Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Journal of Pharmaceutical and Biomedical Analysis 38 (2005) 532–536
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Rapid determination of trace amounts of minoxidil in hamster skin follicles with various formulations using narrow-bore LC/EC

Tiehua Huang *, Michelle E. Garceau, Tore Ramstad, Randall G. Stehle

Pharmaceutical Sciences, Pfizer Inc., 7000 Portage Street, Kalamazoo, MI 49001, USA

Accepted 14 January 2005
Available online 19 March 2005

Abstract

A sensitive liquid chromatographic method with electrochemistry (LC/EC) was developed for the determination of trace of minoxidil in hamster skin follicles after topical administration of the ear using various formulations. The minoxidil in the sebaceous glands of the hamster ear was isolated from the skin and the follicles in different skin layers were treated with aqueous trichloroacetic acid followed by acetonitrile. The supernatant was directly injected into the LC/EC system and minoxidil was detected by oxidation at +800 mV versus Ag/AgCl using a glassy carbon electrode. The analytical recoveries were between 94.4 and103.1% and the linearity was excellent up to 250 μg/ml with a regression coefficient (r^2) of 0.9988. The LC/EC and the widely used radiolabeled scintillation methods agree well and both show high sensitivities. The LC/EC method is rapid and cost-effective with a detection limit of only 1 ng/ml.
© 2005 Published by Elsevier B.V.

Keywords: Minoxidil; Follicles; Formulation; Microbore liquid chromatography; Electrochemical detection

1. Introduction

Hair follicles have a complex and dynamic structure that can contribute significantly to the passive transport of compounds across the skin [1,2]. Minoxidil (Fig. 1) is widely used for stimulating hair growth after topical administration [3]. The concentration of minoxidil in the hamster ear follicles is widely used in the evaluation of new formulations. Because only a small fraction of the applied dose is normally delivered across the skin by the transfollicular route [3,4], a sensitive analytical technique is needed to evaluate various topical minoxidil formulations.

Various analytical techniques have been reported for the determination of minoxidil including capillary isotachophoresis [5], derivative spectrophotometry [6] and differential-pulse polarography [7]. These methods are either insensitive or inconvenient for topical formulation development with minoxidil. Scintillation counting [8] using radiolabeled minoxidil formulations is very sensitive and it is widely used in the pharmaceutical industry for minoxidil formulation development. However, the radiolabeled scintillation counting method is appropriate primarily for solution samples of minoxidil. When dealing with multiple-phase heterogeneous formulation samples such as emulsion or suspension formulations, random distribution of radiolabeled minoxidil cannot be readily achieved by simply adding radiolabeled minoxidil to the 'cold' formulations. The preparation of radiolabeled formulations is expensive and time-consuming, and therefore an alternative rapid analytical technique is needed for developing multiple-phase heterogeneous formulations of minoxidil.

LC with electrochemical detection (LC/EC) was reported for the determination of minoxidil in blood samples [9]; however, the chromatographic and electrochemical detection conditions were not optimized at that time. An improved method is needed for the low-level determination of minoxidil in hair follicles.

In this report, a tape stripping/dissection method was developed for collecting trace amount of minoxidil in hamster

* Corresponding author. Present address: R&D Centre, Bausch and Lomb Inc., 1400 N. Goodman Street, Rochester, NY 14609, USA.
Tel.: +1 585 338 6733; fax: +1 585 338 0277.
E-mail address: ted_huang@bausch.com (T. Huang).

0731-7085/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.jpba.2005.01.032

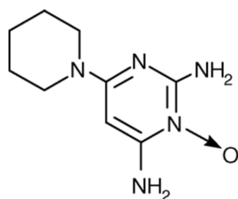

Fig. 1. Structure of minoxidil.

skin follicles and then the minoxidil was dissolved in a trichloroacetic acid (TCA)/acetonitrile solution. The chromatographic and electrochemical detection conditions were optimized for the rapid and sensitive determination of minoxidil in the follicles. This technique is especially useful for the rapid evaluation of a large number of different heterogeneous formulations.

2. Experimental

2.1. Reagents and chemicals

Trichloroacetic acid was obtained from Aldrich (St. Louis, MO, USA) and a 20% TCA solution was prepared by dissolving 20 g of TCA in 80 ml of water. Reagent-grade potassium phosphate monobasic and sodium hydroxide were obtained from Mallinckrodt (USA). HPLC-grade water, acetonitrile and methanol (OmniSolv) were purchased from EM Science (Gibbstown, NJ, USA). USP-grade polyethylene glycol 400 (PEG 400) was obtained from Dow Chemical Co. (Midland, MI, USA) and absolute ethanol was obtained from Aaper Alcohol and Chemical Co. (Shelbyville, KY, USA).

2.2. Chromatographic and electrochemical conditions

The buffer for the mobile-phase consisted of 100 mM potassium phosphate monobasic adjusted to pH 8 with sodium hydroxide solution. The mobile-phase was prepared by mixing the buffer with methanol in the ratio of 73:27. A Luna C8 (2), 150 mm × 2 mm, 5 μm HPLC column (Phenomenex, Torrance, CA, USA) was operated at 0.4 ml/min at ambient temperature. The injection volume was 5 μl using a Sample Sentinel Autosampler obtained from Bioanalytical Systems (BAS, West Lafayette, IN, USA). A BAS PM-80 pump was used along with a BAS LC-4C electrochemical detector containing a glassy carbon electrode. A potential of +800 mV versus Ag/AgCl was applied at a 3 mm glassy carbon electrode in a cross flow cell (BAS). The detector filter was set at 0.1 Hz. The electrode was polished by placing a few drops of polishing alumina onto a water-wetted polishing pad. The electrode was polished by gently rubbing the surface of the glassy carbon electrode in a circular motion for ∼10 s followed by flushing with methanol and then drying with tissue paper. The polished electrode can be used for 3–4 weeks before resurfacing.

2.3. Hamster skin follicle sample preparation

The stratum corneum was removed from the ventral (“inside”) region of the detached hamster ear by multiple tape stripping. The underlying dermis layer was then peeled back to reveal the sebaceous gland bulbs. This peeled-back layer was then placed “bulb-side-up” onto a new microscope slide. The sebaceous glands’ contents were then transferred by scraping without tearing the skin layer and the sample was rinsed into a vial. HPLC-grade water was used as the rinse solution in this stripping/dissection method. The rinses were then adjusted to the predetermined volume of 10.0 ml with water. One millimeter of 20% TCA solution was added to this 10.0 ml collected sample and the tapes and the skin tissues were soaked in this solution. The sample mixture was vortexed for 15 s, followed by adding 5.0 ml of acetonitrile to the solution, and the sample was vortexed for another 15 s. A portion (about 1 ml) of the resulting solution was centrifuged for 2 min at 22,000 × g in an Eppendorf 5415-C clinical centrifuge. Centrifugation resulted in a supernatant that was clear and free of particles; therefore, a filtration procedure was not necessary. Five microlitres of the supernatant was directly injected into the LC/EC system.

3. Results and discussion

3.1. Optimization of follicle sample preparation

Minoxidil is slightly soluble in water, but highly soluble under low pH conditions. In this work, trichloroacetic acid, an excellent denaturing reagent, was used as the acidifying reagent for complete dissolution of minoxidil in the sample preparation solution. Solvents and their mixing ratios were evaluated for the solubility of minoxidil and the ratio of the co-solvents was optimized to ensure that the highest solubility was obtained (Table 1). The results show that methanol and ethanol are less effective solvents for dissolving minoxidil as compared to acetonitrile. Polyethylene glycol 400 is a good solvent for minoxidil, but not as good as acetonitrile, an

Table 1
Solubility of minoxidil in different solutions

Solvent composition[a]	Solubility (mg/ml)
2 ml H_2O, 0.2 ml TCA	0.15
1 ml EtOH, 2 ml H_2O, 0.2 ml TCA	6.99
4 ml EtOH, 2 ml H_2O, 0.2 ml TCA	3.26
1 ml ACN, 2 ml H_2O, 0.2 ml TCA	10.3
1 ml ACN, 2 ml H_2O	5.92
4 ml ACN, 2 ml H_2O, 0.2 ml TCA	15.1
4 ml ACN, 2 ml H_2O	7.74
1 ml MeOH, 2 ml H_2O, 0.2 ml TCA	3.76
4 ml MeOH, 2 ml H_2O, 0.2 ml TCA	16.6
1 ml PEG 400, 2 ml H_2O, 0.2 ml TCA	3.39
4 ml PEG 400, 2 ml H_2O, 0.2 ml TCA	14.5

[a] TCA: trichloroacetic acid in water (20%); EtOH: ethanol; MeOH: methanol; PEG 400: polyethylene glycol 400; CAN: acetonitrile.

efficient deproteinization agent. The solubility of minoxidil was found to be 0.15 mg/ml in TCA solution and 5.92 mg/ml in acetonitrile solution. However, with both TCA and acetonitrile together in the solution, the solubility was significantly increased to 15.1 mg/ml (Table 1). Acetonitrile, water, and 20% TCA in a volume ratio of 4:2:0.2 provides the highest solubility of minoxidil and this ratio was used for dissolving minoxidil in the follicle samples. Since the samples were clear after centrifugation, filtration procedures were not needed. The samples prepared in this fashion were free of matrix interferences.

3.2. Optimization of the LC/EC method for the detection of minoxidil

The electrochemical oxidation of minoxidil is dependent on the pH of the mobile-phase. The higher the pH, the higher is the oxidation current response. However, high pH also resulted in higher noise. For example, the background currents were 11 nA and 18 nA (+800 mV versus Ag/AgCl) when the mobile-phase pH was 9 and 10, respectively. This background current was reduced to 3 nA when the pH was adjusted to 8. The low background current at pH 8 significantly reduced the noise level and enhanced the detection limit. A mobile-phase pH of 8 also has a prolonged column life as compared to pH 9 and above. We therefore selected a pH of 8 for the mobile-phase.

When electrochemical detection is used with liquid chromatography, EDTA is often added to the mobile-phase for reduction of background current by chelating trace amounts of Fe^{2+} ions present in the LC system. In this work, however, the addition of EDTA was not suitable because it resulted in even higher background current due to the oxidation of the amino group in EDTA at the glassy carbon electrode under the basic pH conditions employed. In order to minimize the Fe^{2+} effect on the background current without using EDTA, the LC system was carefully re-passivated using 30% HNO_3 and a low background current was obtained.

Fig. 2 shows the hydrodynamic voltammogram (HDV) of minoxidil. A 250 ng/ml minoxidil standard solution was injected into the LC/EC system while the applied potential was changed from +200 mV to +1000 mV versus Ag/AgCl and the obtained peak areas were measured. The curve reached the diffusion-limited plateau at +900 mV; however, the background current at +900 mV was very high.

Since the minoxidil concentrations were low in some skin sections in the follicular targeting and delivery studies, microbore technology [10,11] was used to improve the detection limit. Columns with 1, 2, and 4.6 mm i.d. were evaluated. A 150 ng/ml minoxidil standard solution was injected onto the columns for comparison. The peak height increased more than 10 times when the column diameter was changed from 4.6 to 1 mm. However, smaller i.d. columns are more susceptible to the extra column dead volume effect resulting in peak broadening. One-millimeter diameter columns are less durable when working with tissue samples. Therefore, as a compromise, the 2 mm i.d. column was chosen for this study.

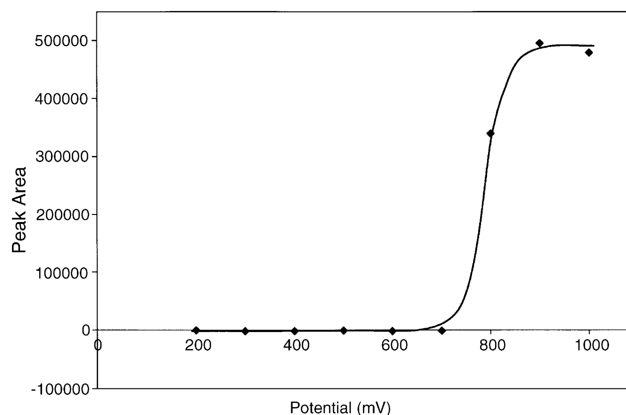

Fig. 2. The hydrodynamic voltammogram (HDV) of minoxidil. The same sample is repeatedly injected while the potential of the working electrode is changed from +200 to +1000 mV vs. Ag/AgCl and the peak areas are measured.

3.3. Analytical results

A typical chromatogram of minoxidil in the hamster ear follicles is shown in Fig. 3. The baseline was flat and clean. There were no interfering peaks in the follicle tissue samples. The detection limit was 1 ng/ml (S/N = 5) and the analytical linearity was excellent. The regression coefficient (r^2) was 0.9990 for concentrations up to 400 ng/ml and was 0.9988 for concentrations up to 250 μg/ml in hair follicle samples. Analytical recovery and precision were evaluated by analyzing control samples prepared by spiking known amounts of minoxidil into the samples. Ten control samples ranging from 30 to 500 ng/ml were analyzed by the LC/EC method. The R.S.D. values were 12.6% (30 ng/ml), 2.6% (50 ng/ml),

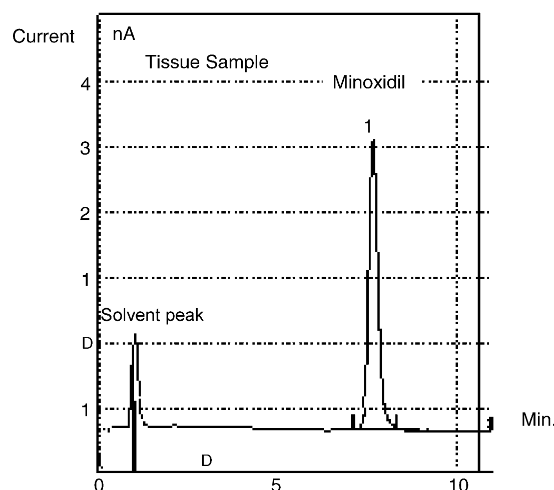

Fig. 3. Typical chromatogram of minoxidil (100 ng/ml) in hamster ear follicles. A potential of +800 mV vs. Ag/AgCl is applied at a 3 mm glassy carbon electrode. A Luna, C8 (2), 100 × 2 mm, 5 μm LC column is used.

Table 2
Precision and analytical recovery with the minoxidil assay

	Spiked (ng/ml)						
	30.0	50.0	125	150	250	300	500
Determined (ng/ml)	28.3	49.6	126	153	245	303	480
	35.0	51.5	127	151	240	299	479
	27.1	47.5	124	147	233	294	478
	34.9	47.5	123	159	232	290	476
	30.4	49.5	124	152	233	293	483
	33.2	48.8	126	152	233	303	486
	31.1	48.9	124	152	233	297	487
	31.2	50.5	124	154	234	300	485
	35.0	48.2	125	151	238	304	490
	23.1	49.0	122	149	237	296	487
Av. (ng/ml)	30.9	49.1	124	152	236	298	483
S.D.	3.9	1.3	1.6	3.1	4.0	4.8	4.5
R.S.D. (%)	12.6	2.6	1.3	2.1	1.7	1.6	0.9
Recovery (%)	103	98.2	99.5	101	94.4	99.3	96.6

Minoxidil is spiked into the follicle samples and the concentration of minoxidil is assayed by the LC/EC method.

1.3% (125 ng/ml), 2.1% (150 ng/ml), 1.7% (250 ng/ml), 1.6% (300 ng/ml), and 0.9% (500 ng/ml). The corresponding analytical recoveries were 103, 98.2, 99.5, 101, 94.4, 99.3 and 96.6%, respectively (Table 2). The method demonstrated an excellent accuracy and precision at these levels.

Day-to-day analytical recovery and precision were obtained by analyzing control samples at concentrations of 25, 50, 100, 125, 150, 200 and 250 ng/ml over a 4-day period (Table 3). The R.S.D. of the 4-day measurements were 2.4, 3.9, 4.0, 5.2, 2.4, 3.9 and 4.4%, respectively. The day-to-day analytical recoveries were 97.8, 93.4, 96.0, 96.3, 97.6, 97.2 and 95.1%, respectively. Table 4 shows the amount of minoxidil in the hamster ear follicles in a formulation evaluation experiment using the LC/EC method.

The LC/EC method was compared to the radiolabeled scintillation counting method by analyzing the same hamster ear follicle samples using the two different methods. Scintillation counting gives the *amount* of minoxidil per sample, while LC/EC gives the *concentration* in the sample. The concentration obtained by LC/EC was then multiplied by the sample volume and the dilution factor to give the total *amount* of minoxidil per sample. The results obtained by the two methods agreed well except for the stratum corneum sample (Fig. 4) in a test for method comparison. During the preparation of the stratum corneum sample, the stripping tapes were not fully soaked in the extraction solution and thus the minoxidil result was low. Without this point of the stratum corneum, the regression coefficient, r^2, of the two methods was 0.9990.

Table 3
Day-to-day precision and analytical recovery with the minoxidil assay

	Spiked (ng/ml)						
	25	50	100	125	150	200	250
Determined (ng/ml)							
Day 1	24.1	44.5	98.6	123	148	200	244
Day 2	23.9	47.5	98.4	125	150	200	247
Day 3	24.6	48.7	96.6	122	147	194	236
Day 4	25.2	46.0	90.4	111	141	184	224
Av. (ng/ml)	24.4	46.7	96.0	120	146	194	238
S.D.	0.6	1.8	3.9	6.3	3.6	7.6	10.4
R.S.D. (%)	2.4	3.9	4.0	5.2	2.4	3.9	4.4
Recovery (%)	97.8	93.4	96.0	96.3	97.6	97.2	95.1

Minoxidil is spiked into the follicle samples and analyzed on four different days.

Table 4
Amount of minoxidil in hamster ear follicles in a typical experiment using LC/EC

Formulation	Hours after the formulation applied on the ear skin	Minoxidil (ng) in the follicles, average of two hamsters
A	1	281
A	3	625
B	1	347[a]
B	3	646
C	20	383
D	20	1610[a]
E	20	2080

The formulations are applied onto the hamster ears and the animals are sacrificed and the follicles are collected.
[a] The data are from one hamster.

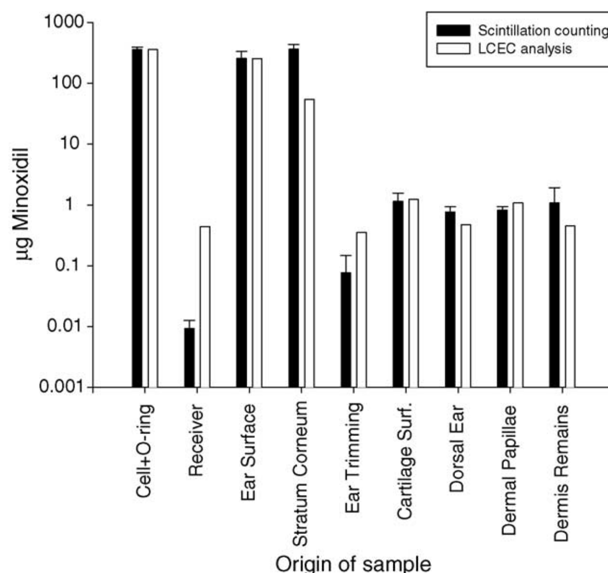

Fig. 4. Comparison of the LC/EC and radiolabeled scintillation methods. The same hamster ear skin samples are analyzed using the two methods in two different laboratories.

4. Conclusion

The liquid chromatographic determination of minoxidil in the hamster ear follicles using electrochemical detection is fast, accurate and sensitive. The LC/EC method is faster, more convenient and cost-effective than the widely used radiolabeled scintillation method. The two methods agreed well in assay results and sensitivity. Unlike the radiolabeled scintillation technique, the LC/EC method can be easily applied

to all types of minoxidil formulations, whether heterogeneous or homogeneous.

References

[1] N. Weiner, Int. J. Pharm. 162 (1998) 29–38.
[2] A. Rougier, C. Lotte, in: V.P. Shaw, H.I. Maibach (Eds.), Topical Drug Bioavailability, Bioequivalence and Penetration, Plenum Press, New York, 1993, pp. 163–182.
[3] A.C. Lauer, in: R.L. Bronaugh, H.I. Maibach (Eds.), Percutaneous Absorption,Drugs-Cosmetics-Mechanisms-Methodology, third ed., Marcel-Dekker, New York, 1999, pp. 427–449.
[4] A.C. Lauer, L.M. Lieb, C. Ramachandran, G.L. Flynn, N.D. Weiner, Pharm. Res. 12 (1995) 179–186.
[5] S. Fanali, M. Cristalli, P. Catellani, J. Chromatrogr. 405 (1987) 385–388.
[6] M.S. Mahrous, M.M. Abdel-Khalek, Y.A. Beltagy, Anal. Lett. 25 (1992) 1673–1686.
[7] L. Amankwa, L.G. Chatten, S. Pons, Analyst 108 (1983) 1221–1226.
[8] J.C. Tsai, N.D. Weiner, G.L. Flynn, J.J. Ferry, Skin Pharmacol. 7 (1994) 262–269.
[9] M.H. Golden, P.H. Zoutendam, J. Pharm. and Biomed. Anal. 5 (1987) 543–551.
[10] T. Huang, R.E. Shoup, P.T. Kissinger, Curr. Sep. 9 (1990) 139–143.
[11] T. Huang, L. Yang, J. Gitzen, P.T. Kissinger, M. Vreeke, A. Heller, J. Chromatogr. B 670 (1995) 323–327.