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The determination of minoxidil in human serum by high-performance liquid chromatography with amperometric detection

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Abstract: An ion-pairing reversed-phase HPLC assay employing amperometric detection has been developed for the determination of free minoxidil (MNX) in human serum. The drug is isolated from the serum and concentrated by solid phase extraction with a disposable cartridge column containing ethylsilane (C2) bonded phase packing material. The average absolute recovery from serum was 85%. The HPLC separation is performed on a Sperisorb-C_8 column, using *n*-octanesulphonic acid as the pairing ion. The method exhibits linear behaviour from 0.3 to 100 ng/ml for spiked serum samples. The average daily relative standard deviations of replicate samples at the 0.75 and 2.0 ng/ml levels were 9.6 and 6.4%, respectively. Utilizing a 1 ml sample, the limit of detection ($S/N \geq 3$) was 0.3 ng/ml.

Keywords: *Minoxidil; ion-pair HPLC; serum; amperometric detection; solid phase extraction.*

Introduction

Minoxidil (2,4-diamino-6-piperdino-pyrimidine-3-oxide) is a potent direct peripheral vasodilator that is used to control hypertension. It is commercially available as a tablet formulation.† Reversible hypertrichosis has been observed in most patients who have received minoxidil for one month or longer [1, 2]. The reversal of male pattern alopecia was reported in a patient receiving oral minoxidil therapy for hypertension [3]. These reports have sparked interest in the topical administration of minoxidil for the treatment of baldness. A number of clinical studies have been performed or are underway and the current status of work in this area has been reviewed recently [4, 5].

A sensitive assay for minoxidil in serum was needed to support these clinical investigations. A radioimmunoassay (RIA) procedure for determining minoxidil in serum has been described [6]. Although this assay is sensitive and specific for minoxidil, it has the disadvantage of requiring the production of antibodies, which is a very time consuming process. An ion-pairing HPLC method for determining minoxidil in tablets has been reported previously [7]; this method employed spectrophotometric detection.

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This report describes the development of an assay for minoxidil in human serum employing solid phase extraction (SPE) and HPLC with amperometric detection (LCEC).

Experimental

Reagents and supplies

All chemicals were reagent grade unless otherwise specified. Acetone, acetonitrile, methanol, methylene chloride and tetrahydrofuran were obtained from Burdick and Jackson (Muskegon, MI). Ethanol (200 proof, dehydrated) was obtained from US Industrial Chemical Co. The mobile phase ion-pairing agent, sodium *n*-octanesulphonate, was obtained from Regis Chemical Co. (Morton Grove, IL). Activated carbon powder (Darco® grade G-60) was obtained from ICI Speciality Chemicals (Wilmington, DE). The water used in all experiments was obtained by purifying deionized water with a Milli-Q reagent water system from Millipore (Bedford, MA). This system was also equipped with a pyrogen removal filter (nominal MW cut-off = 10,000) from Amicon (Danvers, MA). Water and ethanol were treated by mixing one litre of the respective solvents with about 10 g of activated carbon powder and allowing this suspension to stand for 16 h (overnight). These suspensions were then filtered with a Pall Nylon-66 0.2 μm filter to remove the carbon powder. Triethylamine (Sequanol™ grade) was obtained from Pierce (Rockford, IL). The ethylsilane (C2) SPE cartridges (1 ml/100 mg size) were obtained from Analytichem (Harbor City, CA). Minoxidil (MNX) and 2,4-diamino-6-(*N*,*N*-diallylamino)pyrimidine-3-oxide (DAPO) (Fig. 1) were obtained from The Upjohn Company.

Equipment

The LCEC system was composed of either an Altex 100A or LKB pump (Model 2150), a Perkin–Elmer autosampler (Model ISS-100), a Hewlett–Packard recorder (Model HP 7133A), a BrownLee Labs MPLC 250 mm × 2.1 mm, C8 5 μm Spherisorb-column (22 cm analytical + 3 cm guard column), and an electrochemical detector from BAS Inc. (Model LC-4B) (West Lafayette, IN). The BAS Inc. electrochemical transducer (Model TL-8A) consisted of a glassy carbon working electrode, a stainless steel auxiliary electrode, and an RE-1 Ag/AgCl reference electrode. The respective components were powered by house electricity that was filtered through an Electronic Specialists Inc. Magnum isolator (Natick, MA). Cyclic voltammograms were obtained using a BAS Inc. potentiostat (Model CV-1A) and voltammetry cell (Model VC-2). The voltammetry cell utilized an Ag/AgCl reference electrode (Model RE-1), a platinum

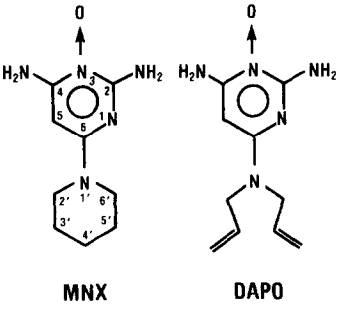

Figure 1
Structures of minoxidil (MNX) and the internal standard (DAPO).

auxiliary electrode (Model PTE), and a glassy carbon working electrode (Model GCE). Voltammograms were recorded with a Houston Instruments X–Y recorder (Model RE0074). The ultracentrifuge used to remove particulates from the reconstituted samples was obtained from Fisher Scientific (Model 235A).

Sample preparation

All serum samples were prepared by a solid phase extraction procedure which utilized C2 Bondelut cartridges. The serum samples were thawed at room temperature and then sonicated for 1 min prior to analysis. The C2 cartridges were prewashed with 2 ml of methanol, followed by 2 ml of purified water. Care was taken not to let the C2 cartridges air dry before the samples were placed on the cartridge. One millilitre of a serum sample was placed in the prewashed C2 cartridge and then 50 microlitres of internal standard (I.S.) (*ca* 212 ng/ml DAPO) was added. The sample and I.S. were then aspirated through the C2 cartridge by vacuum (7–25 cm Hg). The cartridges were subsequently rinsed with 2 ml of water, 1 ml of acetone, and 1 ml of methylene chloride. All of the rinse solutions were dispensed from polypropylene rinse bottles. Elution of minoxidil and the I.S. was accomplished by rinsing the C2 cartridge with three one-millilitre aliquots of purified ethanol containing triethylamine (0.02%, v/v). The triethylamine–ethanol solution was aspirated into 12 mm × 75 mm disposable glass culture tubes. The resultant eluents were reduced to dryness with N_2 at a temperature of 40°C. The residue was then reconstituted with 200 microlitres of mobile phase and was vortex mixed for 20 s. The reconstituted samples were either injected directly (spiked serum samples) or were ultracentrifuged for 2 min to remove particulates. A 100 µl aliquot was injected. No sample carryover was observed. Samples had to be capped with crimp caps to reduce air oxidation.

Grounding

In order to minimize problems caused by electrical ground loops, a Magnum Isolator was used to serve as a common grounding point for all components of the system. The isolator also reduced the amplitude of transient noise spikes that are often present on AC lines. A further safeguard against group loops was employed, which consisted of connecting 1 cm wide ground straps between the potentiostat and the autosampler.

In order to reduce environmentally induced currents, i.e. random spikes, the waste container was housed in a Faraday cage. The EC cell and waste container Faraday cages were connected by a ground strap. Furthermore, the waste line from the electrochemical cell was suspended over, not in, the bulk waste solution. The placement of the waste line out of the bulk waste solution was contrary to what was recommended in the BAS users manual, but was found to be superior in its ability to isolate the system from environmentally induced currents.

Mobile phase preparation

The mobile phase used to chromatograph serum samples consisted of 70% H_2O, 25% acetonitrile, and 5% tetrahydrofuran and contained 0.01 M sodium *n*-octanesulphonate, 0.01 M ammonium citrate and 0.03 M perchloric acid. Ammonium hydroxide was added dropwise to yield an apparent pH of 3.2 ± 0.05. The mobile phase was subsequently filtered by vacuum through a Millipore filtration system which utilized a 47 mm 0.45 µm Nylon-66 filter. It was noticed that exposure to vacuum longer than necessary to filter the bulk mobile phase resulted in significantly increased retention times. The mobile phase

was sparged with helium and maintained under a slight positive pressure with helium during operation. The detector was operated at a potential of +0.925 V versus Ag/AgCl. The volumetric flow rate of the mobile phase was 0.4 ml/min.

Electrode treatment

The working electrode was cleaned and polished once a week. The electrode was cleaned by exposing it for 30 s to a chromic acid solution (200 mg CrO_3, 9.7 ml H_2SO_4 and 0.3 ml water) as described by Anton [8]. Following this step, the electrode was polished on a microcloth polishing pad with a suspension of alumina (0.05 μm) for 1 min. The excess alumina was rinsed off with water and then the electrode was sonicated for 2 min to remove any residual alumina from the electrode surface. After sonication, the electrode was wiped with a soft cloth that was damped with 3A alcohol (anhydrous ethanol denatured with 5% methanol). The cell was subsequently re-assembled and the system was allowed to equilibrate. At this time, the reference electrode was changed to one which had been stored in 3 M NaCl. This routine cycling of the reference electrodes between storage and use, eliminates problems associated with malfunctioning reference electrodes, such as increased noise, and also extends the electrode lifetime.

Results and Discussion

Chromatographic system

The chromatographic system developed for this assay exhibited excellent selectivity, sensitivity and efficiency. Minoxidil is well separated from interfering peaks remaining in the samples after the solid phase extraction and it is well removed from the large group of peaks which elute just after the void volume. Chromatograms of a serum blank and a serum sample spiked with 2 ng/ml of minoxidil are shown in Fig. 2.

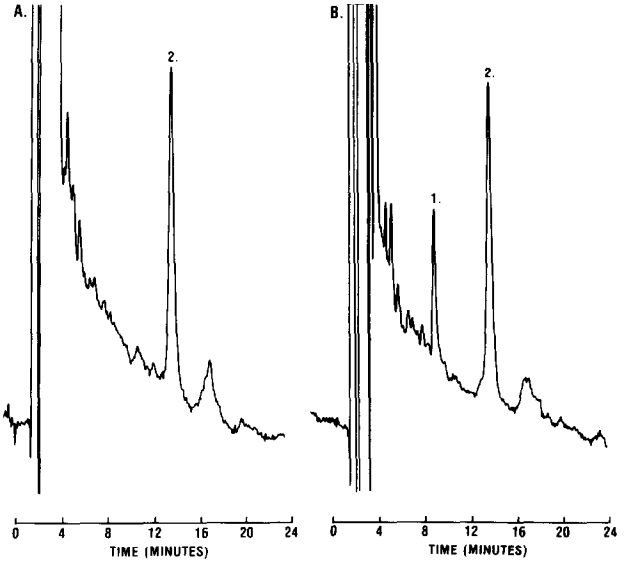

Figure 2
Chromatograms of (A) a serum blank and (B) a serum sample spiked with 2.0 ng/ml of minoxidil. 1 = Minoxidil, 2 = internal standard (DAPO, 11 ng/ml), 2 nA full scale.

The system routinely achieved a theoretical plate number, N, of 6000–9000. The sharp peaks arising from this high efficiency separation in combination with the EC detection result in a very sensitive system. The limit of detection ($S/N \geq 3$) for a minoxidil standard was 50 pg injected on to the column. This was 3–5 times lower than could be obtained with UV detection at 254 nm. Furthermore, EC detection was more selective than UV detection. This was a definite advantage in determining minoxidil in serum because fewer interfering substances were detected. In particular, late eluting peaks which cause dramatic shifts in the baseline when using UV detection were not detected using EC detection.

Several interesting effects were noted during the development of this system. One was the dramatic effect of THF on the chromatographic behaviour of minoxidil. When the acetonitrile was replaced with an equivalent volume of THF, the retention time of minoxidil doubled. Since THF is a stronger eluting solvent than acetonitrile in the reversed-phase mode, a slight decrease in retention was expected. This anomalous behaviour indicates that the THF is interacting in some specific fashion with the minoxidil or the minoxidil–octanesulphonate ion-pair to increase its retention. Alternatively, it may be selectively covering the surface silanols, and reducing any interaction between the amine groups on minoxidil and the silanol groups. Increasing the total organic content of the mobile phase (i.e. adding 5% more acetonitrile) decreased the retention time of minoxidil back to about 9 min. In addition to increasing the retention time, the use of THF caused the number of theoretical plates to increase by approximately a factor of 2. This increase in plate number was maintained when the total organic content was increased to reduce minoxidil's retention time. One disadvantage of using THF in conjunction with EC detection is that it gradually degrades to give electroactive species, i.e. peroxides. This causes a gradual increase in the background current, a problem that could be remedied by sparging the mobile phase with helium and maintaining it under positive helium pressure.

It was also noted that a 100 μl injection volume could be used on this 2.1 mm i.d. system with no loss in efficiency. Sample injections ranging from 10 to 150 μl were made and no loss in efficiency was observed until the injection volume was greater than 100 μl. A 100 μl injection volume is 4–10 times larger than the maximum recommended injection volume. Normally, injection volumes of this size would cause peak broadening on a 2.1 mm i.d. column. The secondary equilibrium involved in ion-pair chromatography reduces the efficiency of such separations. This resultant loss in efficiency is the most likely explanation for the fact that no loss in efficiency is observed with this relatively large injection volume.

Selection of an operating potential

The selection of the appropriate operating potential for amperometric detection for a given substance is determined by the electrochemical activity of that substance. The electrochemical activity of minoxidil was determined by cyclic voltammetry and hydrodynamic voltammetry. A typical cyclic voltammogram of minoxidil in mobile phase is shown in Fig. 3. The observed oxidation peaks are believed to arise from the oxidation of the 2- and/or 4-amino groups. The initial oxidative peak potentials of MNX and DAPO occur at 0.85 and 0.89 V, respectively. The operating potential is normally selected to be on the diffusion limited current plateau, i.e. 50–100 mV beyond the peak potential. Hydrodynamic voltammograms of MNX and DAPO were also obtained which indicated that the limiting current plateau for minoxidil begins at about +0.925 V.

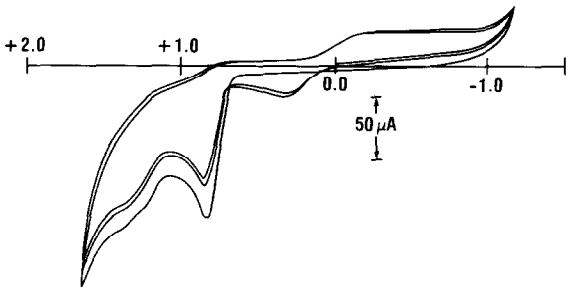

Figure 3
A cyclic voltammogram of minoxidil in mobile phase. Scan rate = 250 mV/s, working electrode = glassy carbon, reference electrode = Ag/AgCl.

Hence, this is the minimum operating potential required to achieve maximum sensitivity. As the potential is increased, more potentially interfering compounds are detected and the background current increases. In the area of +0.9–+1.1 V the background current increases exponentially. The exact rate at which it increases is dependent upon the pH, purity and composition of the mobile phase, and the electrode material. For most applications the background current above +1.0 V is too great to permit detection of trace level components. Hence the selection of an operating potential is a compromise between setting a high enough potential to give maximum signal and as low a potential as possible to minimize the background current, i.e. noise. A potential of +0.925 V was found to be the best compromise for minoxidil.

Sample preparation

A variety of sample preparation methods including liquid–liquid extraction, protein precipitation followed by direct injection, and solid phase extraction (SPE) were investigated. Only SPE gave both good recovery and a sufficiently clean sample for trace determinations. SPE columns packed with both reversed- (CN, Phenyl, C2, C8, C18) and normal phase (Diol, Si) materials were evaluated.

The relatively weak reversed-phase materials (CN and Phenyl) extracted minoxidil from aqueous solutions, but exhibited drug loss when rinsed with organic solvents. The relatively strong reversed-phase materials (C8 and C18) exhibited very strong retention of minoxidil. Hence, the drug could not be quantitatively recovered. The DIOL and SI cartridges retained minoxidil from aqueous solutions and yielded quantitative recovery, but serum chromatograms contained too many interferences. The packing material that performed the best was C2, i.e. ethylsilane bonded phase material.

A variety of solvents were evaluated for use as rinse or elution solvents. Optimum performance was obtained with the three rinse solvents and the elution solvent selected, as described in the Experimental section. These rinse solvents cover a wide range of polarities and have differing selectivities. The water removes highly polar serum components such as salts and polar compounds. The acetone removes medium polarity serum components and produces serum samples clean enough to permit the determination of minoxidil at sub-nanogram per millilitre levels. The methylene chloride is used to remove highly retained lipophilic species. The mixed elution solvent, 0.02% (v/v) triethylamine–ethanol is necessary to obtain acceptable recoveries (>70%). Elution with pure ethanol does not yield consistent recoveries. This sample clean-up procedure gives very clean chromatograms, as was illustrated in Fig. 2.

Linearity and recovery

The detector response, as defined by the peak height ratio (PHR) of minoxidil to the internal standard (I.S.), was linear over three orders of magnitude. The minoxidil concentration ranged from 1 to 1000 ng/ml while the I.S. concentration was 11 ng/ml. The actual amount of minoxidil that was injected on column ranged from 100 pg to 100 ng. Linear regression analysis yielded an intercept not significantly different from zero (−0.029) and a slope of 0.239 (n = 10). The correlation coefficient for this line was 0.9999. In order to test the linearity of the sample preparation procedure, spiked serum samples from 0.3 to 100 ng/ml minoxidil (11 ng/ml I.S.) were prepared and assayed. The peak height ratios over this concentration range were linear, with an intercept that was not significantly different from zero (correlation coefficient = 0.9999, intercept = 0.065, slope = 0.217, n = 9). Individual peak height ratios and absolute recoveries of minoxidil and the I.S. are listed in Table 1. The average absolute percent recoveries for minoxidil and the I.S. over this range were 84.7 and 69.0%, respectively. Minoxidil recoveries ranged from 73.5 to 118%, while I.S. recoveries ranged from 65.0 to 73.3%.

Table 1
Sample preparation linearity and recovery — 1 ml spiked serum samples

			Absolute recoveries	
ng/ml Minoxidil	PHR (MNX/I.S.)		MNX (%)	I.S. (%)
0.3	0.103		118	70.0
0.5	0.134		94.4	70.6
1.0	0.221		77.1	67.8
2.0	0.469		85.7	71.1
5.0	1.02		77.6	73.3
10.0	2.35		82.5	67.8
20.0	4.54		75.6	65.0
50.0	11.1		73.5	65.0
100.0	21.7		77.6	70.0
No. of pts.	9	$\bar{x}$ =	84.7	69.0
Slope	0.2174	RSD =	16.6	4.0
Intercept	0.0647			
Corr. coeff.	0.9999			

The practical limit of detection (LOD) for 1 ml samples is 0.3 ng/ml for minoxidil. For approximately one in every two injections, concentrations of 0.1 ng/ml were detected, but accuracy and reproducibility at this level were questionable.

In an attempt to lower the LOD, 2, 3, and 5 ml samples (1.0, 0.4, and 0.4 ng/ml minoxidil in serum, respectively) were assayed. The 3 and 5 ml samples exhibited low recoveries (41 and 42%, respectively). The 2 ml sample, however, showed about 80% recovery. This prompted a 2 ml linearity study that covered the range of concentrations from 0.1 to 10 ng/ml (0.1, 0.3, 0.5, 1.0, 5.0 and 10.0 ng/ml). Over this range of concentrations, the response was linear (slope = 0.390, intercept = 0.085, correlation coefficient = 1.000, n = 6), and the average absolute percent recovery for minoxidil was 108%. Therefore, the LOD was decreased by a factor of two (150 pg/ml minoxidil) when 2 ml of serum was used.

Table 2
Working curve data and results of linear regression analysis

	Peak height ratio	
ng/ml	Day 1	Day 2
0.3	0.098	0.066
0.5	0.100	0.088
1.0	0.238	0.144
5.0	0.976	0.937
10.0	1.748	1.737
Slope	0.173	0.176
Intercept	0.052	0.004
Corr. coeff.	0.9985	0.9989

Table 3
Precision data — 0.75 ng/ml minoxidil low control

	Day 1		Day 2	
	Amount found (ng/ml)	Relative recovery (%)	Amount found (ng/ml)	Relative recovery (%)
1	0.73	97.2	0.70	92.8
2	0.75	100.1	0.77	103.2
3	0.87	116.7	0.80	107.2
4	0.69	92.1	0.74	99.1
5	0.71	94.7	0.63	84.0
Avg.	0.75	100.1	0.73	97.3
RSD	9.7%	9.7%	9.4%	9.4%
		Amount found	Relative recovery	
Interday avg. (N = 10)		0.74	98.5%	
Interday RSD (N = 10)		8.9	8.9%	

Table 4
Precision data — 2.0 mg/ml minoxidil high control

	Day 1		Day 2	
	Amount found (ng/ml)	Relative recovery (%)	Amount found (ng/ml)	Relative recovery (%)
1	1.90	95.0	2.05	102.5
2	1.93	96.5	2.28	114.0
3	1.88	94.0	2.03	101.5
4	1.70	85.0	2.04	102.0
5	1.61	80.5	2.06	103.0
Avg.	1.80	90.2	2.09	104.6
RSD	7.8%	7.8%	5.0%	5.0%
		Amount found	Relative recovery	
Interday avg. (N = 10)		1.95	97.5%	
Interday RSD (N = 10)		9.9%	9.9%	

Working curves

Working curves consisting of five points covering the range from 0.3 to 10 ng/ml were run daily. The working curve data and results of linear regression analyses are listed in Table 2. The working curves exhibit good linearity (average correlation coefficient = 0.9987) and have intercepts that are not significantly different from zero (average intercept = 0.028).

Precision and accuracy

Precision and accuracy data were generated by assaying five low and high spiked serum controls (0.75 and 2.0 ng/ml, respectively) on two different days. The amount found was determined from that day's working curve. This data is presented in Tables 3 and 4. The average RSDs for the low and high controls were 9.6 and 6.4%, respectively. The average relative recovery for the 0.75 and 2.0 ng/ml controls were 98.7 and 97.5%, respectively. On a daily basis, a minimum of two samples each of the low and high spiked serum controls are run. Over seven days the relative standard deviations of the low and high controls were 13.1% (n = 25) and 13.4% (n = 22), respectively.

Conclusion

The method described here is linear over a wide concentration range and exhibits good accuracy, reproducibility and precision. The sample clean-up procedure yields good recovery and very clean chromatograms. The limit of detection for this assay is 0.3 ng/ml, when 1 ml of serum is used. The method is suitable for determining free minoxidil in serum from patients administered minoxidil either topically or orally.

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