Journal of Chromatography, 248 (1982) 297–302
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,107

LIQUID CHROMATOGRAPHIC SEPARATION AND DETECTION OF EPINEPHRINE AND ITS OXIDATION PRODUCTS, ADRENOCHROME AND ADRENOLUTIN

ERIC C. JUENGE*, PAUL E. FLINN and WILLIAM B. FURMAN
National Center for Drug Analysis, Food and Drug Administration, 1114 Market Street, St. Louis, MO 63101 (U.S.A.)
(Received June 11th, 1982)

SUMMARY

High-performance liquid chromatographic and silica gel thin-layer chromatographic methods were developed for the separation and simultaneous detection of adrenochrome, adrenolutin and epinephrine. The latter method includes a convenient plate treatment to preclude decomposition of substances during chromatography. A simple column chromatographic method that separates mixtures of adrenochrome and adrenolutin is also described.

INTRODUCTION

Chromatographic techniques for separation and detection of adrenochrome and adrenolutin must be carefully chosen because these compounds often readily decompose under usual test conditions. Adrenochrome is subject to catalytic conversion to adrenolutin by certain heavy metals and alkali[1]. Adrenochrome and adrenolutin undergo oxidation or catalytic oxidation, which generates polymeric products. High-performance liquid chromatographic (HPLC) techniques have been used to detect epinephrine in pharmaceutical formulations[2], but their use to detect the common decomposition products[1] adrenochrome and adrenolutin in "aged", "pink" or oxidized solutions of epinephrine is not reported. This paper presents an HPLC method for the simultaneous detection of epinephrine, adrenochrome and adrenolutin.

Paper chromatography (PC) of adrenochrome and adrenolutin can be accomplished provided the paper has been carefully prewashed with dilute acid to remove heavy-metal ions and prevent catalytic decomposition caused by their presence[3,4]. A similar separation of adrenochrome and adrenolutin on acetylated paper has also been described[5]. Detection of epinephrine and adrenochrome on polyamide thin-layer chromatographic (TLC) plates has been reported[6].

The silica gel TLC system described in this paper can be used for simultaneous separation and detection of epinephrine, adrenochrome and adrenolutin, and is also useful for automated and multiple-spotting[7] TLC techniques.

0021-9673/82/0000–0000/$02.75

This study also yielded a simple, fast, column chromatographic (CC) method that will separate small quantities of adrenochrome and adrenolutin. The authors are unaware of any previously published HPLC or silica gel TLC methods for simultaneous separation and detection of adrenochrome and adrenolutin or their combination with epinephrine. These chromatographic techniques can be used in the study of epinephrine solutions contaminated by adrenochrome (pink solutions) and adrenolutin (yellow-green fluorescent solutions).

EXPERIMENTAL

l-Epinephrine was USP grade (J. H. Walker and Co., Mount Vernon, NY, U.S.A.): adrenochrome[8] and adrenolutin[9] were synthesized according to published procedures. Adrenolutin was also obtained from I.C.N./K&K Life Sciences Group (Irvine, CA, U.S.A.). Deionized water was used for all operations, and all other chemicals and solvents were reagent grade.

TLC plates (2.5 × 7.5 cm) of silica gel bonded permanently to glass, Permakotes I[10], and 1-μl microcaps (Drummond Scientific, Broomall, PA, U.S.A.) were used for TLC. Waters Assoc. (Milford, MA, U.S.A.) chromatographic columns and an alkyl phenyl (P/N 27198) μBondapak stainless-steel column (30 cm × 3.9 mm I.D.) were used for HPLC, and 1-cm Sep-Pak C_{18} cartridges for CC.

Just before the HPLC study, the synthesized adrenochrome was recrystallized from methanol[8], and the synthesized adrenolutin was recrystallized from water containing sodium dithionite to prevent oxidation[9]. A sample of the adrenolutin was also sublimed (18 h, 180°C, 1 mmHg). Adrenochrome and adrenolutin show an increased tendency for decomposition when in solution. Stock solutions were not prepared; all solutions were freshly prepared from the solid materials kept in the dark and refrigerated until use.

Samples were examined for purity by melting point (m.p.), PC[3,4] and infrared (IR) and nuclear magnetic resonance (NMR) spectrometry. IR spectra were taken of mineral oil mulls of samples on KBr plates.

Instruments

A Model 3500B high-performance liquid chromatograph (Spectra-Physics, Santa Clara, CA, U.S.A.), including an ultraviolet (UV) detector with a fixed wavelength of 254 nm, was used with a Servo/Riter II recorder (Texas Instruments, Houston, TX, U.S.A.). A Model 337 grating IR spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.), a Model T-60A 60-MHz NMR spectrometer (Varian Instrument Division, Palo Alto, CA, U.S.A.) and a Fisher-Johns melting-point apparatus (Fisher Scientific, St. Louis, MO, U.S.A.) were used.

High-performance liquid chromatography

The mobile phase was aqueous methanol. The methanol–water ratio was adjusted to optimize separation of epinephrine, adrenochrome and adrenolutin. Solvents were degassed by vacuum filtration through a 0.6-μm Type BD filter (Millipore, Bedford, MA, U.S.A.). Samples were injected with a 2-ml loop and were eluted at a flow-rate of 2.4 ml/min at 2140 p.s.i. The chart speed was 5 mm/min.

To prepare the sample, the free base l-epinephrine was dissolved in water by addition of dilute hydrochloric acid to give a solution of 112 μg in 75 μl. To this solution were added 18 μg of adrenochrome in 10 μl of the mobile solvent and 20 μg of adrenolutin in 50 μl of the mobile solvent. The entire sample prepared in this manner was injected.

Thin-layer chromatography

Several Permakote silica gel plates were soaked overnight in 3 l of 2% acetic acid, soaked several hours in the same volume of deionized water, rinsed under running deionized water and finally air-dried for 24 h. Adrenochrome and adrenolutin were spotted as aqueous and methanol solutions, respectively, at origins 8 mm from the base of the plate and chromatographed with deionized water by upward equilibrated development until the solvent ascended to within 2–3 mm from the top. Alternatively, solutions for spotting combinations of epinephrine, adrenochrome and adrenolutin were made with methanol and dilute hydrochloric acid mixtures.

Adrenochrome and adrenolutin were detected under white light. Adrenolutin was also detected by irradiation with long-wavelength UV light. Epinephrine was detected by spraying the plate with 5% potassium ferricyanide in 0.1 *N* potassium dihydrogen phosphate.

Column chromatography

Sep-Pak C_{18} cartridges were used as obtained, or two 1-cm Sep-Pak C_{18} cartridges were connected in tandem for separation of larger quantities of materials. For the latter, the cartridges were connected by an 8- to 9-mm length of 4-mm O.D. glass tubing; the ends of the cartridges were trimmed so that their plastic tubes overlapped by 2–3 mm the ends of the glass tube and nearly touched each other. The column was prewet with 2 ml of methanol per Sep-Pak used and then flushed with about 10–15 ml of deionized water.

Deionized water was added to 5 mg of adrenochrome and methanol was added to 5 mg of adrenolutin with swirling until dissolution occurred. Equal amounts of solvents, not to exceed 250 μl, were used. The solutions were filtered and combined. The resulting clear solution was transferred with a capillary tube to the surface of a single C_{18} Sep-Pak cartridge. Only the upper half of the Sep-Pak was colored by this operation. The solution was washed completely onto the column by draining the capillary tube to the surface of the packing, rinsing the tube with a drop of water and repeating the process. Adrenochrome was eluted essentially with the solvent front by forcing water through the column with an all-glass syringe. Air pockets were avoided by filling the column brimful before attaching the loaded syringe. Adrenolutin was eluted with methanol with the solvent front in a similar fashion.

RESULTS AND DISCUSSION

Just before the HPLC study, the recrystallized samples of adrenochrome and adrenolutin were examined for purity. The m.p.s. of adrenochrome and adrenolutin were 114–115°C (decomp.) and 237–238°C, respectively, in agreement with those reported in the literature[11]. Adrenolutin melted sharply under a fast rate of temperature increase (5–8°C/min) but at a rate of 1°C/min it decomposed slowly without

TABLE I

R_F VALUES AND RETENTION TIMES FOR ADRENOCHROME, ADRENOLUTIN AND EPINEPHRINE

Bonded silica gel Permakote TLC plates and Whatman No. 1 chromatography paper were prewashed with 2% acetic acid, and chromatograms were developed with deionized water. An alkyl phenyl μBondapak column and 15% methanol as mobile phase were used in HPLC for a convenient elution pattern.

Compound	R_F			*Retention time (min)*
	TLC	*PC*		*HPLC*
		Found	*Refs. 3 and 4*	
Adrenochrome	0.52	0.85	0.80	1.0
Adrenolutin	0.90	0.45	0.45	6.2
Epinephrine	0.05	–	–	0.6

melting. The IR spectra of these substances, as well as that of a sublimed sample of adrenolutin, showed the major peaks reported[4] for adrenochrome and adrenolutin, and PC analyses[3,4] produced the same R_F values (Table I) and color or fluorescence characteristics as reported for these materials[3]. The commercial sample of adrenochrome, obtained at the onset of this study, was essentially decomposed material; it was black, failed to match adrenochrome by IR examination and gave mostly a black precipitate and a dark brown solution when added to water instead of the deep red solution expected for adrenochrome. On the other hand, a commercial sample of adrenolutin was pure material with a melting point that agreed with the literature value[11], and with IR and NMR (in [$^2H_6$]dimethyl sulfoxide) spectra which matched those of the synthesized sample.

Separation and resolution in the HPLC were optimized by varying the methanol–water ratio of the mobile phase. The use of 25% methanol resulted in peak crowding, and baseline resolution was not quite achieved. Good separation and baseline resolution of epinephrine, adrenochrome and adrenolutin, eluted in that order, resulted when 15% methanol was used as the mobile phase (Fig. 1). This mobile phase gave convenient retention times for adrenochrome and adrenolutin (Table I) while washing out the epinephrine near the solvent front. Furthermore, the free base epinephrine is sparingly soluble in water and insoluble in methanol, whereas adrenochrome and adrenolutin are extremely soluble in these solvents, respectively. These solubilities permit the facile solvent extraction and concentration of adrenochrome and adrenolutin from large samples of epinephrine drug products before HPLC. For example, since epinephrine is sparingly soluble in the mobile phase, one may examine the bulk drug epinephrine directly for the presence of adrenochrome and adrenolutin by extraction of the solid with the mobile phase.

Initial attempts to conduct TLC of adrenochrome and adrenolutin on silica gel gave only black streaks with decomposition similar to that observed in PC of these substances by Heacock and co-workers[3,4], who found it essential to use acid-washed (2% acetic acid in water) chromatographic paper to obtain good chromatograms. Their PC technique, when repeated with freshly synthesized samples of adrenochrome and adrenolutin, gave essentially reproducible values (Table I). The washing

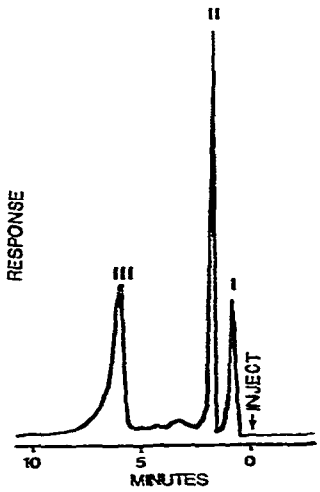

Fig. 1. Chromatogram of epinephrine (I) and its oxidation products, adrenochrome (II) and adrenolutin (III), on an alkyl phenyl μBondapak column.

of ordinary silica gel plates with aqueous acids is precluded; such treatment causes stripping of silica gel from the plate. The bonded, permanent-surface plates (Permakote) provide a silica gel layer which cannot be removed by washing or mild friction. These plates are suitable for prewashing. Chromatograms of adrenochrome, adrenolutin and epinephrine gave sharp spots, with R_F values as reported in Table I. Adrenochrome and adrenolutin were observed as red and yellow spots, respectively, under white light, and adrenolutin was seen as an intense fluorescent blue-green spot under long-wavelength UV light. Epinephrine was observed as a red spot after the plate was sprayed with 5% potassium ferricyanide in 0.1 *N* potassium dihydrogen phosphate. Impure samples of adrenochrome and adrenolutin may contain black water-insoluble melanin-like or polymeric oxidation products or may generate brownish solutions. TLC of such impure products resulted in immobile dark spots at the origin.

CC with C_{18} Sep-Paks allowed a quick separation of mixtures of adrenochrome and adrenolutin. A single Sep-Pak column was used to separate about 5 mg of each material, and twice this amount was separated by two columns connected in tandem. Water eluted the bulk of the adrenochrome as 2–4 drops of deep red solution immediately following the solvent front. The orange eluate which followed was discarded, as was the subsequent water wash, until the eluate no longer had an orange or reddish tinge. The mobile solvent was changed to methanol, and a yellow adrenolutin band eluted immediately following the solvent front. The yellow solution also exhibited a strong yellow-green fluorescence, which was especially intense under long-wavelength UV irradiation. PC or TLC on bonded silica gel (Permakote) plates showed the expected R_F values for adrenochrome and adrenolutin, and each chromatogram showed only small traces, or none, of the other component. No spots remained at the origins in either PC or TLC, suggesting that the substances were free of polymeric oxidation products.

ACKNOWLEDGEMENTS

The authors express their thanks to Matthew Dow and Patrick Carberry for their helpful suggestions in the course of this work.

REFERENCES

1 R. A. Heacock, *Chem. Rev.*, 59 (1959) 181.
2 C. Fu and M. J. Sibley, *J. Pharm. Sci.*, 66 (1977) 425.
3 R. A. Heacock, C. Nerenberg and A. N. Payza, *Can. J. Chem.*, 36 (1958) 853.
4 R. A. Heacock and M. E. Mahon, *Can. J. Chem.*, 36 (1958) 1550.
5 R. A. Heacock and M. E. Mahon, *J. Chromatogr.*, 6 (1961) 91.
6 J. D. Sapira, *J. Chromatogr.*, 42 (1969) 134.
7 P. E. Flinn, *J. Chromatogr.*, 82 (1973) 117.
8 A. Lund, *Acta Pharmacol. Toxicol.*, 5 (1949) 75.
9 J. Harley-Mason, *J. Chem. Soc., London*, (1953) 1276.
10 *Applied Science Technical Bulletin No. 15*, Applied Science Labs., State College, PA, 1972.
11 M. Windholz, S. Budavari, L. Y. Stroumtsos and M. N. Fertig (Editors), *The Merck Index*, Merck & Co., Rahway, NJ, 9th ed., 1976, p. 161.