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QUANTITATION OF N,N,N′,N′-TETRAKIS(2-HYDROXYPROPYL)-ETHYLENEDIAMINE IN PLASMA BY GAS CHROMATOGRAPHY

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SUMMARY

A wide-bore capillary gas chromatographic method with nitrogen-selective thermionic detection is described for the quantitative analysis of N,N,N′,N′-tetrakis(2-hydroxypropyl)ethylenediamine (Quadrol) in plasma. N,N,N′,N′-tetrakis(2-hydroxybutyl)ethylenediamine is used as an internal standard. Rat or human plasma samples (0.5 ml) are mixed with internal standard, adjusted to alkaline pH and subjected to a single extraction with dichloromethane. Quadrol recovery from plasma typically exceeds 90%. The method is linear over the range 1.0–50 μg/ml. The working detection limit is 0.5 μg/ml and the analysis time is under 7 min. The procedure has been used to obtain plasma concentration versus time data for the evaluation of Quadrol pharmacokinetics in rats.

INTRODUCTION

Aliphatic N-hydroxyalkyldiamines have been shown to possess useful biological properties. Wilkinson and co-workers [1–3] have reported that *d*-2-2′-(ethylenediimino)-di-1-butanol (Ethambutol, I, Fig. 1) exhibits highly selective antituberculin activity. Ethambutol hydrochloride is currently used as a therapeutic agent, typically in conjunction with isoniazid. Another compound, N,N-dioctadecyl-N′,N′-bis(2-hydroxyethyl)propane-1,3-diamine (Avridine, II, Fig. 1), was first reported as a macrophage stimulator and interferon inducer in mice [4] and was later shown to enhance nasal interferon production in rhinovirus-infected humans [5] and reduce associated symptoms [6]. Avridine has also been used as an adjuvant in the treatment of herpes simplex virus [7] and simian malaria [8].

Recently, it was reported that N,N,N′,N′-tetrakis(2-hydroxypropyl)ethylenediamine (Quadrol, I, Fig. 2) stimulates macrophage spreading and enhances phagocytotic activity in vitro [9,10]. Bhide et al. [11] also observed that Quadrol increases collagen deposition in wounds during wound healing experiments involving both normal and diabetic mice. Because Quadrol exhibits

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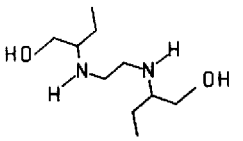
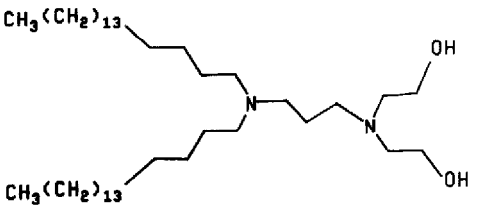
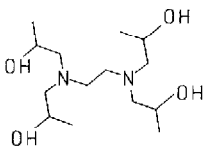


I. ETHAMBUTOL

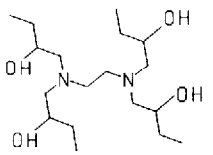


II. AVRIDINE

Fig. 1. Chemical structures of Ethambutol and Avridine.



I. QUADROL



II. INTERNAL STANDARD

Fig. 2. Chemical structures of Quadrol and the internal standard.

biological activity, an investigation of its pharmacological and pharmacokinetic properties was initiated. Consequently, a method was needed to assay plasma Quadrol. A review of the literature revealed no suitable quantitative plasma assay for Quadrol. Several gas chromatographic (GC) methods have appeared [12–15] for the quantitation of Ethambutol, however, the procedures typically involve the formation of trimethylsilyl or trifluoroacetyl derivatives and the use of packed-column technology. This paper describes a simple (single extraction and no derivatization), rapid capillary GC procedure employing nitrogen-selective thermionic detection for the quantitative analysis of Quadrol in plasma.

EXPERIMENTAL

Reagents

Dichloromethane, toluene, diethyl ether, methanol, hexane, anhydrous sodium sulfate and sodium hydroxide were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Ethylenediamine, propylene oxide and 1,2-epoxybutane (gold label grade) were purchased from Aldrich (Milwaukee, WI, U.S.A.). Anhydrous ethanol was obtained from Quantum Chemical (Cincinnati, OH, U.S.A.) and dimethyldichlorosilane was purchased from Supelco (Bellefonte, PA, U.S.A.).

N,N,N′,N′-Tetrakis(2-hydroxypropyl)ethylenediamine (Quadrol) was synthesized by slowly adding propylene oxide (2 g, 34 mmol) to 3 ml of a solution of ethylenediamine (0.2 g, 3.4 mmol) in ethanol. The reaction mixture was contained in a flask with an attached dry ice condenser and maintained at 90°C for 6 h. After concentration in vacuo, the resultant oil was dissolved in diethyl ether and dried over anhydrous sodium sulfate. After filtration and concentration in vacuo, a colorless oil was obtained (0.9 g, 3.1 mmol) in 90% yield. The product was purified by fractional distillation at 175°C at 0.8 mmHg. NMR (^2H_2O), δ 3.9 (4H, hextet), 2.1–2.4 (12H, m) and 1.1 (12H, d).

The internal standard, N,N,N′,N′-tetrakis(2-hydroxybutyl)ethylenediamine (HBQ, II, Fig. 2), was prepared by mixing ethylenediamine (4.36 g, 75 mmol) with 2 ml of ethanol in a four-necked flask and slowly adding 1,2-epoxybutane (50 ml, 580 mmol) over 2.5 h under a nitrogen atmosphere at 90°C. After complete addition of the epoxide, the mixture was refluxed for 20 h. The work-up was the same as described for Quadrol. The product, a colorless oil (24.9 g, 71 mmol), was obtained in 95% yield. NMR (C^2HCl_3), δ 4.7 (4H, broad), 3.6 (4H, m), 2.2–2.5 (12H, m), 1.4 (8H, pentet), 0.9 (12H, t).

Stock solutions

Stock solutions of both Quadrol and the internal standard (1 mg/ml) were prepared separately by dissolving 1.00 g of each into a total volume of 1 l of deionized water. A 100 μg/ml stock solution of Quadrol in human plasma was prepared by adding 5.0 ml of the stock aqueous Quadrol solution to drug-free human plasma to make 50 ml of solution. Working plasma standards (containing 1, 5, 10, 20 and 50 μg/ml Quadrol) were prepared by appropriate serial dilutions of the stock plasma standard. All plasma standards were frozen until used. A working aqueous internal standard solution (0.3 mg/ml) was prepared by appropriate dilution of the stock HBQ solution.

Sample preparation

All glassware was silanized with a 5% (v/v) solution of dimethyldichlorosilane in toluene. After soaking for 10 min in the silanization reagent, glassware was immediately rinsed with toluene and then methanol.

Plasma (0.5 ml) was transferred into a culture tube (100×13 mm) containing 100 μl (30 μg HBQ) of the working internal standard solution and 200 μl of 20% (w/v) aqueous sodium hydroxide solution. After vortex-mixing briefly, 5 ml of dichloromethane were added and the tube was sealed with a PTFE-lined cap.

Samples were extracted by repeated inversion for 5 min, and then the phases were separated by centrifugation for 10 min at 2000 *g*. The aqueous layer was removed by aspiration, and the organic solvent was decanted into a clean 100×13 mm culture tube. The organic phase was treated with anhydrous sodium sulfate, centrifuged for 5 min at 2000 *g* and then carefully decanted into a 5-ml conical microreaction vial (Pierce, Rockford, IL, U.S.A.). The vials were placed in a Reacti-Therm III unit (Pierce) adjusted so that water placed in the wells of the aluminum blocks was at 40°C. The solvent was evaporated to dryness under a stream of dry nitrogen. The remaining residue was reconstituted with 100 μl of a 50% (v/v) ethanol–methanol mixture and mixed well. A 0.3–0.5 μl aliquot was injected on the column.

Recovery of Quadrol from plasma

Two plasma standards (25 ml each), one spiked with 10 μg/ml and the other with 50 μg/ml Quadrol (Q), were prepared. Triplicate 0.5-ml aliquots of each standard were mixed with 0.2 ml of either 0.5 *M* phosphate buffer (pH 7), 0.5 *M* bicarbonate buffer (pH 10) or 20% sodium hydroxide (pH > 13) and then extracted with 5.0 ml of either toluene, diethyl ether, hexane or dichloromethane (DCM). The sample was then processed as indicated under *Sample preparation*. The extraction residue was reconstituted with 100 μl of 50% (v/v) ethanol in methanol containing 0.3 mg/ml HBQ. The peak-height ratio, $(Q/HBQ)_S$, was determined for each aliquot assayed. A '100 recovery' reference sample for each standard was prepared by adding an appropriate volume of a methanolic Quadrol solution (50 μg/ml) to a microreaction vial, evaporating the methanol under a stream of dry nitrogen and reconstituting the residue as indicated for the plasma standards. The peak-height ratio of the reference sample, $(Q/HBQ)_R$, was measured and compared to $(Q/HBQ)_S$ for the plasma standards, and the percentage recovery was calculated as $[(Q/HBQ)_S/(Q/HBQ)_R] \times 100$.

Chromatography

A Hewlett-Packard Model 5890 gas chromatograph equipped with a nitrogen–phosphorus thermionic detector and a HP 3396A recording integrator (Avondale, PA, U.S.A.) was used. The gas chromatograph was fitted with a methylsilicone (HP-1) wide-bore capillary column (5 m×0.33 mm I.D., 2.65 μm film thickness, Hewlett Packard) and a silanized 3.2 mm I.D. injection port liner. The helium carrier gas was channeled through a high-capacity heated gas purifier (Supelco) placed in-line near the injection port. The column head pressure was set at 130 kPa at 150°C and this produced a carrier flow-rate of 25 ml/min. The oven temperature was ramped from 150 to 180°C at 5°C/min. The injection port and detector were set at 250 and 300°C, respectively. The nitrogen–phosphorus detector bead voltage was set between 15 and 20 V. Samples were injected into the gas chromatograph with a 1.0-μl capacity Hamilton syringe (Supelco).

Quantitation

A calibration curve was prepared by plotting peak-height ratio Q/HBQ versus plasma Quadrol concentration (μg/ml) for a series of spiked plasma standards

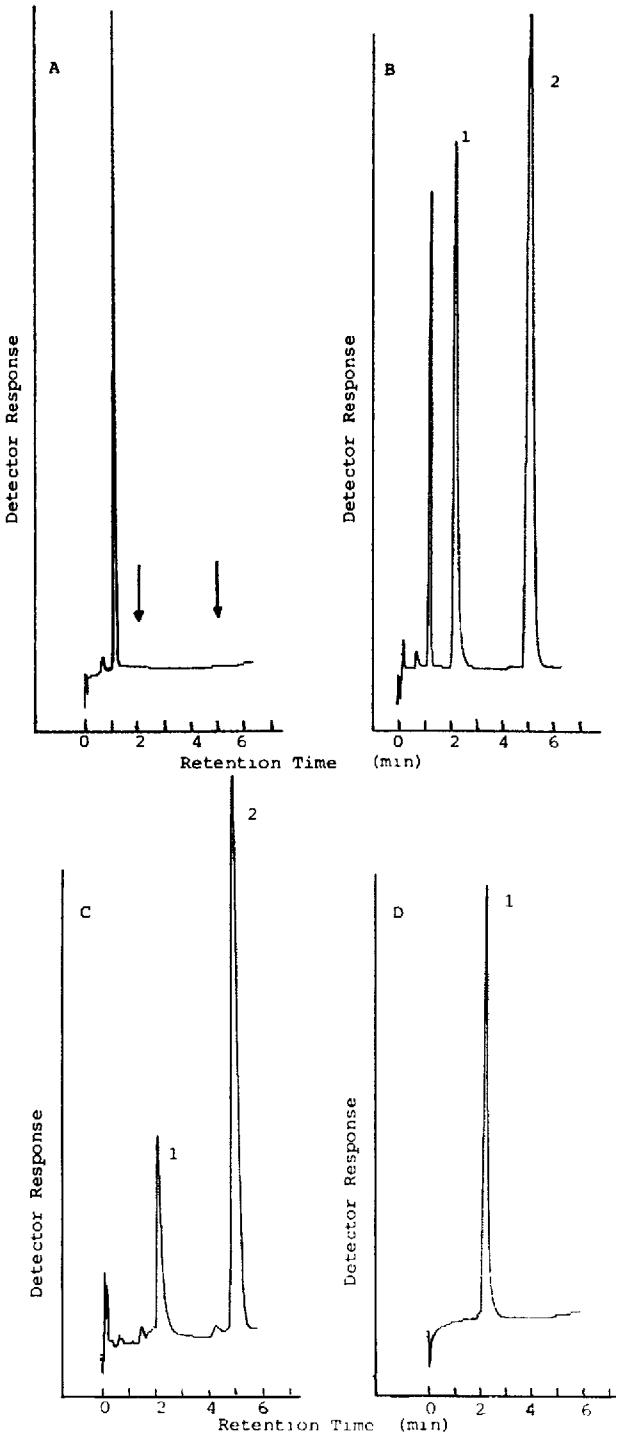


Fig. 3. Chromatograms of (A) blank human plasma, (B) 20 μg/ml spiked plasma sample, (C) rat plasma sample 20 min after a 100 mg/kg oral dose of Quadrol and (D) pure Quadrol. Peaks: 1 = Quadrol; 2 = internal standard (HBQ). Note: blank rat plasma is missing the peak eluting at 1 min in blank human plasma; otherwise, the chromatograms are essentially the same.

(1.0–50 μg/ml). The linear regression slope from the calibration curve was used to calculate the Quadrol concentration in plasma samples obtained from Quadrol-dosed rats. Within-day and between-day precision was evaluated with multiple analyses of plasma standards over a fourteen-day period.

RESULTS AND DISCUSSION

Representative chromatograms obtained from extracted plasma samples and purified Quadrol are presented in Fig. 3. Retention times for Quadrol and the internal standard (I and II, Fig. 2) were 2.0 and 5.0 min, respectively. The peaks were sharp, well resolved and symmetrical. No substances were observed to co-elute with either Quadrol or the internal standard when blank rat or human plasma samples was analyzed.

The calibration curve exhibited good linearity over the range of concentrations from 1.0 to 50 μg/ml Quadrol. The results are shown in Table I. Precision data for within-day and between-day analyses are summarized in Table II.

Quadrol recovery from plasma after a single extraction using various organic

TABLE I

LINEARITY OF THE QUADROL ASSAY

Spiked concentration (μg/ml)	Quadrol/HBQ peak-height ratio (mean ± S.D.)	Calculated mean concentration[a] (μg/ml)
1.0	0.038 ± 0.005	0.87
5.0	0.203 ± 0.008	4.7
10.0	0.203 ± 0.006	9.6
20.0	0.838 ± 0.026	19.2
50.0	2.17 ± 0.080	49.8

[a]Mean concentrations calculated based on the linear regression slope; linear regression analysis: slope, 0.043; intercept, −0.015; r^2, 0.997.

TABLE II

PRECISION OF THE QUADROL ASSAY

Aliquots (0.5 ml) of plasma samples spiked at the indicated concentrations were analyzed.

Quadrol concentration (μg/ml)	Quadrol/HBQ peak-height ratio (mean ± S.D.)	Coefficient of variation (%)
Within-day (n = 10)		
5.0	0.21 ± 0.013	6.2
20 0	0.83 ± 0.041	4.9
Between-day (n = 5)		
5.0	0.23 ± 0.017	7.4
20.0	0.84 ± 0.064	7.6

TABLE III

QUADROL RECOVERY FROM PLASMA

Plasma Quadrol concentration (μg/ml)	Plasma pH	Recovery (mean ± S.D., $n=3$) (%)			
		Ether	Toluene	Hexane	DCM
10	7	1.2 ± 0.3	0.0	0.0	0.8 ± 0.4
	10	8.7 ± 1.9	3.4 ± 1.1	0.9 ± 0.4	0.8 ± 0.4
	13	95 ± 7.9	12 ± 2.8	2.7 ± 1.2	96 ± 4.7
50	7	2.6 ± 0.9	0.0	0.0	1.6 ± 0.9
	10	9.8 ± 1.6	4.6 ± 1.0	1.1 ± 0.3	71 ± 7.1
	13	97 ± 8.4	16 ± 3.9	4.4 ± 0.9	95 ± 6.6

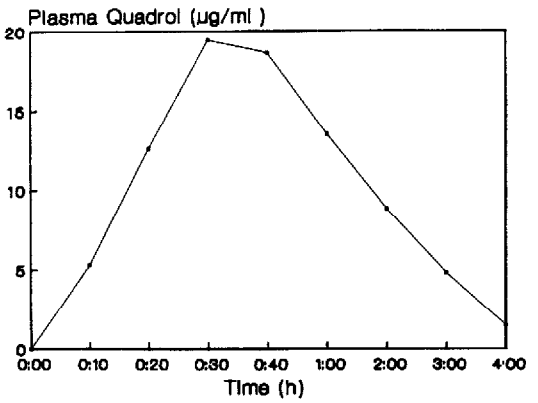


Fig. 4. Representative plasma concentration (C_p) versus time profile of Quadrol following on oral dose of 100 mg/kg in a male Sprague–Dawley rat.

solvents was evaluated at both 10 and 50 μg/ml (see Experimental). The data are presented in Table III. The results indicate that Quadrol is efficiently transferred into diethyl ether or DCM when the pH > 13. Toluene and hexane are poor extraction solvents (recovery < 20%) even at high plasma pH. Quadrol extracted poorly from plasma below pH 10 regardless of the solvent chosen. DCM was chosen over diethyl ether for this study due to its lower volatility and flammability.

The working detection limit was 0.5 μg/ml (twice the signal-to-noise ratio) using the normal range (4 on the GC) and attenuation (3 on the HP 3396). By decreasing the reconstitution volume and the attenuation setting, as little as 0.1 μg/ml could be detected. If the sample size is increased to 1.0 ml, greater sensitivity is possible. However, obtaining 1.0-ml serial plasma samples from rats is not practical during short time intervals.

This method has been used to obtain plasma concentration (C_p) versus time data from rats given oral doses of Quadrol. A typical C_p versus time plot after a single 100 mg/kg oral dose of Quadrol is shown in Fig. 4. The plasma Quadrol typically reached a maximum between 25 and 40 min post-dose, and the half-life ($t_{1/2}$) was 1.0–1.5 h. C_p versus time data for intraperitoneal, subcutaneous and

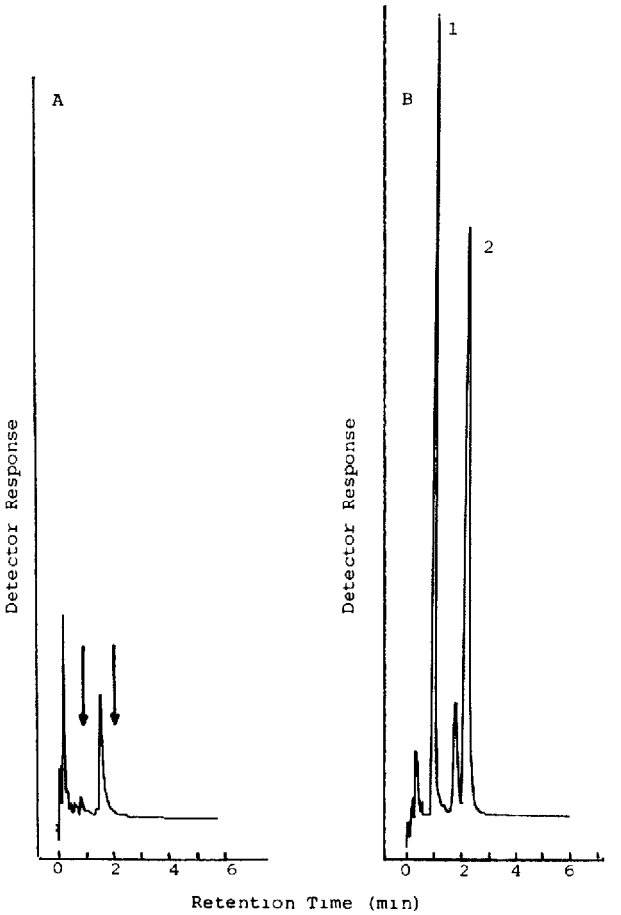


Fig. 5. Chromatograms of (A) urine extract from a control (undosed) male Sprague–Dawley rat and (B) extract of a 24-h urine sample from a male Sprague–Dawley rat after a 100 mg/kg oral dose of Quadrol. Peaks: 1 = suspected Quadrol metabolite; 2 = Quadrol.

intravenous routes of administration are currently being collected for thorough pharmacokinetic studies of Quadrol in rats (results to be published later). Additionally, evidence suggesting the existence of a Quadrol metabolite is presented in Fig. 5. GC analysis of the urine from Quadrol-dosed rats (100 mg/kg) indicates the presence of an early-eluting substance (Fig. 5B, peak 1) which is not present in urine from control rats. An investigation designed to identify and characterize the possible metabolite is currently in progress.

This paper describes a simple and rapid procedure for the quantitation of Quadrol in plasma. The assay has been used in the evaluation of Quadrol pharmacokinetics in rats and may be applicable to the analysis of Quadrol in other biological fluids.

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