)	308 (25)	292 (100)	281 (45)	250 (50)	143 (90)
2-Nitro-2-methoxyaniline	G	168 (100)	153 (18)	137 (20)	58 (26)		
2-Methyl-3-(2′-hydroxy-4′-aminophenyl)-4(3*H*)-quinazolinone	H	267 (100)	252 (33)	251 (26)	225 (60)	143 (20)	

of acetic anhydride was added, and the mixture refluxed for 30 min. The reaction mixture was allowed to cool then neutralized and extracted with ether. The extract was dried on anhydrous sodium sulfate and evaporated. The residue was recrystallized from methanol. White crystals (mp 256°) were obtained, yield 82%. The NMR spectrum showed a singlet at δ = 2.15 integrating for three protons proving the presence of an acetyl group in the molecule. The mass spectrum from this compound (Table II) confirmed the proposed structure.

Anal.—Calc. for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00. Found: C, 66.91; H, 5.21; N, 13.09.

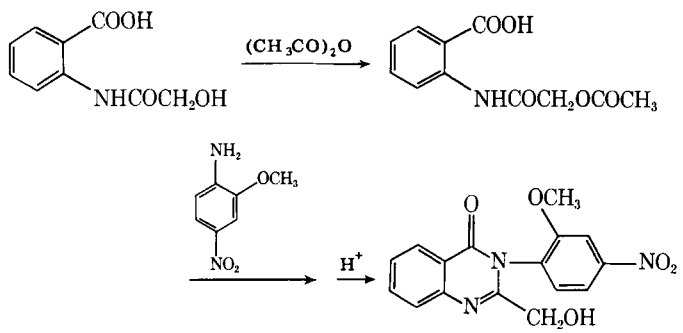
2-Hydroxymethyl-3-(2′-methoxy-4′-nitrophenyl)-4(3H)-quinazolinone—The synthesis of an analogous methaqualone metabolite (Scheme II) was carried out using a procedure described previously (19). Glycolic acid was taken up in an excess of thionyl chloride and the mixture kept at room temperature for 25 hr. The excess thionyl chloride was then evaporated and the residue taken up in dry toluene, along with an equivalent amount of anthranilic acid, and refluxed for 1 hr. The solvent was evaporated under vacuum, and the residue was taken up in acetic acid and treated with sodium acetate and acetic anhydride in slight excess [1.5 mole of both for 1 mole of *N*-(hydroxyacetyl)anthranilic acid]. The mixture was heated to 100° for 2 hr and then concentrated *in vacuo*. The residue was taken up in chloroform and washed with 5% sodium carbonate. The chloroform was evaporated under vacuum and the resulting *N*-(acetoxyacetyl)anthranilic acid condensed with 2-methoxy-4-nitroaniline in the usual way (15).

The obtained ester was dissolved in a 100:25 mixture of dioxane–5 *M* HCl. The mixture was boiled for 10 min then cooled and the hydrochloride filtered off. The base was isolated by dissolving the hydrochloride in chloroform and adding 0.2 *N* NaOH. The chloroform was evaporated and the residue crystallized from methanol–water. White crystals with mp 169–170° were obtained, yield 10%. The NMR spectrum of this compound showed a singlet at δ = 4,2 integrating for two protons replacing the singlet at δ = 2,2 integrating for three protons in nitromethaqualone. An additional broad singlet integrating for one proton was observed at δ = 3,4 corresponding to an hydroxyl proton.

Anal.—Calc. for $C_{16}H_{13}N_3O_5$: C, 58.72; H, 4.00; N, 12.84. Found: C, 58.62; H, 3.94; N, 12.77.

2-Hydroxymethyl-3-(2′-methoxy-4′-aminophenyl)-4(3H)-quinazolinone—This compound was prepared by alkaline reduction of 2-methyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone in alkaline medium with sodium dithionite. The reaction product was extracted with a mixture of chloroform–2-propanol (85:15). White crystals (mp 290°, dec.) were obtained, yield 92%. The NMR spectrum of the compound showed an additional broad singlet at δ = 4,9 in comparison to the starting compound proving the presence of an amino group. The last gives a positive Bratton–Marshall reaction proving its aromatic nature. The mass spectrum (Table II) confirms the proposed structure.

Anal.—Calc. for $C_{16}H_{15}N_3O_3$: C, 64.64; H, 5.09; N, 14.13. Found: C, 64.69; H, 5.13; N, 14.22.



Scheme II—Synthesis of 2-hydroxymethyl-3-(2′-methoxy-4′-nitrophenyl)-4(3H)-quinazolinone.

RESULTS

Rat Experiments—*Identification of Urinary Metabolites*—After counting the individual urine samples, the residual collected samples were pooled and extracted on columns[8], and 21–25% of the total urinary radioactivity was extracted. TLC of the extract using Solvent System C separated a compound with R_f = 0.30 (Metabolite I). The remaining radioactivity was located at the start of the chromatogram. Intact [^{14}C]nitromethaqualone (R_f = 0.6) could not be detected. The same plate was now run with Solvent System B allowing the detection of two additional radioactive zones with R_f = 0.7 (Zone II) and 0.61 (Zone III). This made it possible to locate on a plate the corresponding zones of metabolites in urine extracts of rats treated with unlabeled nitromethaqualone. The latter were applied in 10-cm bands across the plates. One centimeter of this band was overspotted with the extract from [^{14}C]nitromethaqualone-treated rats and the plates run in Solvent System B. After localizing the radioactive zones, the corresponding nonradioactive zones were scraped off and extracted with methanol. The solvent was evaporated and the residue examined by GC and GC–MS.

Metabolite I—The metabolite isolated from Zone I appeared to consist of one single spot on TLC with all solvent systems, A, B, and C. GC of the product, however, showed a broad peak of two maxima with R_t = 13.4 and 13.6 min, respectively. Following silylation with trimethylsilylimidazole[14], only one homogeneous peak was observed. The mass spectrum of the product recorded *via* solid probe (Table II,A) was different from the product recorded by GC–MS indicating that the metabolite decomposed under the described gas chromatographic conditions. The mass spectrum of Metabolite I recorded by the direct inlet method indicated a molecular ion at *m/z* 327 representing an addition of one oxygen to nitromethaqualone. The mass spectrum of the trimethylsilyl derivative (Table II,B) showed one silylated function, indicating the presence of an hydroxyl group in the molecule. This, however, is not located on one of the phenyl nuclei as could be shown from the fragmentation pattern of both silylated and underivatized metabolites. This metabolite proved to be identical with the synthesized 2-hydroxymethyl-3-(2′-methoxy-4′-nitrophenyl)-4(3*H*)-quinazolinone.

Metabolites II, III, and IV—The product isolated from Zone II was examined by GC and showed one homogeneous peak with an R_t value of 12.8 min (Metabolite II). The mass spectrum obtained with GC–MS (Table II,C) revealed a molecular ion at *m/z* 281 and a fragment ion at *m/z* 250 indicating the loss of a methoxy fragment. The metabolite showed a positive Bratton–Marshall reaction. All those characteristics

[14] Pierce Eurochemie NV, Rotterdam, Holland.

Table III—Excretion of Total Radioactivity in Urine and Feces of [^{14}C]Nitromethaqualone-Treated Rats [a]

Excretion, End of Period, hr	Dose Excreted, % Urine Rat I	Urine Rat II	Feces Rat I	Feces Rat II
24	10.0	10.2	9.8	13.1
48	23.5	18.2	45.5	26.3
72	26.0	22.0	56.8	48.1
96	27.0	24.0	58.7	54.2
120	27.2	24.4	59.7	54.9
144	27.3	24.6	59.9	55.0

[a] Expressed as cumulative percent.

Table IV—Percent of [^{14}C]Nitromethaqualone and Metabolites Present in the Direct Extracts from Rat Urine

Drug and Metabolites	Recovered Radioactivity (Extract, %)[a]
Nitromethaqualone	0
Metabolite[b] I	8
Metabolite II	40
Metabolite III	19
Metabolite IV	14
Not identified	19

[a] The values are averages of results on two rats. [b] Symbols correspond to metabolites given in the text.

were identical with those of synthesized 2-methyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone.

Zone III, isolated with Solvent System B, was reexamined with Solvent System A and consisted of two distinct products with R_f values of 0.45 and 0.52. In this way both compounds of Zone III were separated on a preparative scale and examined separately with GC and GC–MS. The zone with R_f = 0.52 on GC showed different peaks when analyzed without derivatization. Following silylation with trimethylsilylimidazole, one homogeneous peak eluted with R_t = 16.6 min. The mass spectrum of this product obtained with the direct inlet method indicated a molecular ion at *m/z* 297 (Table II,E). The silylated derivative showed one silylated hydroxyl function (Table II,D). Fragmentation patterns of both the underivatized metabolite and silylated derivative showed that this is not located on one of the phenyl nuclei of the molecule. The metabolite showed also a positive Bratton–Marshall reaction. These characteristics suggest 2-hydroxymethyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone as a possible structure for this metabolite. This structure was confirmed by synthesis of the expected metabolite (Metabolite III). The zone with an R_f value of 0.45 in Solvent System A showed one homogeneous peak with an R_t value of 43 min on GC (Metabolite IV).

The mass spectrum recorded by GC–MS indicated a molecular ion at *m/z* 323 (Table II,F). The transition *m/z* 292 to *m/z* 250 proved by the presence of a metastable peak corresponds to the loss of ketene and indicates the presence of an acetyl group in the molecule. The metabolite showed only a positive Bratton–Marshall reaction on the plate following acid hydrolysis, indicating the presence of an aromatic acetylamino group. Those characteristics suggest that Metabolite IV is the acetylated form of Metabolite II. The structure could be confirmed by synthesis of the metabolite. Table I represents the TLC and GC data of nitromethaqualone and its metabolites.

Quantitation of the Metabolites—The cumulative urinary and fecal excretion of nitromethaqualone after a single oral dose of 10-μCi [^{14}C]-nitromethaqualone is given in Table III. Within 6 days, 55–60% of the dose is excreted in the feces while 25–27% is excreted in the urine.

Aliquots of the collected urine samples were extracted on kieselguhr columns. In this way 21 and 25%, respectively, of the radioactivity present in the urine of the two rats could be extracted.

The concentrated extracts were chromatographed on silica gel plates using the described solvent. The radioactive zones were scraped off and counted as described. The quantitative amounts of the different metabolites present in definite areas are given in Table IV. Metabolite II is the major metabolite (40%). Unchanged nitromethaqualone could not be detected. The measured radioactivity (19%) remained at the start of the chromatogram and was not further examined. From the kieselguhr columns used for extraction, remaining polar metabolites, probably consisting of glucuronides, could not be eluted. Following enzymatic hydrolysis with β-glucuronidase of urine samples previously extracted with chloroform and ethyl acetate at pH 3.0 and 9.0, only Metabolites

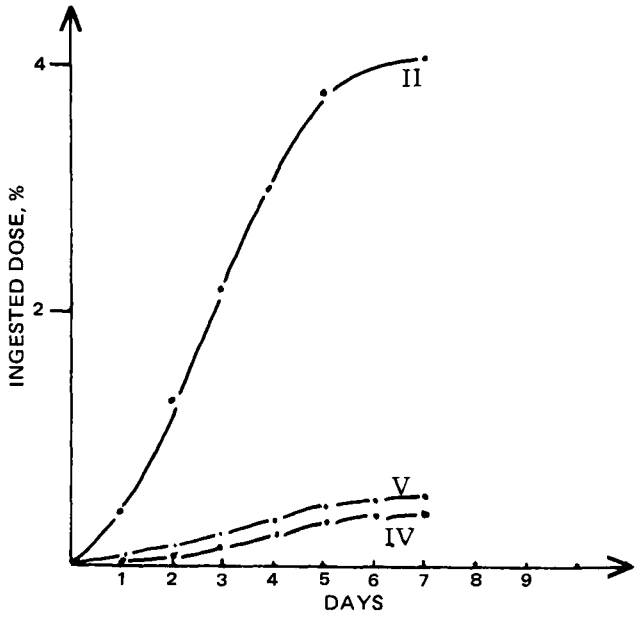


Figure 1—*Cumulative excretion graph of nitromethaqualone Metabolites II, IV, and V in humans.*

I and III could be identified in the same way as described above, proving their excretion as conjugates with glucuronic acid. No quantitative data, however, could be obtained due to the instability of the amino compound during the reaction procedure.

Human Studies—The metabolism in humans was studied only with unlabeled nitromethaqualone. Aliquots of the different urine samples were pooled and extracted as described. The extracts were examined by analytical and preparative TLC as for the study on urine from rats. Solvent System C was used for detecting unchanged nitromethaqualone and the hydroxymethyl derivative (Metabolite I). Neither nitromethaqualone nor Metabolite I could be detected in human urine. TLC with Solvent System B resulted in three spots showing a positive Bratton–Marshall reaction corresponding to R_f values of 0.90, 0.70, and 0.16 and one additional spot at R_f = 0.61 reacting positively only following acid hydrolysis on the plate. Preparative TLC allowed isolation of all four metabolites. The zone with R_f = 0.90 showed on GC one single homogeneous peak at 170°. The mass spectrum (Table II,G) revealed a molecular ion at *m/z* 168. Loss of 31 indicates the presence of an intact methoxy group.

Furthermore, a positive Bratton–Marshall reaction indicates the presence of an aromatic amino group. The spectrum is compatible with 2-methoxy-4-nitroaniline as a possible structure of this metabolite (Metabolite V). This product could be obtained from a commercial source[15] and proved to be identical with the isolated metabolite. The zone

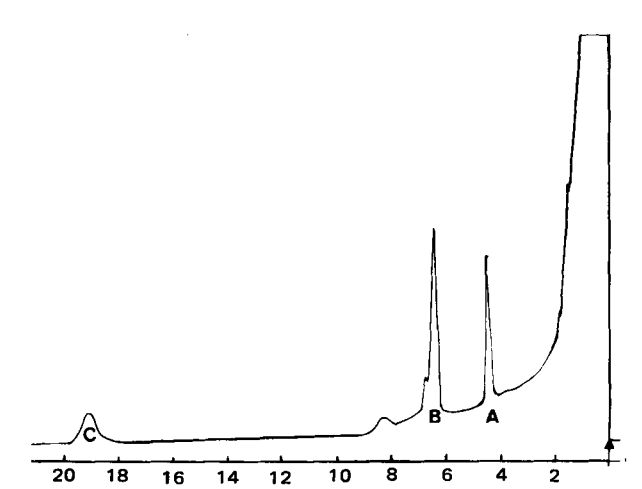
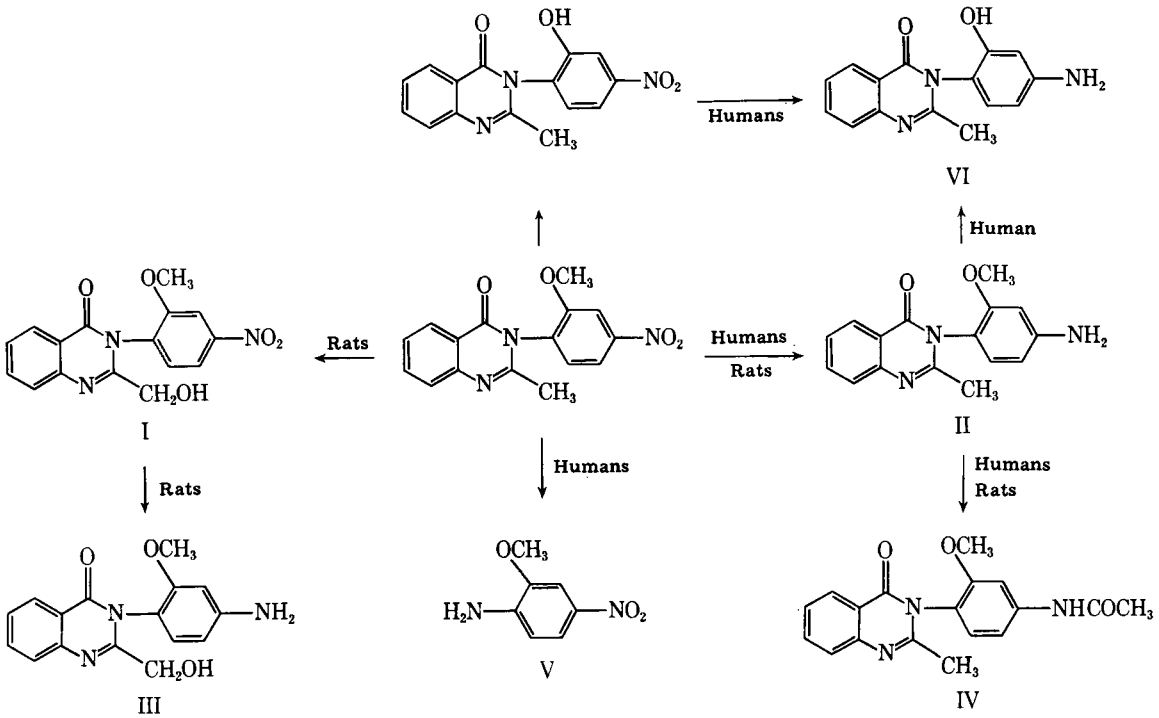


Figure 2—*Typical gas chromatogram from a urine extract. Key: (A) internal standard; (B) Metabolite III; (C) Metabolite IV.*

[15] Riedel-de-Haën AG, Seelze, Hannover, West Germany.



Scheme III—Metabolic pathways of nitromethaqualone in humans and rats.

with an R_f value of 0.70 showed one peak on GC at 260°. The mass spectrum proved to be identical with the spectrum of Metabolite II already identified in the urine of treated rats. Likewise, the zone with R_f = 0.61 appeared to be identical with Metabolite IV. The zone with R_f = 0.16 reacts not only positively with the Bratton–Marshall reagent but also with the Folin–Ciocalteu reagent. The product could only be analyzed by GC following methylation with diazomethane. The resulting peak showed the R_f value of Metabolite II along with an identical mass spectrum. The most important fragment ions of the mass spectrum of the metabolite prior to methylation are represented in Table II,H. The molecular ion shows at m/z 267. All these characteristics suggest 2-methyl-3-(2′-hydroxy-4′-aminophenyl)-4(3*H*)-quinazolinone as a possible structure (Metabolite VI). This structure was proved to be correct by synthesis. After enzymatic hydrolysis with β-glucuronidase, Metabolite VI could be detected in the extract. No quantitative data could be obtained, since the metabolite was not stable in the course of the reaction.

Cumulative excretion curves (Fig. 1) in humans were established for Metabolites II, IV, and V. Therefore, following administration of nitromethaqualone, urine samples were collected at regular intervals and the metabolite concentration per milliliter determined. Upon multiplication by the volume of urine excreted during the collecting period, the amount of metabolites was obtained. The additive amounts excreted for each collecting period were then plotted *versus* time of collecting intervals.

Metabolites II and IV were determined by GC. Therefore, 20-ml urine samples, to which 2-methyl-3-(4′-nitrophenyl)-4(3*H*)-quinazolinone had been added as an internal standard, were extracted as described above. The concentrated extracts were injected on a mixed phase 1.5% OV-17, 1.5% QF_1 column at 260° and detected with a nitrogen-phosphorus detector. Figure 2 shows a typical gas chromatogram of a urine extract. Metabolite V was determined by TLC. The violet color of the spots following a Bratton–Marshall reaction was measured on the plate in comparison with known amounts of the reference compound.

DISCUSSION

Nitromethaqualone is eliminated from rats and humans by biotransformation. No unchanged drug can be detected in urine samples. In both rats and humans, 2-methyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone is quantitatively the most important unconjugated metabolite. Both species excrete this metabolite partially in the acetylated form. In rats but not in humans, oxidation of the 2-methyl group to 2-hydroxymethyl-3-(2′-methoxy -4′ -nitrophenyl)-4(3*H*)-quinazolinone along with reduction of the nitro function of the latter to 2-hydroxymethyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone represents two other important metabolites, both being excreted also as conjugates with glucuronic acid. The slow urinary excretion along with the protracted fecal excretion of carbon 14 in the rats suggests an extensive enterohepatic circulation. In addition, human subjects excrete 2-methoxy-4-nitroaniline indicating cleavage of the quinazolinone nucleus. *O*-Demethylation followed by reduction of the nitro group is a minor metabolic pathway in humans (Scheme III). The slow urinary excretion in the urine of the formed metabolites in humans may be related to a continuous reabsorption of the biliary fraction. The method, as described for the extraction and analysis of urine extracts, using Solvent System B and the reagents for the Bratton–Marshall reaction, makes it possible to demonstrate nitromethaqualone intake even in a therapeutic dose. For legal practice, a GC–MS examination for the presence of the major metabolite in humans [2-methyl-3-(2′-methoxy-4′-aminophenyl)-4(3*H*)-quinazolinone] should confirm the TLC results.

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Guinea Pig Ear as a New Model for *In Vivo* Percutaneous Absorption

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Abstract □ A new animal model for *in vivo* percutaneous absorption utilizing the hairless, relatively thick skin of the guinea pig ear is proposed. Topical absorption studies were carried out with [^{14}C]hydrocortisone and [^{14}C]testosterone. Systemic studies were also conducted to correct for incomplete urinary excretion. In addition, a single stratum corneum correction factor was developed from published data to enable the guinea pig ear skin to be directly compared with human forearm skin. A comparison of human percutaneous absorption with the corrected guinea pig ear absorption shows a high correlation for both hydrocortisone and testosterone. The effects of ambient changes in relative humidity are also discussed with respect to *in vivo* percutaneous absorption.

Keyphrases □ Guinea pig ear—model for *in vivo* percutaneous absorption, hydrocortisone, testosterone □ Absorption—percutaneous, guinea pig ear, *in vivo* model, hydrocortisone, testosterone □ Hydrocortisone—guinea pig ear, model for *in vivo* percutaneous absorption, testosterone □ Testosterone—guinea pig ear, model for *in vivo* percutaneous absorption, hydrocortisone

Various animal species have been used to study percutaneous absorption *in vivo*. Percutaneous absorption rates of common laboratory rodents such as rats and rabbits have been studied (1). These studies have shown that the back skin of these animals absorb a selected number of substances much more rapidly than the ventral surface of the human forearm (2, 3). Higher species of mammals including hairless dogs (4), rhesus monkeys (5), and miniature swine (1) have also been studied. Of these species, it has been concluded (6) that the rhesus monkey and the miniature swine percutaneous absorption characteristics correlated best with human penetration. The obvious disadvantage of using swine and monkeys include cost and the need for special animal facilities.

One important determinant of percutaneous absorption is the thickness of the stratum corneum. If two areas of skin are sufficiently similar, then one would expect percutaneous absorption to be inversely proportional to the number of stratum corneum layers in the skin. The percutaneous absorption of hydrocortisone from a number of different anatomical sites in humans has been determined previously (7). The number of stratum corneum layers at these sites has also been determined (8). If a proportional relationship between thickness and absorption can be established in humans, this provides a rationale to correct animal model data for thickness. However, the problem with most animal models is that skin with fur generally has a thin stratum corneum (9). Consequently, changes of one or two layers could cause corrections as much as 50% if the stratum corneum is very thin. An ideal animal surface would have to be relatively large and essentially hairless. The guinea pig ear appeared to meet the desired criteria.

EXPERIMENTAL

Male albino guinea pigs, 250–300 g, were individually acclimated for 1 day in metabolism cages[1]. Water and guinea pig chow were given *ad libitum*. For topical application of [^{14}C]hydrocortisone (I) or [^{14}C]testosterone (II), the animals were lightly anesthetized in a bell jar with methoxyflurane[2]. One ear of an animal was sandwiched with medium pressure between two halves of Karush-type dialysis chambers without caps[3]. A 4 μg/cm^3 volume of one of the ^{14}C-labeled compounds reconstituted in acetone was applied in a 40-μl volume to a 1.77-cm^2 area with a flat tip 50-μl syringe[4] to the dorsal surface of the ear. After the acetone had evaporated, the dialysis chambers were removed, and the animals were returned to their metabolism cages. For systemic administration, the ^{14}C-compounds were solubilized first in 50 μl of ethanol and then in 1 ml of sterile saline. The animals were then dosed intraperitoneally with a 0.5-ml volume.

Urine samples were collected daily. The urine was acidified with glacial acetic acid to a pH of 5 to solubilize the magnesium and calcium phosphate precipitates. A 1-ml aliquot was counted on a liquid scintillation counter[5] using 10 ml of liquid scintillation cocktail[6]. The internal standard method using [^{14}C]toluene[7] was used to correct for quench. Relative humidity readings were taken daily using a wet–dry thermometer.

RESULTS

Thickness of the Stratum Corneum and Absorption—Table I presents the determination of:

$$N \times A = K \qquad \text{(Eq. 1)}$$

where N is the number of cell layers in the stratum corneum (8), A is the percent of drug absorbed in 5 days (7), and K is the inverse proportionality constant. These results from human data show that K (forearm) differs from K (back) by only 9.8%. It would be desirable to have more data to confirm this result for different compounds besides hydrocortisone in humans. Nevertheless, one form of verification would be to use a single correction factor to relate guinea pig ear data to the human forearm for a number of different compounds. If Eq. 1 holds, then this

[1] Nalge Co., Rochester, N.Y.
[2] Abbott Lab., North Chicago, Ill.
[3] Belco Glass Co., Vineland, N.J.
[4] Hamilton.
[5] Model 3385 Tri-Carb.
[6] Fisher Scientific Co., Fair Lawn, N.J.
[7] New England Nuclear, Boston, Mass.

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