Journal of Chromatography, 148 (1978) 453–457

CHROM. 10,496

ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF A MULTICOMPONENT ANTICHOLINERGIC DRUG FORMULATION

NESBITT D. BROWN, LOVELYN L. HALL, H. KENNETH SLEEMAN, BHUPENDRA P. DOCTOR and GALE E. DEMAREE

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C. 20012 (U.S.A.)

(Received June 7th, 1977)

SUMMARY

N,N'-Trimethylene-bis-(pyridinium-4-aldoxime)dibromide, 4-pyridine aldoxime, atropine sulfate, benactyzine hydrochloride, methyl paraben and propyl paraben are separated by ion-pair high-performance liquid chromatography. The method is specific for detecting and quantifying each compound in a complex mixture without solvent extraction or pretreatment. Levels as low as 1 ng on column are quantifiable by the procedure. All components are eluted within 9 min subsequent to the initial injection. Because of the simplicity of the method, the procedure is suitable for routinely monitoring the stability of the various compounds in the formulation during storage.

INTRODUCTION

N,N'-Trimethylene-bis-(pyridinium-4-aldoxime) dibromide (TMB-4), benactyzine hydrochloride and atropine sulfate have each enjoyed some popularity during the past 10–15 years as a therapeutic modality in the treatment of poisoning by organophosphate-inhibited cholinesterases[1–5]. Grob *et al.*[6] have used oximes in the treatment of intoxication by anticholinesterase compounds in normal subjects. Benactyzine hydrochloride has been also used as a mild anticholinergic drug in special cases of nerve agent poisonings. Chew *et al.*[7] have studied the therapeutic efficacy of atropine sulfate in the management of organophosphorus insecticidal poisonings.

In light of the supporting evidence, extolling the therapeutic values of these reactivators of phosphorylated cholinesterases, much of the current research has dealt primarily with the efficacy of these drugs in simple chemical formulations. The same has been true with the analytical methods involving the analyses of these compounds.

However, data obtained recently from a series of reports[8–10] have made it possible to separate several anticholinergic drugs in a complex mixture. The use of the described method to quantify such a wide variety of different compounds in a multicomponent formulation is unique to the field of liquid chromatography. Where formerly the separation of a complex mixture was based totally on the homogeneity

of each compound in the analyzed sample, the use of this new analytical method offers a technique contrary to this basic rule. In ion-pair high-performance liquid chromatography (HPLC), all compounds are chromatographed as neutral species of the analytical sample.

To this extent, ion-pair HPLC might possibly be classified as the "universal separating technique".

MATERIALS* AND METHODS

Apparatus and reagents

All separations were made using a Waters Model APC/GPC–204 liquid chromatograph, equipped with two Model 6000A high-pressure pumps, a 660 solvent programmer, a U6K loop injector, a 254 nm UV detector, a Houston Instrument Omni-Scribe A5000 dual pen recorder and a Columbia Scientific Industries Supergrator-3-integrator.

Spectroquality acetonitrile (Eastman-Kodak, Rochester, N.Y., U.S.A.) mixed with PIC B-7 reagent (1-heptane sulfonic acid, Waters Assoc., Milford, Mass., U.S.A.) was used as the mobile phase. TMB-4 and 4-pyridine aldoxime were obtained from the Analytical Chemistry Branch, Research Labs., (Edgewood Arsenal, Md., U.S.A.). Benactyzine hydrochloride and sulfanilic acid were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). Atropine sulfate, methyl paraben and propyl paraben were supplied by U.S.P.C. (Rockville, Md., U.S.A.). Stock standard solutions of each compound were used to prepare the synthetic multicomponent anticholinergic reference standard. A commercially contracted preparation of a multicomponent anticholinergic mixture (Cartrix Parenteral Systems, St. Louis, Mo., U.S.A.) was also analyzed by the procedure.

Procedure

A prepacked 300 × 3.9 mm I.D., μBondapak C_{18} column (Waters Assoc.) was used to chromatograph a series of anticholinergic compounds present in the multicomponent samples. The mobile phase consisted of 0.01 *M* 1-heptane sulfonic acid combined with acetonitrile. The PIC B-7 reagent was prepared by mixing 20 ml of the prepackaged reagent with 480 ml of glass distilled water. A 35% solution of acetonitrile mixed with 65% of the PIC reagent was isocratically pumped through the column. Flow-rate for the dual pumping system was 1.5 ml/min. Column pressures ranged between 1200 to 1500 p.s.i. All separations were performed at ambient temperatures. Samples were introduced into the column through a continuous-flow loop injector. The peak areas were measured by an on-line computing integrator.

RESULTS AND DISCUSSION

Few reports have been published discussing the efficacy of multicomponent anticholinergic mixtures in reversing the effects of organophosphate-inhibited cholinesterase compounds. Many investigators have indicated significant therapeutic

* The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

effects from the various individual anticholinergic drugs. However, the possibility has always existed that a multicomponent drug formulation might better enhance the effective therapeutic activity of the various drugs when such a group of compounds are administered in a therapeutic bolus. This being the case, a new antidote has been developed and tested as a reactivator of various types of organophosphate poisonings.

At the same time, an attempt was made to develop a specific method to separate and quantify the chemical components of this mixture. From our previous work[8–10] several anticholinergic compounds and some of their degradative products were chromatographed by ion-pair HPLC. Incorporating the various separation parameters, including the respective retention times for each compound, a simple and specific method was developed for chromatographing this multicomponent formulation. In addition, methyl paraben and propyl paraben, which were added as preservatives and fungistatic agents were chromatographed by this procedure.

Our prime objective for initiating this study was to develop a method which could be used for observing the stability of the anticholinergic antidote during its manufacture and later, during its storage. The correct concentration of each compound in the mixture is a necessary requirement in order that the antidote works effectively as a therapeutic modality against organophosphate poisoning.

A mixed reference standard of the various anticholinergic compounds, including methyl and propyl paraben was prepared. Upon injection the following chromatogram was produced. Fig. 1 represents the separation of the standard mixture,

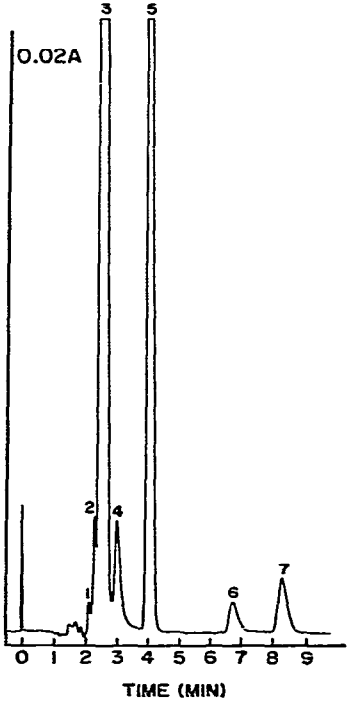

Fig. 1. Separation of an anticholinergic reference standard, containing (1) P-hydroxybenzoic acid, (2) 4-pyridine aldoxime, (3) TMB-4, (4) atropine sulfate, (5) methyl paraben, (6) benactyzine hydrochloride and (7) propyl paraben. Column: 300 × 3.9 mm I.D. μ-Bondapak C_{18}.

which was prepared by dissolving each compound into 0.002 *N* HCl. The pH of the solution was 2.70. In the chromatogram, seven peaks were observed. All were quantifiable by the method. The concentrations of the various compounds ranged from 1.5 ng/μl to 2 μg/μl. Even with such a wide variation in the respective concentrations of these compounds, the accuracy and precision of the method were excellent. Except for the presence of trace amounts of P-hydroxybenzoic acid and 4-pyridine aldoxime, the mixture remained stable for an indefinite period of time, when refrigerated at 4°.

Analyzing the commercially prepared antidote, a slightly different picture was observed in the chromatographic profile of this formulation. Fig. 2 depicts the separation.

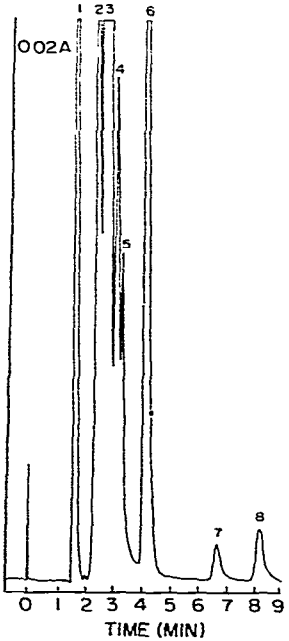

Fig. 2. Separation of a commercially prepared anticholinergic antidote, containing (1) sulfanilic acid (internal standard), (2) 4-pyridine aldoxime, (3) TMB-4, (4) atropine sulfate + unknown, (5) unknown, (6) methyl paraben, (7) benactyzine hydrochloride and (8) propyl paraben. Column temperature: 18°, flow-rate: 1.5 ml/min. Mobile phase: 35% acetonitrile and 65% PIC reagent. Chart speed: 1 cm/min.

Aside from the presence of a large amount of 4-pyridine aldoxime, two additional peaks appeared in the chromatogram. Their identities are unknown. One of these unknown peaks masked the exact area in which atropine sulfate was to appear. The presence of these unknown compounds did not interfere with the separation of the other compounds. More than 500 analyses were made during this study. The precision of the method for a representative number of samples is given in Table I. Sulfanilic acid was used as an internal standard for maintaining quality control.

TABLE I

PRECISION OF ION-PAIR HPLC METHOD FOR QUANTIFYING A MULTICOMPONENT DRUG FORMULATION (20 SAMPLES)

Compounds	*Reference standard*			*Commercial preparation*		
	Mean (ng/μl)	*SD*	*% CV*	*Mean (ng/μl)*	*SD*	*% CV*
TMB-4	2049	± 34.0	1.60	1980	±35.0	1.81
Atropine sulfate	50	± 1.1	2.36	—	—	—
Methyl paraben	40	± 0.7	1.82	41	± 0.9	2.43
Benactyzine HCl	173	± 3.9	2.26	172	± 11.0	6.52
Propyl paraben	1.6	± 0.06	4.37	1.5	± 0.06	4.37

From this study, involving the use of ion-pair HPLC to analyze a multicomponent drug formulation, the results obtained gave credence to the employed method, as one, which will fulfill our projected needs. In the near future, a more comprehensive investigation will be launched to seek more definitive answers to the stability of this therapeutic antidote when the mixed formulation is subjected to various experimental procedures.

ACKNOWLEDGEMENT

We thank Ms. Margot M. Stevens for her excellent secretarial assistance in the preparation of this manuscript.

REFERENCES

1 J. H. Feisher, H. O. Michel, L. Yates and C. S. Harrison, *J. Pharmacol. Exp. Ther.*, 129 (1960) 31.
2 E. Bay, S. Krop and L. F. Yates, *Proc. Soc. Exp. Biol. Med.*, 98 (1958) 107.
3 W. J. Hayes, *J. Amer. Med. Assoc.*, 192 (1965) 49.
4 V. M. Sim, *J. Amer. Med. Assoc.*, 192 (1965) 403.
5 J. Schenk, W. Loeffler and N. Weger, *Arch. Toxikol.*, 36 (1976) 71.
6 D. Grob and R. J. Johns, *Amer. J. Med.*, 24 (1958) 497.
7 L. Chew, K. T. Chee and J. M. Yeeo, *Singapore Med. J.*, 12 (1971) 80.
8 N. D. Brown and H. K. Sleeman, *J. Chromatogr.*, 138 (1977) 449.
9 N. D. Brown and H. K. Sleeman, *J. Chromatogr.*, 140 (1977) 300.
10 N. D. Brown and H. K. Sleeman, *J. Chromatogr.*, in press.