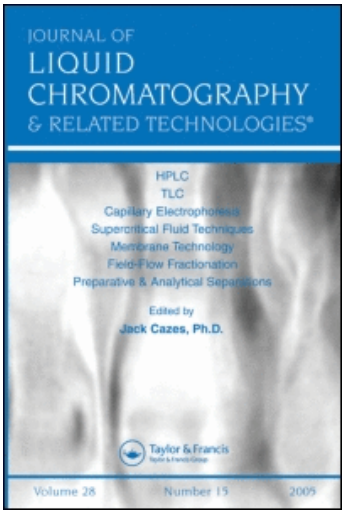

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Online publication date: 14 May 2010

To cite this Article Fernández, P. , Rodríguez, P. , Bermejo, A. M. , López-Rivadulla, M. and Cruz, A.(1994) 'Simultaneous Determination of Cocaine and Benzoylecgonine in Vitreous Humor by HPLC', Journal of Liquid Chromatography & Related Technologies, 17: 4, 883 — 890
To link to this Article: DOI: 10.1080/10826079408013375
URL: http://dx.doi.org/10.1080/10826079408013375

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(4), 883-890 (1994)

SIMULTANEOUS DETERMINATION OF COCAINE AND BENZOYLECGONINE IN VITREOUS HUMOR BY HPLC

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ABSTRACT

An isocratic HPLC system for quantifying benzoylecgonine (BE) and cocaine (COC) in vitreous humour (VH) was developed. Following solid-phase extraction, the two drugs are chromatographed on a column filled with octadecyl silica packing, eluted with 75% methanol in phosphate buffer of pH 7, and sensed by UV spectrophotometry at 235 and 275 nm, simultaneously. The detection limit thus achieved was 0.5 μg drug per millilitre of VH. By using tetracaine as internal standard, BE and COC can be determined with a coefficient of variation of 6 and 7%, respectively.

INTRODUCTION

Cocaine (COC) is a powerful stimulant and one of the most widely abused drugs by all social classes at present. Cocaine-related deaths are reportedly on the increase [1,2]. Consequently, analyses for COC in biological specimens have major social and legal implications. One of the major metabolites for COC is benzoylecgonine (BE), which must thus also be assayed jointly with the parent drug in comprehensive analyses.

Vitreous humour (VH) is frequently the best or even the sole type of sample available for post-mortem analyses on severely burned, embalmed or

bleeding-shocked bodies. It is a fairly simple matrix compared to blood and urine and is scarcely prone to major post-mortem alteration. The lack of metabolic activity in the eye suggests that drug levels may provide a more accurate indication of body drug concentrations at the time of death.

Only a few of the many analytical procedures available for the determination of COC and BE by using RIA [3], GC–MS [4] and HPLC [5] are concerned with VH [6]. High performance liquid chromatography (HPLC) is particularly effective for the isolation of polar, non-volatile substances such as BE and related products with no derivatization.

This paper reports a straightforward, sensitive reversed-phase HPLC method for the joint determination of COC and BE in VH, based on solid-phase extraction and UV spectrophotometric detection.

MATERIALS

Apparatus

The isocratic HPLC system used was composed of a Model 501 pump, a U6K injector and a 490 programmable multiwavelength UV detector, all from Waters Associates (Mildford), and was interfaced via an appropriate module to a Digital-Professional 350 personal computer for recording.

Reagents

COC and BE hydrochlorides were obtained from the Spanish Ministry of Health. Tetracaine (TET) hydrochloride was complimentarily supplied by Ifesa S.A. Laboratories. HPLC-grade methanol from Merck (Darmstad) and water distilled through a Milli-Q apparatus from Millipore Corporation were also used. A phosphate buffer of pH 7 was made by adding 320 mL of 0.02 M *di*-potassium hydrogen phosphate to 680 mL of 0.02 M potassium dihydrogen phosphate.

Standards

COC and BE stock solutions containing 1 mg/mL of either drug in methanol were used to make working strength solutions of 0.5, 1, 2, 4, 8 and 10 μg/mL by appropriate dilution.

The TET internal standard was made by dilution to 20 μg/mL of a previously prepared stock solution containing 1 mg/mL of the compound.

Vitreous humour samples were prepared as follows: a VH pool was made from drug-free post-mortem specimens that were mixed, centrifuged and refrigerated at 4°C prior to use. Aliquots of 0.5 mL were spiked with 0.5, 1, 2, 4, 8 and 10 μg/mL COC and BE, plus 20 μg/mL TET internal standard. All these solutions were prepared in triplicate.

METHODS

Extraction Procedure

The solid-phase extraction procedure employed used 3-mL Bond-Elut certify™ columns from Analytichem Int. that were held in a Vac-Elut™ system. The sorbent was prepared by adding 2 mL of methanol, 1 mL of water and 0.5 mL of 10 mM phosphoric acid at pH 3.4. The sample was applied after mixing 0.5 mL of COC- and BE-spiked VH with 0.25 mL of 10 mM phosphoric acid. The washing step was effected by adding 1 mL of 10 mM phosphoric acid, 0.5 mL of 0.1 M acetic acid, 0.5 mL of methanol and 0.5 mL of 0.3 M ammonium hydroxide. Elution was done with 2 mL of methanol and the eluate was finally concentrated by evaporation to dryness under a nitrogen atmosphere at 70°C, followed by reconstitution with 500 μL of 20 μg/mL TET in methanol. The pressure used for passing liquids through solid phase cartridge was 2 in of mercury.

Chromatographic Procedure

The analytical column used was a 125 × 4 mm ID reversed-phase column packed with 5-μm Lichrospher 100 RP-18 (Merck, Darmstad). A guard column (4x4 mm) with the same packing was also employed. The mobile phase was prepared from methanol and 0.02 M phosphate buffer in a 75:25 ratio. The flow-rate was 0.6 mL/min and the working pressure 1,000–1,200 psi. Detection was carried out by UV spectrophotometry at 235 and 275 nm simultaneously (0.03 AUFS). The overall analysis time was 11 min and the injected volume 25 μL.

RESULTS

As can be seen in figures 1 and 2, the chromatograms obtained under the above described conditions, at 235 and 275 nm respectively, showed three peaks corresponding to as many compounds with retention times of 2.45 (BE), 6.40 (COC) and 9.40 min (TET).

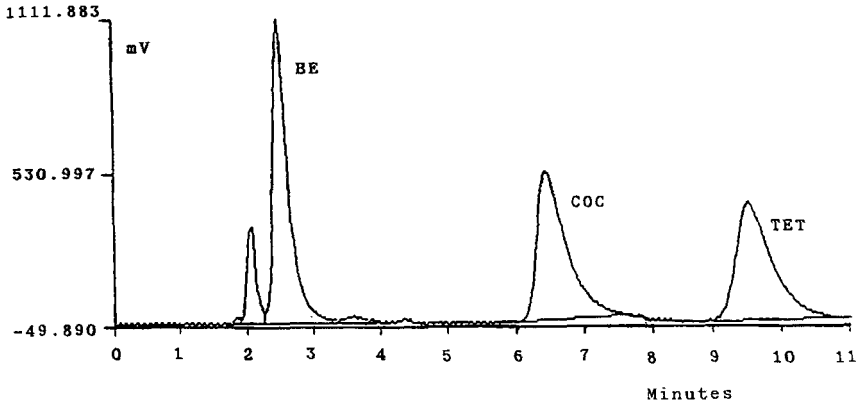

FIGURE 1 — Chromatogram obtained at 235 nm for 10 μg/mL BE (Area: $1.73.10^7$) and COC (Area: $1.80.10^7$) in methanol, and 20 μg/mL TET (Area: $1.54.10^7$) as internal standard.

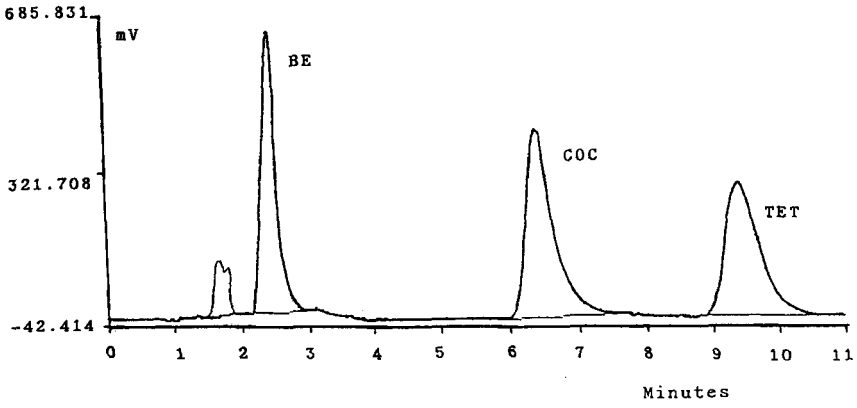

FIGURE 2 — Chromatogram obtained at 275 nm for 10 μg/mL BE (Area: $1.13.10^7$) and COC (Area: $1.32.10^7$) in methanol, and 20 μg/mL TET (Area: $1.42.10^7$) as internal standard.

TABLE 1
Statistical parameters for the determination of COC and BE

	r		Slope		Intercept		SEE	
Compound	MeOH	VH	MeOH	VH	MeOH	VH	MeOH	VH
COC	0.999	0.996	0.131	0.074	-0.035	-0.017	0.009	0.023
BE	0.999	0.997	0.113	0.080	-0.008	-0.008	0.019	0.024

SEE = standard error of estimate

Calibration curves were run by analysing mixtures of methanol and VH spiked with COC and BE at concentrations between 0.5 and 10 μg/mL. The peak area ratios of BE and COC to internal standard (TET) were found to be linearly related to the drug concentrations. Table 1 summarizes the results of the regression analysis performed.

No peaks were obtained at the retention times of BE and COC following solid-phase extraction. Figures 3 and 4 show the chromatograms obtained for a blank of VH containing TET and another of VH spiked with 10 μg/mL BE and COC.

The mean absolute recoveries or extraction efficiencies were 75 and 65% for BE and COC, respectively.

The reproducibility of the proposed method was determined by analysing a sample of VH spiked with 4 μg/mL BE and COC in sixtuplicate. The coefficients of variation for BE and COC were quite acceptable (6 and 7%, respectively).

The detection limit achieved was 0.5 μg of BE or COC per millilitre of VH.

DISCUSSION

The mobile phase used was a modification of that employed by Masoud [7]. After assaying various pH values and methanol contents, we finally chose pH 7 and 75% methanol as optimal for separation of the analytes (Fig. 1).

Of the three compounds tested as internal standards, *viz.* lidocaine, procaine and tetracaine, the last was found to be the most suitable as it was readily

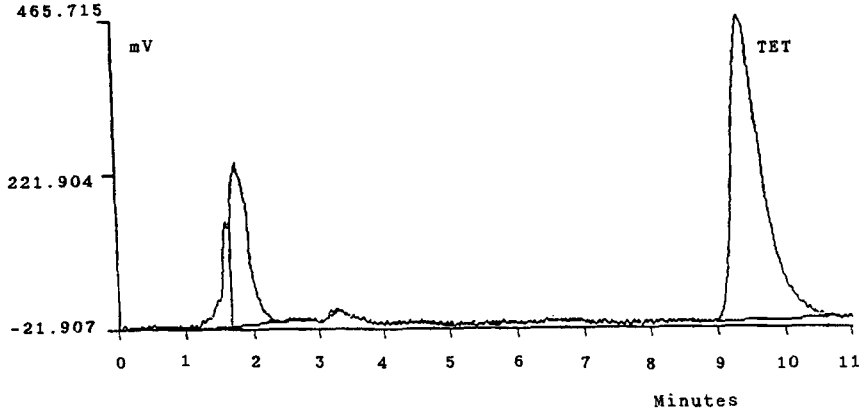

FIGURE 3 — Chromatogram obtained at 235 nm for a blank of VH containing 20 μg/mL TET (Area: $1.56.10^7$).

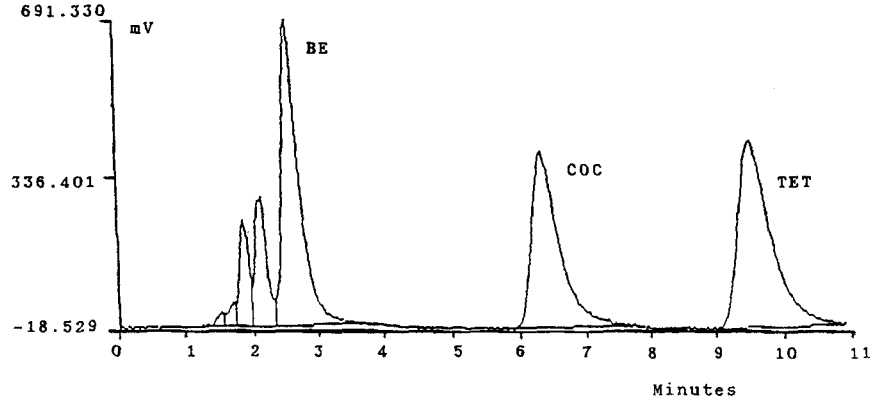

FIGURE 4 — Chromatogram at 235 nm for VH spiked with 10 μg/mL BE (Area: $1.35.10^7$) and COC (Area: $1.24.10^7$) plus 20 μg/mL TET (Area: $1.50.10^7$).

separated from BE and COC and exhibited adequate absorption at 235 nm in the form of a consistent peak.

Of the two optimal wavelengths, 235 and 275 nm, the former resulted in stronger signals (figures 1 and 2), notwithstanding which we chose to use both simultaneously in order to obtain height peak ratios to ascertain the presence of BE and COC in the samples.

Ecgonine methyl ester, which was also assayed, exhibited inadequate absorption for UV spectrophotometric detection.

Solid-phase extraction (figure 4) offers the advantage that it prevents emulsion formation. Also, it has so proved to be more expeditious than traditional liquid-liquid extraction, which usually involves using large solvent volumes, time-consuming centrifugation, back-extraction and evaporation to make the sample ready for analysis.

The detection limit of the method proposed (0.5 μg of drug/mL of VH) happened to be adequate at the sight of the results obtained in our laboratory, where twelve cases of fatal poisoning caused by heroine and cocaine or cocaine alone were studied. This work, not yet published, was carried out using radioimmunoassay and the COC and BE levels ranged between 1.04 and 26.14 μg/mL of VH.

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Received: June 6, 1993
Accepted: August 10, 1993