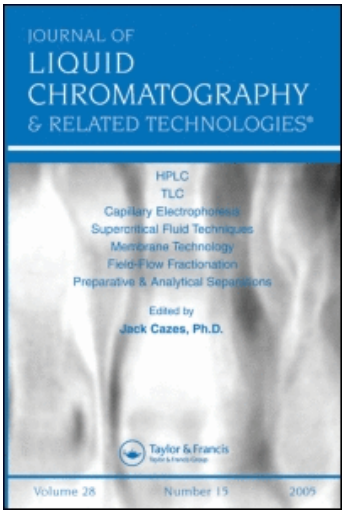

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J. Muztar[a]; G. Chari[a]; R. Bhat[a]; S. Ramarao[a]; D. Vidyasagar[a]
[a] Department of Pediatrics, The University of Illinois at Chicago Chicago, Illinois

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A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE SEPARATION OF COCAINE AND SOME OF ITS METABOLITES FROM ACEPROMAZINE, KETAMINE, AND ATROPINE FROM SERUM

J. MUZTAR, G. CHARI, R. BHAT, S. RAMARAO, AND D. VIDYASAGAR
Department of Pediatrics
The University of Illinois at Chicago
Chicago, Illinois 60612

ABSTRACT

A high-performance liquid chromatographic procedure for the separation of cocaine and its metabolites from such non-test drugs as acepromazine, ketamine and atropine in serum is described. The method involves the use of a SemiPermeable Surface (SPS) C8 column with a mobile phase constituted of 3.25% tetrahydrofuran and 96.75% 0.0025 M potassium phosphate buffer, v/v, containing 0.0025% triethylamine, the final pH of the mobile phase being 2.75. The flow rate was 0.5 ml/min. A photodiode array detector equipped with a computer software was used to monitor the analyte peaks. Retention times for cocaine (COC), benzoylecgonine (BE), benzoylnorecgonine (BN), norcocaine (NOR) and bupivacaine (BV) were 15.5, 7.4, 12.25, 21.0 and 24 minutes, respectively. The non-test drugs ketamine and atropine co-eluted at 10.0 minutes while acepromazine eluted with the solvent front (3.8 min.). The sensitivity of this assay for each of COC, BE, BN and NOR, at a signal to noise (S/N) ratio of greater than 6.0, was 1.0 ng/ml while that for BV was 5ng/ml at the same S/N value.

INTRODUCTION

There are numerous published methods for the determination of cocaine (COC) and one or more of its metabolites in biological fluids and tissue, using high-performance liquid chromatography (HPLC) (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11). However, none of these methods have

addressed the issue of separating COC and its metabolites from acepromazine (ACE), ketamine (KET) and atropine (ATR). Experiments investigating the distribution of COC and its metabolites in tissues and biological fluids of larger animal species, particularly the non-human primates (monkeys, baboons, etc.), commonly involve administration of these non-test drugs at frequent intervals before surgery and during sampling in order to sedate the animal and thus permit safe handling (12, 13, 14).

Due to the basic nature of these drugs and those of COC and its metabolites, all these are expected to demonstrate very similar chromatographic properties and hence , not surprisingly we were not able to separate them using the existing HPLC methods (Muztar et al , unpublished results).

The adverse pharmacological effects of COC and its active metabolite benzoylecgonine (BE) on the fetus of pregnant women abusing the drug are well established (15, 16). Therefore, the need for an analytical method that can distinctly separate COC and its metabolites from the non-test drugs can hardly be overemphasized.

In the present study, we have developed an HPLC method which separates COC and some of its metabolites, norcocaine (NOR) , BE and benzoylnorecgonine (BN) from the non-test drugs (ACE, KET and ATR).

MATERIALS AND METHODS

Reagents and standards

HPLC grade tetrahydrofuran (THF) and reagent grade triethylamine (99%) were purchased from Fisher Scientific (Itasca, Illinois, U.S.A.).

(-) Cocaine hydrochloride, BE, and bupivacaine (BV) were purchased from Sigma (St. Louis, Missouri, U.S.A.). (-) Benzoylnorecgonine hydrochloride and (-) norcocaine were supplied by NIDA, National Institute of Health , Rockville, Md. Benzethonium chloride and benzyl alcohol were purchased from Aldrich Chemicals (Milwaukee, U.S.A). Atropine sulfate was a product of

American Reagent Laboratories (Shirley, NY, U.S.A.), while acepromazine maleate and ketamine hydrochloride were made by Aveco Co. (Fort Dodge, Iowa, U.S.A.) and Parke-Davis (Morris Plains, New Jersey, U.S.A.), respectively.

Stock solutions (1.0 mg/ml) of COC , BN and NOR were made in water. BE was taken in 0.1 M $NaHCO_3$. Appropriate dilution of the stock solutions were made for running HPLC standards and to spike drug-free serum except for BN which was spiked at four times the above concentrations because of its very poor recovery. The internal standard BV was used at a concentration of 0.5, 0.1, 0.2, 0.4, 0.8 and 1.0 µg/ml.

Chromatographic conditions

A Waters 600E multi-solvent HPLC pump was used to provide solvent at a flow rate of 0.5 ml/min. Tetrahydrofuran was used as the organic modifier. A 0.0025 M potassium phosphate buffer mixed with THF (96.75: 3.25, v/v) containing 0.0025% triethylamine with the final pH adjusted to 2.7 to 2.8 with 85% orthophosphoric acid formed the mobile phase.

A SemiPermeable Surface (SPS) C8 column (Regis Chemicals, Morton Grove, IL, U.S.A.) with dimensions of 15-cm length and 4.5-cm i.d. was used for the analysis of the drugs. The HPLC system was equipped with a U6K injector (Water-Millipore, Milford, MA) and a 100-µl loop. The eluting drugs were detected with a Waters 991 photodiode array detector at 4 different wavelengths.

Extraction of the drugs

Solid phase extraction of the drugs form spiked serum samples was carried out using 3-ml Clean Screen Columns (Worldwide Monitoring, Horsham, PA, U.S.A). The cartridges were placed on a 24-station VAC-Elut (Varian, Harbor City, CA, U.S.A.) manifold and vacuum applied from 1 to 5 mmHg or as necessary. The cartridges were first conditioned by washing with methanol (2 x 2 ml), then with water (3 ml) and 0.01 M phosphate buffer, pH 3.0 (3 ml). A 1-ml serum mixed with 0.5 ml of a 0.01 M phosphate buffer, pH 3.0, was then applied to the

extraction cartridge and air dried for about 30 s. This was then washed with 3 ml of phosphate buffer, 3 ml of 100 mM HCL and finally with 3 ml of methanol. The elution of the drugs was performed by using 2 ml of $CHCl_3$- Isopropyl alcohol- NH_4OH (77:20,5:2.5). The extract was evaporated at 30° C by passing N_2 gas using the Meyer N-Evap analytical evaporator (Organomation Assoc., Berlin, MA, U.S.A.) and taken in 100μl of the mobile phase for injection into the HPLC column.

RESULTS

The calibration was performed using seven concentrations (50 ng/ml to 1.0 μg/ml) of COC, BE, NOR in serum. The mean coefficient of variation (CV) was 10.4 %. Linear correlations were done by regression analysis. The correlation coefficients for each standard were > 0.998. Similar correlations were obtained for BN with four times greater concentrations. The intra-assay precisions were done over 5 days for 500 ng/ml and 1μg/ml concentrations. The CVs ranged from 4.2 to 7.6 % for COC, BE, NOR and BN. The inter-assay precision CVs ranged from 4.8 to 7.3 %. Since quantitation of the non-test drugs ACE, KET and ATR was not the focus of this study, the precision of the HPLC measurements for these drugs was not determined.

A standard chromatogram (Figure 1) obtained with a mixture of the test drugs COC and its three metabolites along with the non-test drugs KET, ACE and ATR demonstrated a clear separation of COC and its three metabolites from the non-test drugs. Retention times for BE, BN, COC and NOR were 7.4, 12.25, 15.5 and 21.0 min., respectively and that for the internal standard BV was 24.0 min. Acepromazine eluted at 3.8 min.

Serum was spiked with 10 ng (injected volume 10 μl) of both the test and non-test drugs as well as the internal standard and extracted with Clean Screen column as described in the experimental section. HPLC analysis (Figure 2) showed a clear separation of COC and its three metabolites as well as the internal standard from the non-test drugs. Also noticeable was the fact

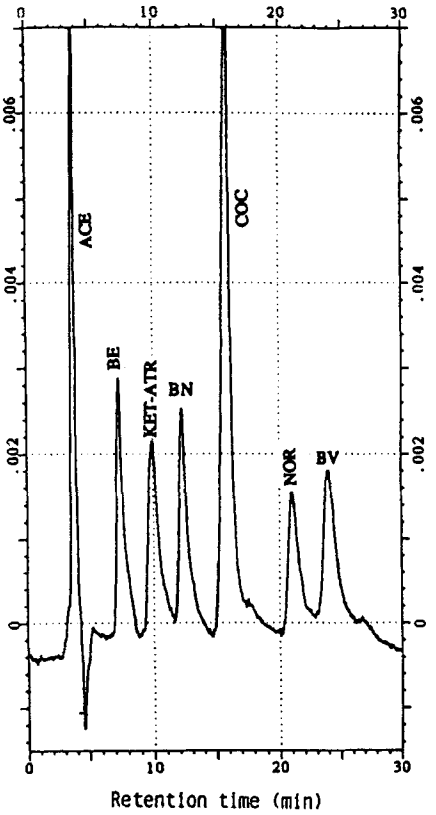

FIGURE 1: Chromatogram showing the separation of a standard mixture of the test drugs COC, BE, BN, NOR and BV from the non-test drugs , ACE, KET and ATR.

that there was no peaks of the additives, benzethonium chloride and benzyl alcohol present in ketamine and atropine. Apparently these were washed out from the SPE cartridge during the sample clean-up steps. Figure 4 shows a chromatogram of a serum sample obtained from a baboon injected with 1 mg/kg of COC.

Comparing differences of areas under the curve of extracted and unextracted samples for the same concentrations, recoveries of BE , COC and NOR were 95, 97 and 96% respectively and of BV was 95%. Recovery of BN was very poor (<2%). Limit of detection for the COC and its

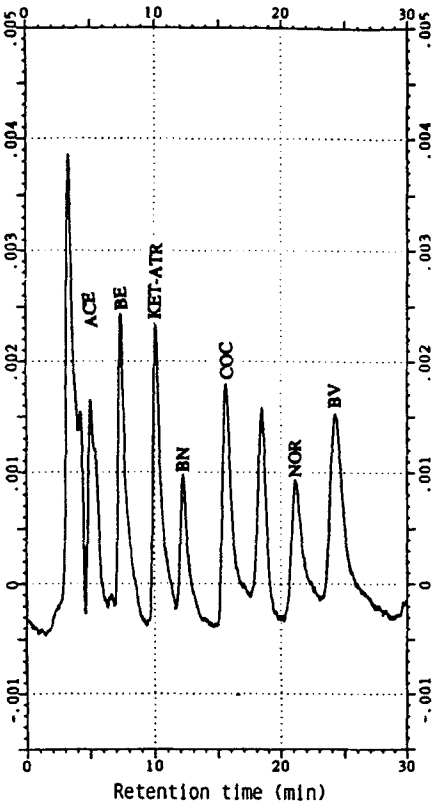

FIGURE 2: Chromatogram showing the separation of a serum extract mixture of 10 ng (10 µl injection) of the test drugs cocaine (COC), benzoylecgonine (BE), benzoylnorecgonine (BN), norcocaine (NOR) with the internal standard bupivacaine (BV) plus the non-test drugs acepromazine (ACE), ketamine (KET) and atropine (ATR).

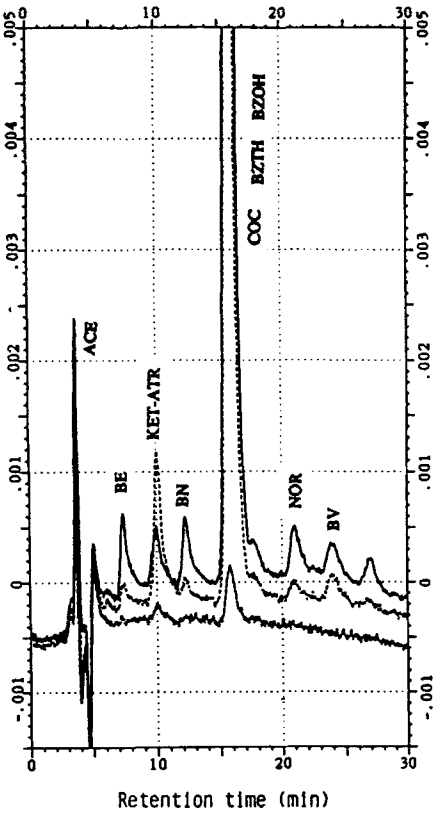

FIGURE 3: Shows the absorbance spectra of a standard mixture of the test drugs and the non-test drugs at three different wavelengths, 235 (------), 255 (------) and 280 nm (.....). BZTH *refers to benzethonium* chloride and BZOH to benzyl alcohol. For other abbreviations see Figures 1 & 2.

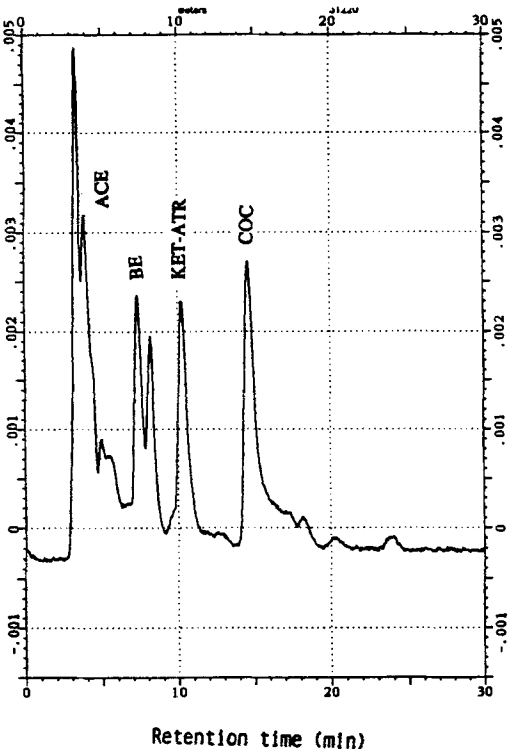

FIGURE 4: Chromatogram obtained from a baboon serum sample injected with 1 mg/kg of COC.

three metabolites was 1.0 ng/ml at a signal to noise (S/N) ratio of greater than 6.0, while for BV was 5.0 ng/ml at the same S/N ratio. Since quantitation of the non-test drugs ACE, KET and ATR was not the focus of this study, recovery data was not obtained.

DISCUSSION

In theory, the non-test drugs can be thought of being eliminated during sample preparation in the solid phase extraction (SPE). However, it is important to note that SPE procedures rely heavily on the gross differences in such physico-chemical parameters as pKa and hydrophobicity,

and thus lack manipulation capability and the subtle selectivity of an analytical method such as HPLC. For example, BE (pKa 2.6), which is far apart from that of COC (pKa 8.6) and the non-test drugs, ACE (pKa 9.3), KET (pKa 7.5) and ATR (pKa 9.9), yet attempts to elute BE alone from the SPE cartridge using an eluent mixture of pH 8.0 to 10.0 results in very poor or no recovery (Muztar et al, unpublished results). On the other hand, when using an eluent mixture of pH $\geq$ 11.5, over 90% of BE and COC are eluted and so are the non-test drugs (ACE, KET and ATR).

Using the existing HPLC procedures we were unable to separate BE and BN in serum samples obtained from animals administered with COC and non-test drugs because of the overlapping retention times. Further, our attempts to accurately quantitate BE and BN in serum by analyzing chromatograms using differential wavelength scanning produced very little success since the non-test drugs, particularly ATR and KET, show significant absorption over the entire absorption wavelength of both BE and BN (Figure 3). For reason of clarity, absorption spectra is shown only for three wavelengths (235, 255 and 280 nm). However, the 200 through 210 nm region causes KET and ATR to absorb as much as BE and BN while the 215 through 235 nm zone demonstrates relatively less absorption. On the other hand, in the 240 through 280 nm region KET and ATR absorb much more than BE and BN with absorption increasing with an increase in the wavelength. In the present separation method the absorption of ACE with respect to the wavelength is not in sequence since it is found to elute with the solvent front. From these results it is apparent that the non-test drugs absorb much less than the test drugs between the wavelengths of 215 through 235 nm.

Initially, a number of mobile phases consisting of various proportions of acetonitrile to varying molarities of phosphate buffer were tried but none separated KET and ATR from BE while certain proportions caused coelution of BN and COC. Finally, acetonitrile was replaced with tetrahydrofuran. Using 0.0025 M potassium phosphate buffer (96.75%) to THF (3.25%), v/v, and 0.0025% triethylamine with the final pH adjusted to 2.7 to 2.8 with 85% orthophosphoric

acid, we were able to clearly separate both BE and BN from ACE, ATR and KET as evident from Figures 1 and 2.

It was also evident from the test chromatogram (Figure 2) that the additives benzethonium chloride and benzyl alcohol, contained in the non-test drugs, are eliminated during the SPE treatment as indicated by the absence of their respective peaks. Absence of these peaks from the chromatogram was confirmed by extracting serum spiked with a known amount of these two compounds. The disappearance of the additive peaks post-extraction is not surprising because these differ substantially in structure from COC and its metabolites as well as the non-test drugs and hence should exhibit significantly different physico-chemical properties during sample clean-up using the Clean Screen solid phase extraction cartridge.

Another important aspect of this separation method is that, it not only distinctly separates the COC and its metabolites from the non-test drugs, but also provides us with a very sensitive HPLC procedure (detection limit of 1.0 ng/ml, S/N ratio > 6.0).

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