Journal of Chromatography, 381 (1986) 127—135
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3195

MINOXIDIL ANALYSIS IN HUMAN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

APPLICATION TO PHARMACOKINETIC STUDIES

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(First received October 31st, 1985; revised manuscript received April 10th, 1986)

SUMMARY

A method is described for determination of minoxidil in human plasma using high-performance liquid chromatography with electrochemical detection. The method is specific and sensitive (500 pg/ml), however, minoxidil and minoxidil sulfate cannot be differentiated due to rapid autohydrolysis of minoxidil sulfate to minoxidil. The extraction procedure employs a C_{18} preparatory column to remove endogenous plasma constituents which would interfere with the assays. The calibration curves are linear for concentrations from 500 pg to 10 ng/ml. Within-day and between-day reproducibility are satisfactory with coefficient of variation less than 5.7% for all concentrations. Sample recovery from extraction is consistent at 45 to 55% at low and high concentrations, respectively. A pharmacokinetic study in a hypertensive volunteer receiving two different oral doses of minoxidil (1.25 and 2.5 mg) on different occasions demonstrates the utility of the method.

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0378-4347/86/$03.50

INTRODUCTION

Minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide) is a potent vasodilator used primarily in the treatment of severe refractory hypertension [1]. In addition, it has recently undergone evaluation as a topical agent in hair regrowth [2, 3]. The renewed interest in the prolonged hypotensive effect of minoxidil in relationship to its presence in the plasma has led to the discovery of an active metabolite [4, 5], minoxidil sulfate. The only method available for analysis of minoxidil in human serum has been radioimmunoassay [6], but unfortunately, extent of cross-reactivity of minoxidil metabolites and endogenous substances has been impossible to establish. Therefore, a sensitive and specific assay has long been needed for the determination of minoxidil. With continued advances in detecting picogram amounts of substances by an electrochemical detector, and with evidence in the literature that minoxidil can be oxidized or reduced by differential pulse polarography [7], we have developed a sensitive and selective method of detection of minoxidil by high-performance liquid chromatography (HPLC) using electrochemical detection in the oxidative mode.

EXPERIMENTAL

Apparatus and chromatographic conditions

The HPLC system consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model A-6000 solvent delivery system with a Waters Assoc. Model U6K sample loop. The separation system was a 30 cm × 3.9 mm stainless-steel (10 μm) μBondapak C_{18} reversed-phase column (Waters). The detector was an ESA Model 5100 A (Environmental Sciences Assoc., Bedford, MA, U.S.A.) dual-electrode coulometric electrochemical detector. The guard cell was ESA Model 5020 and the detector cell ESA Model 5011. Guard cell voltage was +0.90 V, detector 1 of the dual-electrode analytical cell +0.30 V, and detector 2, +0.80 V. Signal from detector 2 was used for detector output, which was quantitated on a Fisher (Fair Lawn, NJ, U.S.A.) Recordall Model 5000 chart recorder. Mobile phase (distilled water—acetonitrile, 98:11, adjusted to pH 3.0 with phosphoric acid) was run at a flow-rate of 1.5 ml/min. All analyses were performed at room temperature.

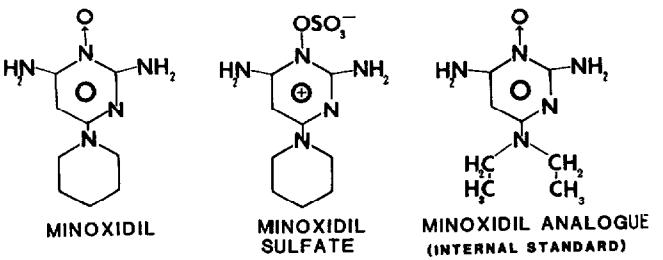

Fig. 1. Molecular structures of minoxidil, minoxidil sulfate and internal standard.

Reagents

Minoxidil, minoxidil sulfate, and an analogue of minoxidil were kindly provided by the Upjohn Company (Kalamazoo, MI, U.S.A.) (Fig. 1). All other reagents of analytical grade or better were purchased from commercial sources without further purification. Water was deionized, double-distilled, and the prepared mobile phase was filtered through a 0.2-μm filter (Rainin Instrument Co., Woburn, MA, U.S.A.) and degassed under vacuum.

Reference standards

Standard solution was prepared by dissolving 10 mg of minoxidil in 100 ml methanol, 5 mg of minoxidil sulfate in 50 ml methanol, and 2.8 mg of the internal standard in 50 ml of methanol. Sequential dilutions of minoxidil and minoxidil sulfate to 0.1 μg/ml were done with water. Minoxidil was further diluted to 0.01 μg/ml. Internal standard was diluted to 0.056 μg/ml with water. Stock solutions of minoxidil and the minoxidil analogue were stored at 4°C and were stable for four months. Minoxidil sulfate is unstable in aqueous or methanolic solution, and undergoes autohydrolysis within 24 h.

Preparation of sample

A close structural analogue of minoxidil was used as an internal standard (Fig. 1). A 50-μl volume of the 0.056 μg/ml stock solution containing 2.8 ng of the internal standard was added to a series of glass borosilicate disposable culture tubes (13 mm × 100 mm) (Fisher, Fair Lawn, NJ, U.S.A.). A 0.5- or 1.0-ml volume of unknown plasma sample was added to each tube. All tubes were agitated for 15 s on a vortex mixer (medium speed). A minoxidil calibration curve was performed by adding the following concentrations: 500 pg/ml (0.01 μg/ml stock), 1 ng, 2 ng, 5 ng and 10 ng (0.1 μg/ml stock) of minoxidil to consecutive tubes containing 1 ml of blank plasma. One blank sample, taken from the patient being studied prior to drug administration, was analyzed with the calibration standards and each set of unknown samples to ensure endogenous plasma contaminants did not interfere with internal standard or minoxidil chromatographic peaks.

Extraction procedure

After tubes were vortexed, a Fisher C_{18} Prepsep extraction column was conditioned with 2 volumes of methanol, followed by 2 vols. of distilled water. The samples were then transferred from the glass culture tubes to the Prepsep columns via borosilicate pasteur pipets.

Samples were adsorbed to the column by applying mild vacuum. The column was then washed with 2 volumes of distilled water. Following this, the column was washed with 6 ml acetone to remove non-polar contaminants. Minoxidil and the internal standard were then eluted with ten 200-μl fractions of methanol. Eluent was collected in clean glass culture tubes (13 mm × 100 mm) and dried at 50°C under a stream of nitrogen. Residue was reconstituted with 100 μl of mobile phase and vortexed for 10 s. Of this solution 10—60 μl were injected directly into the liquid chromatograph.

Clinical pharmacokinetic study

A hypertensive male patient received single oral doses (1.25 and 2.5 mg) of minoxidil on two occasions after giving written informed consent. Plasma samples were obtained at multiple time points following drug administration. Plasma was stored at −20° C until time of analysis. Samples were extracted as described above and plasma minoxidil concentrations determined. Elimination half-life was determined by linear regression analysis and area under the plasma concentration—time curve by the trapezoidal method. Derived pharmacokinetic parameters are shown in Table I.

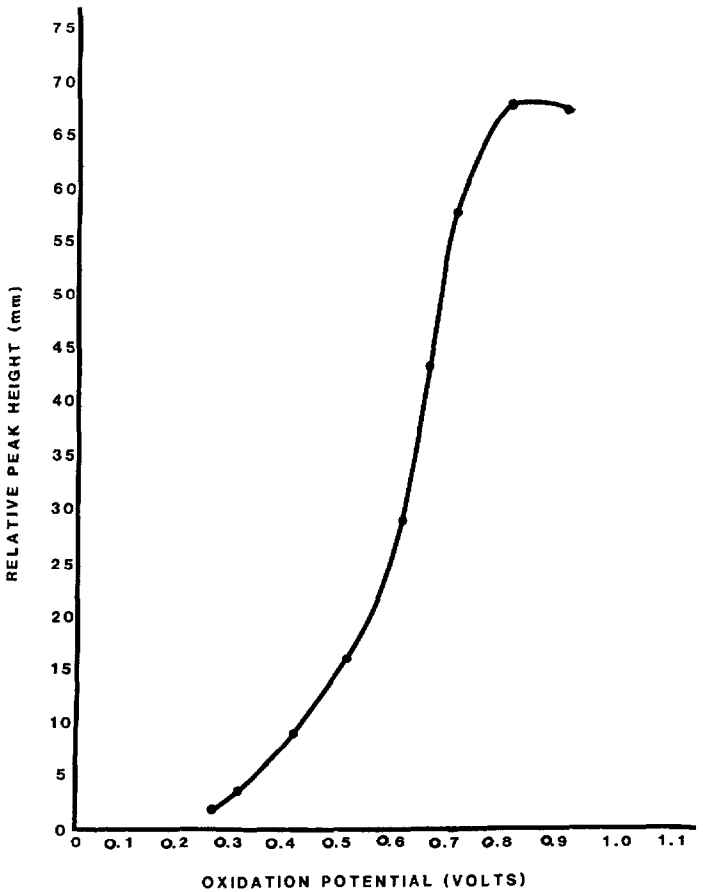

Fig. 2. Extent of electrochemical oxidation with increasing oxidation potential of the analytical cell for minoxidil.

RESULTS

Evaluation of method

Minoxidil and the internal standard both are maximally oxidized at +0.80 V (Fig. 2). The retention times of internal standard and minoxidil are 11 and 13 min, respectively, with good peak resolution (Fig. 3).

Minoxidil sulfate cannot be determined separately or used in the calibration curve because it undergoes autohydrolysis back to minoxidil within 24 h at room temperature or −20°C. Fig. 4 shows the presence of two peaks after the injection of 20 μl of minoxidil sulfate. The first peak corresponds with minoxidil, while the second peak is consistent with pure minoxidil sulfate. Within the next few hours, the minoxidil peak increases and the minoxidil sulfate peak rapidly decreases in relative peak height when minoxidil sulfate is

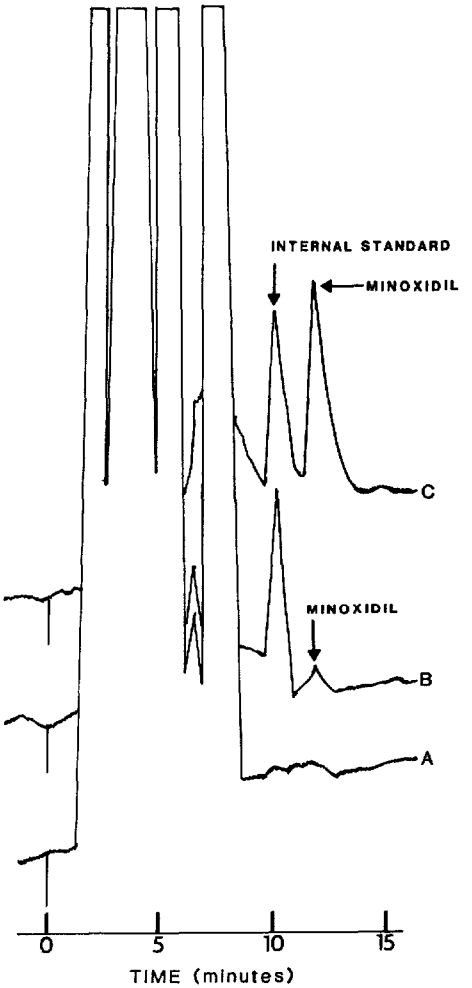

Fig. 3. Chromatograms of extract of 1 ml of blank plasma (A), an extract of 1 ml of plasma to which internal standard and 500 pg of minoxidil had been added (B); and unextracted standards of minoxidil and internal standard (C).

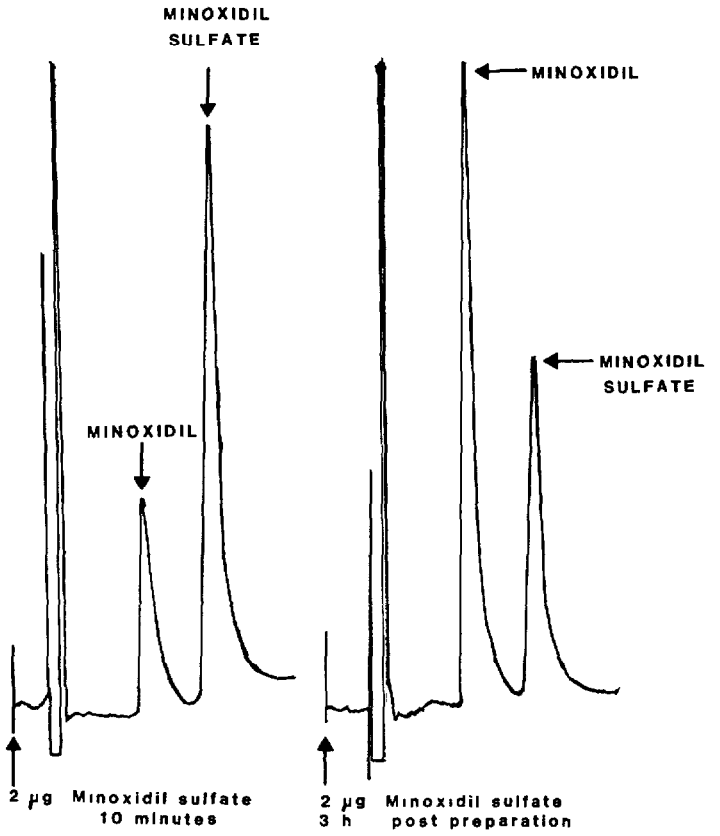

Fig. 4. Chromatograms of minoxidil and minoxidil sulfate showing rapid autohydrolysis of minoxidil sulfate during chromatography.

simply left in aqueous solution at room temperature of at −20°C. Therefore, under the conditions of storage and handling, all minoxidil sulfate in patient plasma samples has hydrolyzed to the parent drug prior to analysis. This was verified by spiking blank plasma with minoxidil sulfate, extracting immediately, at which time a large peak for minoxidil sulfate and a small peak for minoxidil were noted. The sample was then stored at −20°C overnight and reanalyzed 24 h after the initial preparation. At this time, only minoxidil was detected in a quantitatively appropriate amount. Fig. 5 shows the linear increase in minoxidil, and decrease in minoxidil sulfate peak height obtained from a series of injections over 3 h. The minoxidil sulfate sample used was established pure by melting point determination (reported minoxidil sulfate m.p. 188°C, our sample, 186—188°C; minoxidil m.p. 251°C).

Nine standard curves performed over a two-month period indicated a correlation coefficient was always greater than 0.998. Day-to-day coefficient of variation (C.V.) in the slope of the calibration curve was 5.7%. The limit of detection of this method is 500 pg/ml of a 1-ml extracted plasma sample. Within-day C.V. values for identical samples were at 500 pg/ml, 5.1% (n = 4); 1 ng/ml, 5.2% (n = 4); 2 ng/ml, 4.2% (n = 4); 5 ng/ml, 1.5% (n = 5); and 10 ng/ml, 0.69% (n = 5). Residue analysis indicated the extraction of minoxidil

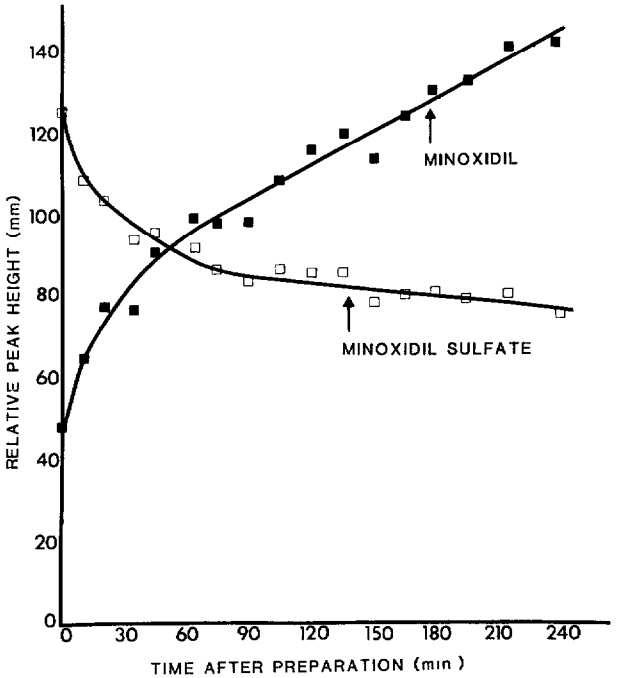

Fig. 5. Time course of disappearance of minoxidil sulfate and appearance of minoxidil with sequential injections of an aqueous minoxidil sulfate solution.

at 500 pg/ml was 46.4% and at 10 ng/ml was 54%; extraction of the internal standard at the concentration employed (2.8 ng/ml) was 47.1%.

Finally, the pharmacokinetic data on the two sets of plasma samples obtained from administration of 1.25- and 2.5-mg oral doses to the same hypertensive volunteer demonstrate parallel terminal elimination slopes (0.394 h^{-1}, 0.395 h^{-1}) and identical terminal elimination half-life (1.76 h for both doses) (Fig. 6). Area under the plasma concentration—time curve was 60.5 ng/ml · h for the 1.25-mg dose and 116 ng/ml · h for the 2.5-mg dose (Table I).

DISCUSSION

The described method for the determination of minoxidil in human plasma has a limit of detection of 500 pg and is selective for minoxidil. Minoxidil concentration determined represents the sum of concentrations of minoxidil and minoxidil sulfate that was initially present in the plasma due to the rapid autohydrolysis of minoxidil sulfate back to minoxidil in aqueous solutions. A number of methods were utilized to try to prevent this hydrolysis (rapid sample preparation, freezing of samples) all without success. Using the described chromatographic conditions, minoxidil sulfate is separated from the parent drug and internal standard; therefore, any residual minoxidil sulfate in the extracted sample would be detected and the sample would then be left at room temperature overnight until hydrolysis was complete. We have currently analyzed approximately 150 patient samples using this method and have not yet detected minoxidil sulfate without any further hydrolysis procedure.

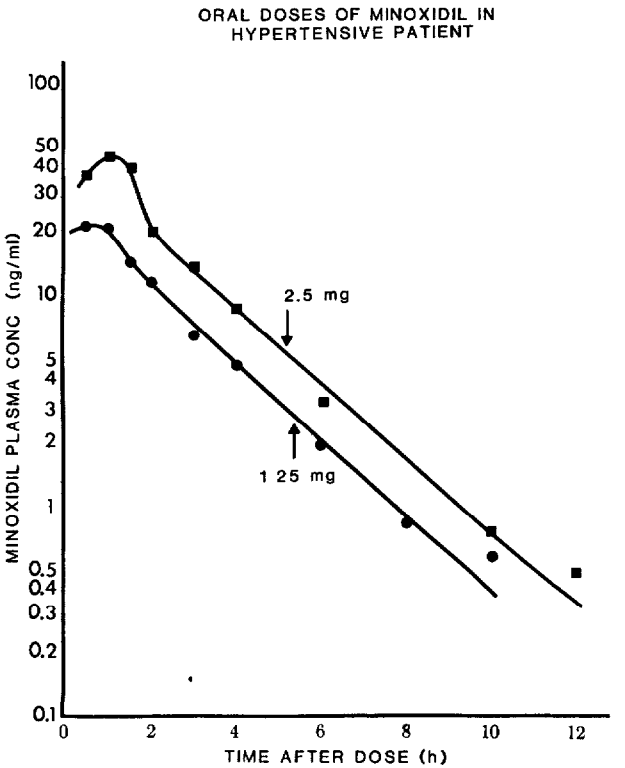

Fig. 6. Plasma minoxidil concentrations and the derived pharmacokinetic functions following oral minoxidil (1.25 and 2.5 mg) doses to a hypertensive male volunteer. See Table I for derived pharmacokinetic variables.

TABLE I

MINOXIDIL PHARMACOKINETIC VARIABLES AFTER SINGLE ORAL DOSES TO A 61-YEAR-OLD HYPERTENSIVE MALE

Parameter	Dose (mg)	
	1.25	2.5
Peak plasma concentration (ng/ml)	22.4	46.5
Time to peak concentration (h)	0.5	1.0
Elimination half-life (h)	1.76	1.76
Area under the curve (ng/ml · h)	60.5	116
Oral clearance (ml/min)	345	359
Oral volume of distribution (l)	53.2	54.5

The extraction procedure of minoxidil and internal standard from human plasma is straightforward and reliable. Over a two-month period, the slopes from calibration curves were reproducible (C.V. 5.7%) with consistent recoveries (45—55%) from spiked plasma samples. Furthermore, within-day concentration replicates had a C.V. of 5.2% or less. The major limitation of the method is the lengthy injection times (30—40 min between injections of

sample) because of prolonged retention times of endogenous plasma constituents which appear as chromatographic peaks using the described conditions.

Finally, the usefulness of this method is demonstrated in pharmacokinetic results from a patient who received two different oral doses of minoxidil on different occasions. On each occasion, the terminal elimination rate constant and the half-life of minoxidil are similar, and the area under the plasma concentration time curve is a linear multiple of the dose administered.

ACKNOWLEDGEMENTS

The authors are grateful for the support and encouragement of Dr. Jerry R. Mitchell, Dr. Josephine Egan and Dr. Elizabeth L. Todd, and to Ms. Rebecca Fullen for manuscript preparation. This work was supported in part by Grant GM34120 from the United States Public Health Service.

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