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DETERMINATION OF COCAINE AND SELECTED METABOLITES IN CANINE AND HUMAN SERUM BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON COUPLED CYANOPROPYL AND SILICA COLUMNS

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SUMMARY

A high-performance liquid chromatographic procedure for the determination of cocaine and selected metabolites (benzoylecgonine, norcocaine and benzoylnorecgonine) from human and canine serum has been developed The analytes are extracted from 0 5-ml serum samples using strong cation-exchange and octadecylsilane solid-phase extraction columns Chromatographic separation was accomplished on coupled cyanopropyl and silica columns using acetonitrile–6 25 m*M* phosphate buffer, pH 2 9 (80 20 v/v) as eluent and a flow-rate of 1 1 ml/min Low-wavelength UV detection (228 nm, cadmium lamp) allowed detection of 1 ng/ml for each analyte The assay was linear from 0 to 200 ng/ml ($r^2 = 0\ 997$–$0\ 999$, $n = 15$ for each analyte) and showed good precision (relative standard deviation = 0 66–13 8%, $n = 6$) and accuracy (98–115% relative recovery)

INTRODUCTION

There is an interest in these laboratories in a study of cocaine and norcocaine pharmacokinetics in dogs. Apart from its legitimate use as a local anesthetic in surgery and endoscopic procedures [1], cocaine has become the drug of choice for recreational abuse in the U S.A [2,3] Cocaine is rapidly and extensively metabolized to ten known metabolites mainly by non-specific serum esterase and/or base-catalyzed hydrolysis and oxidative deamination [4–8] Benzoylecgonine, one of the major metabolites, is usually selected as the analyte of choice for drug monitoring in urine [5] Norcocaine is the only pharmacologically active metabolite [9,10] and benzoylnorecgonine is the major metabolite of norcocaine There are numerous references in the scientific lit-

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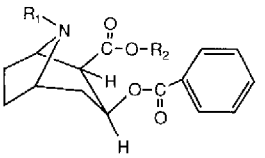

Fig 1 Structures of cocaine ($R_1=R_2=-CH_3$), benzoylecgonine ($R_1=-CH_3$, $R_2=-H$), norcocaine ($R_1=-H$, $R_2=-CH_3$) and benzoylnorecgonine ($R_1=R_2=-H$)

erature describing the analysis of cocaine and/or selected metabolites in biological fluids Gas chromatographic (GC) procedures in conjunction with nitrogen–phosphorus detection [11], electron-capture detection [12] or mass spectrometric (MS) detection [13] appear to be the most favored methods due to their sensitivity and selectivity However, the GC procedures usually require chemical derivatization of the analytes for successful chromatography and there is a substantial investment in MS equipment and expertise

The reported high-performance liquid chromatographic (HPLC), thin-layer chromatographic (TLC) and immunoassay methods suffer from a lack of selectivity and/or sensitivity and do not allow the concurrent analysis of cocaine and any of the other desired metabolites from serum samples [8,14–20] In this paper, an HPLC assay for the simultaneous determination of cocaine, benzoylecgonine, norcocaine and benzoylnorecgonine in human and canine serum is reported The structures of the individual compounds are shown in Fig. 1 The compounds are separated from the serum matrix using solid-phase extraction techniques and are chromatographed on tandem cyanopropyl–silica columns in the reversed-phase mode The method will be used in our study of cardiovascular pharmacokinetics of cocaine and norcocaine in dogs.

EXPERIMENTAL

Chemicals and reagents

Cocaine hydrochloride and tolazoline hydrochloride were obtained from Sigma (St Louis, MO, U S A) Benzoylecgonine was synthesized using a modification of the procedure by Findlay [21] Cocaine hydrochloride (500 mg) was dissolved in 50 ml of 10% sodium bicarbonate and extracted three times with 25 ml of diethyl ether The ether solution was then washed with distilled water, dried with anhydrous magnesium sulfate, and the ether was evaporated to dryness under vacuum The residue containing cocaine base was refluxed for 24 h in 50 ml of HPLC water The aqueous solution was then allowed to cool to ambient temperature, washed twice with diethyl ether and then stored at 5 °C overnight in a refrigerator. White needles of benzoylecgonine were collected, recrystallized from HPLC water and dried over phosphorus pentoxide in a drying pistol. A melting point (m.p.) of 193 °C (lit. 195 °C) was obtained. The synthesis of norcocaine was initially attempted using recent

literature references [22,23], but it was finally synthesized according to a procedure described by Werner et al [24] [m.p 79–81°C (lit. 82°C)]. Benzoylnorecgonine was synthesized in a similiar manner as described above for benzoylecgonine except that norcocaine was used as the starting material The molecular weights and structures of all the synthesized analytes were confirmed by direct-probe MS and ^{1}H NMR All compounds were shown to be chromatographically pure, the analytes were injected into the herein described HPLC system at a concentration of 1 μg/ml at the most sensitive detector setting with no extraneous peaks being observed.

Acetonitrile, methanol, chloroform, toluene and water were HPLC grade (J.T Baker, Phillipsburg, NJ, U S A) Monobasic sodium phosphate, concentrated phosphoric acid, glacial acetic acid, sodium carbonate, sodium bicarbonate, sodium fluoride, phosphorus pentoxide and diethyl ether were Baker-analyzed reagents Chromerge was obtained from Fisher Scientific (Fairlawn, NJ, U S.A) and hexamethyldisilazane was purchased from Aldrich (Milwaukee, WI, U S A)

Instrumentation

Chromatography was performed on an HPLC system consisting of a Varian Model 2510 HPLC pump (Walnut Creek, CA, U S A), a Rheodyne Model 7125 injector equipped with a 50-μl injection loop (Cotati, CA, U S.A) and a Beckman Model 160 UV detector (San Ramon, CA, U.S A.) fitted with a cadmium lamp (228 nm; UVP, San Gabriel, CA, U.S A). Separation was accomplished on tandem 5-μm cyanopropyl and silica columns (Brownlee Labs , Santa Clara, CA, U.S.A., 100 mm × 4.6 mm I D.) Two 7-μm silica precolumns, 15 mm × 4.6 mm (Brownlee Labs) were also used; one was inserted before the injector and the other was placed before the analytical columns The mobile phase consisted of acetonitrile–6.25 m*M* aqueous sodium dihydrogen phosphate (80 20 v/v), pH adjusted with concentrated phosphoric acid to 2 9 The flow-rate was set at 1 1 ml/min

Data collection and manipulation

The UV detector was set at its maximum sensitivity (0 001 a u f s) with no digital filtering and a 0.5-s time constant The 10 mV full scale detector output was fed into a preamplifier/analogue filter with a gain of 1000 (fabricated in-house, circuit diagram and flow chart for data acquisition are shown in Fig 2) The resulting 10-V signal was collected and digitized with an IBM data acquisition and control Adapter (12 bit resolution, Boca Raton, FL, U S A) on an AT compatible computer (Jameco Electronics, Belmont, CA, U S A.) Data reduction and analysis were performed using Unkelscope 2+ software (Unkel Software, Lexington, MA, U.S A) and a Spectra-Physics Model SP 4290 computing integrator (San Jose, CA, U S.A.) Data were sampled and digitized at

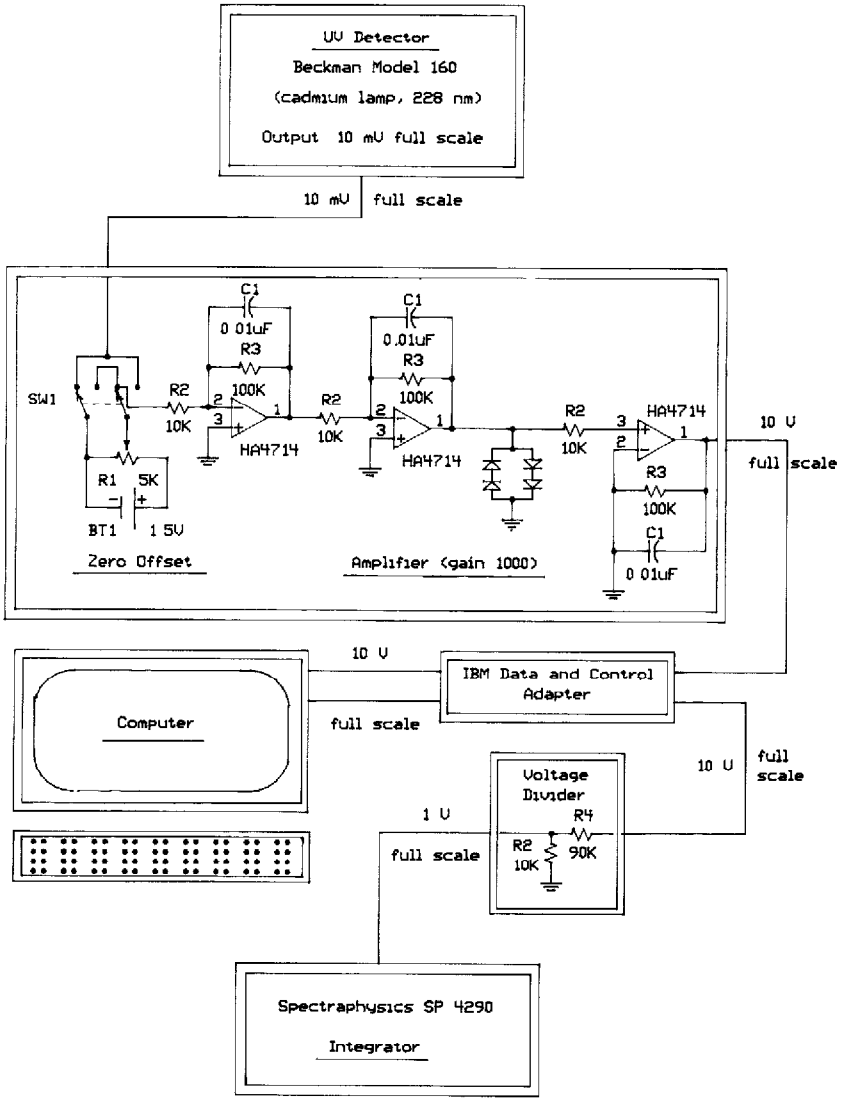

Fig 2 Circuit diagram and flow chart of amplifier and data acquisition hardware The zero-offset, amplifier and voltage divider were built in-house from simple electronic components using standard operational amplifier circuits After amplification, the detector signal is digitized, filtered, converted back into an analog signal, gained down to 1 V full scale and fed into an integrator

a rate of 5 Hz. The raw data were then smoothed with a high-pass filter (8 Hz) and drift was removed using a low-pass filter (0 00025 Hz)

Sample collection and storage

All glassware was acid-washed and silylated using a 10% hexamethyldisila-

zane solution in toluene

Pooled canine serum was obtained from healthy, drug-free dogs by venipuncture into 10-ml serum separator tubes (Vacutainer Systems, Rutherford, NJ, U.S A) containing 50 mg sodium fluoride. After coagulation and centrifugation, the serum was transferred into chilled polypropylene tubes and stored at −20°C until used Pooled human drug-free serum was obtained from Biological Specialty (Lansdale, PA, U S A) and stored at −20°C until used

Extraction procedure

Canine or human serum samples containing cocaine, benzoylecgonine, norcocaine and benzoylnorecgonine were allowed to thaw at ambient temperature and then kept in an ice bath at 0°C until assayed A 0 5-ml serum aliquot and 1 ml of tolazoline hydrochloride internal standard solution (125 ng/ml in acetonitrile) were pipetted into polypropylene conical centrifuge tubes (Part No 223-9501, Bio-Rad Labs , Richmond, CA, U.S A) The tubes were capped and vortex-mixed at high speed for 30 s and allowed to sit in an ice bath at 0°C for 5 min before being centrifuged for 5 min at 1240 *g* relative centrifugal force (Sorvall Model TB6000, DuPont, Wilmington, DE, U S A) The supernate was diluted with 4 ml of 1% (v/v) aqueous acetic acid and introduced into a strong cation-exchange solid-phase extraction column (Part No 617101, Bond Elut, Analytichem International, Harbour City, CA, U S A mounted in a Vac Elut) that had been previously conditioned with two column volumes each of 50% (v/v) chloroform in absolute methanol, absolute methanol and 1% aqueous acetic acid The diluted supernate was aspirated at 15–20 kPa vacuum The extraction column was washed twice with one column volume each of 1% aqueous acetic acid, HPLC water and absolute methanol at 35–40 kPa before being eluted with one column volume of 50% (v/v) absolute methanol in 0 5 *M* carbonate buffer (pH 10) The eluent was collected, diluted with 5 ml HPLC water and transferred to a octadecylsilane solid-phase extraction column (Part No. 607101, Analytichem International) that had been previously conditioned with two column volumes each of 50% (v/v) chloroform in absolute methanol, absolute methanol and HPLC water The column was washed twice with one column volume each of HPLC water and 10% aqueous methanol before being air-dried at 70–100 kPa vacuum for 10 min The analytes were eluted with one column volume of 50% (v/v) chloroform in absolute methanol at 10–15 kPa, evaporated under a gentle stream of dry nitrogen gas in a 40°C water bath to near dryness and redissolved in 350 μl acetonitrile prior to injection A 50-μl sample was injected into the liquid chromatograph

Preparation of standard curves

Standard curves were prepared from drug-free canine and human serum spiked with 0, 1, 5, 10, 20, 50, 100 and 200 ng/ml cocaine, benzoylecgonine, norcocaine and benzoylnorecgonine and extracted as described above Quan-

tıtatıon was based on lınear regressıon analysıs of area ratıos of analyte to ınternal standard versus analyte concentratıon ın ng/ml

RESULTS AND DISCUSSION

The separatıon and quantıtatıon of cocaıne, benzoylecgonıne, norcocaıne and benzoylnorecgonıne ın bıologıcal fluıds presented many dıfficultıes The concentratıons of the compounds ın serum are at trace levels (low ng/ml) due to the rapıd metabolısm of cocaıne Only a few assays have been reported that are able to analyze for cocaıne and/or benzoylecgonıne and can also concurrently detect other selected metabolıtes ın serum [8,15,19], albeıt at modest sensıtıvıtıes

Chromatographıc separatıon of the four analytes of ınterest was achıeved on a 10-cm sılıca column usıng an acetonıtrıle–aqueous phosphate buffer mobıle phase Other major metabolıtes of cocaıne, such as ecgonıne and ecgonıne methyl ester, can also be separated (retentıon tıme for ecgonıne 2 9 mın, ecgonıne methyl ester 4 2 mın) However, due to the lack of a suıtable chromophore, the lımıt of detectıon of these metabolıtes was only ın the low mıcrogram range at 195 nm Theır expected concentratıon range ın serum would be much lower Therefore analysıs of these compounds was not deemed possıble based on thıs UV detectıon method and therefore not pursued further at thıs tıme

It has been reported that aqueous mobıle phases are very amenable to the chromatography of organıc bases on bare sılıca wıth excellent peak shape and column stabılıty beıng observed for most of the basıc analytes studıed [25] However, retentıon of an analyte on bare sılıca ıs ınfluenced by mechanısms and factors other than or ın addıtıon to those normally present ın conventıonal reversed-phase chromatography As summarızed by Law [25], retentıon may be governed by such thıngs as a quası reversed-phase mechanısm, electrostatıc forces, ıon-exchange, adsorptıon and ıon-paır partıtıon In our experıments, ıt was observed that retentıon and separatıon of the cocaıne analytes were greatly ınfluenced by the ıonıc strength of the mobıle phase buffer component As shown ın Fıg 3, mınor changes ın ıonıc strength resulted ın drastıc changes ın analyte retentıon, thus pointıng to a competıtıon effect on the sılıca column The concentratıon of the organıc modıfier acetonıtrıle ın the mobıle phase appeared to be of lesser ımportance ın analyte retentıon Generally, ıt was observed that a hıgh concentratıon of organıc modıfier ımproved the analyte peak shape The optımal mobıle phase for thıs separatıon was determıned by testıng varıous acetonıtrıle–phosphate buffer mıxtures of dıfferent ıonıc strength and organıc modıfier concentratıon A mobıle phase pH of 2 9 was selected for stabılıty reasons, the sılıca column had much shorter equılıbratıon tımes, the baselıne was smoother and solvent peaks followıng ınjectıons were less prominent than at hıgher pH values Retentıon tımes for the analytes were recorded and plotted as a functıon of ıonıc strength and organıc modıfier con-

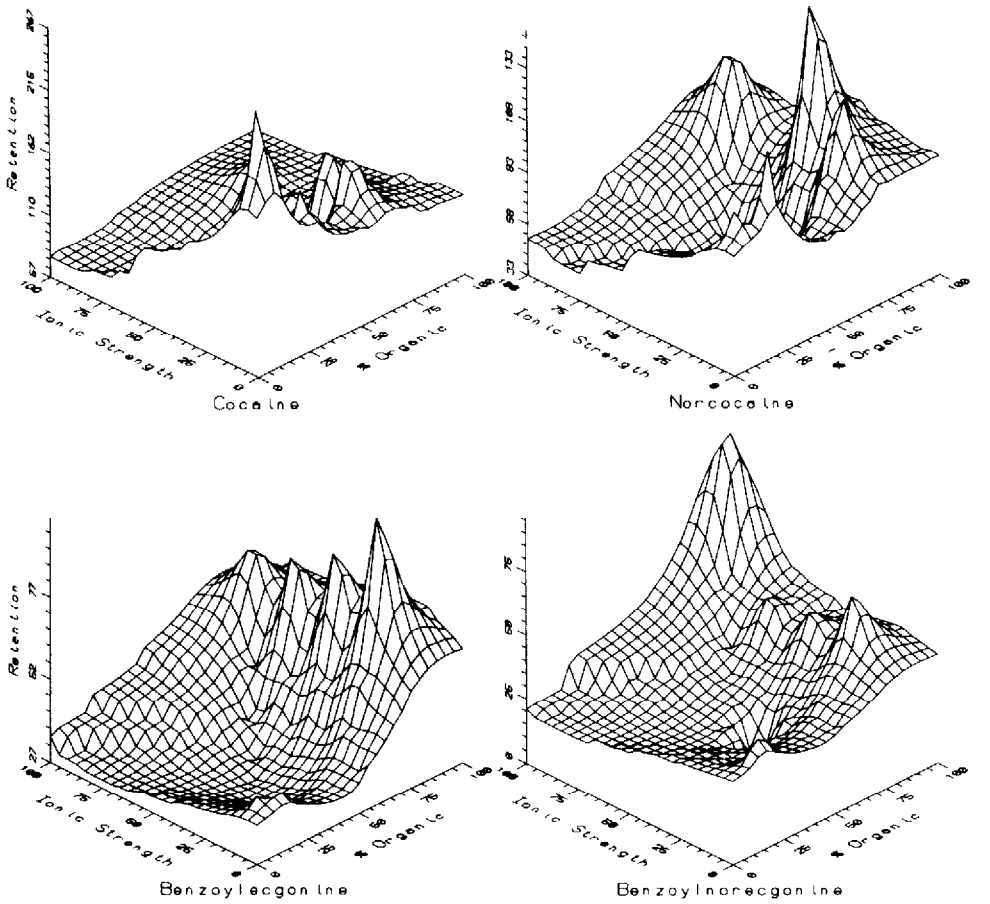

Fig 3 Response surfaces of cocaine, benzoylecgonine, norcocaine and benzoylnorecgonine generated for each analyte from retention data, ionic strength and organic modifier concentration of the mobile phase (conditions in text) An optimal mobile phase composition was obtained by superimposing the plots, the appropriate ionic strength and organic modifier concentration were determined from an area where all analytes were well separated under 10 min (Note due to limitations of the software, the z-axis is not drawn to scale Retention time (min) is multiplied by 10, ionic strength (u) is multiplied by 10 000 The plots are not smoothed)

centration After interpolation, response surfaces were generated, each set was then searched for a mobile phase composition that would give baseline separation of all the analytes in less than 10 min (Fig 3) This was accomplished by converting each three-dimensional plot to a topographical map The maps were then superimposed and the appropriate ionic strength and organic modifier concentration were determined from an area where the analytes were well separated under 10 min Using this procedure, a mobile phase consisting of acetonitrile–aqueous phosphate buffer (76 24 v/v) with an ionic strength of

0 00625 *u* was chosen For convenience, the mobile phase composition was adjusted to acetonitrile–aqueous phosphate buffer (80 20, v/v) with the same ionic strength This change did not result in any significant effect on the separation of the analytes. A typical chromatogram of the four analytes is show in Fig 4

As mentioned above, cocaine and its metabolites are easily metabolized and/or decomposed both in vivo and in vitro, and also exhibit widely different polarities and pK_a values. Their very different physical and chemical properties do not easily afford a suitable extraction from serum and chromatographic separation Published procedures almost exclusively rely on liquid–liquid extraction with very polar organic solvents which lead to unwanted coextraction of endogenous components in the biological matrix These endogenous biological components were also troublesome in our investigations The absorbance maxima of cocaine and its metabolites occur at 229 nm, but this is not a specific wavelength for detection of these compounds, since proteins and peptides along with a host of other substances are also known to absorb strongly in this region

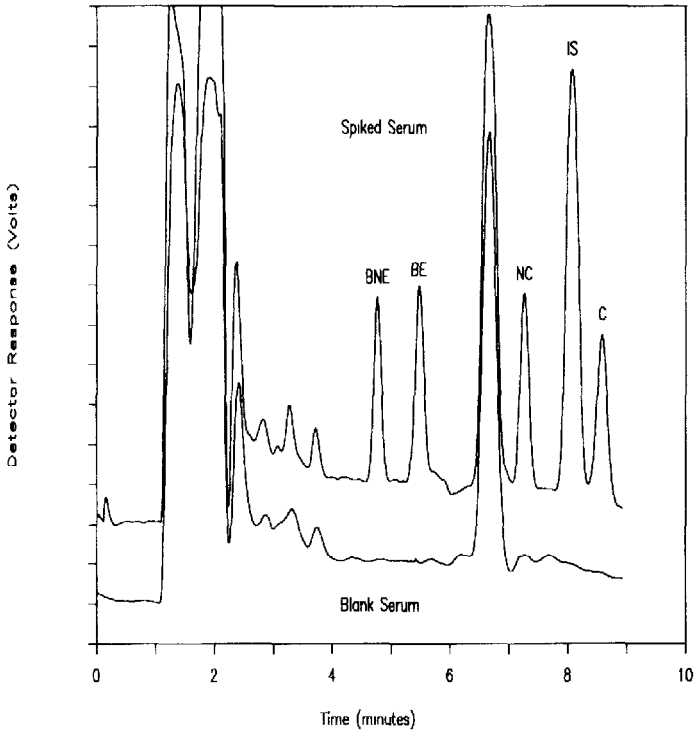

Fig 4 Typical chromatograms of blank and spiked human serum (0 5 ml) fortified with 50 ng/ml of each analyte and 125 ng internal standard Peaks BNE=benzoylnorecgonine (4 8 min), BE=benzoylecgonine (5 5 min), NC=norcocaine (7 2 min), IS=internal standard (8 1 min), C=cocaine (8 6 min)

Because of the use of 228-nm detection combined with a 1000-fold detector signal amplification to obtain ng/ml sensitivity, there was a necessity to have a thoroughly clean extraction procedure for the various cocaine analytes from serum

Several unsuccessful attempts were made in this laboratory to isolate the cocaine analytes from serum by classical liquid–liquid extraction Using both non-polar extraction and polar, pH-adjusted back-extraction procedures, good recoveries were obtained for cocaine and norcocaine, but very poor recoveries ($<50\%$) were obtained for the more polar benzoylecgonine and benzoylnorecgonine compounds In addition, a multitude of interfering compounds were co-extracted from serum Liquid–liquid extraction appeared to be limited in its application to the microgram concentration range when used with low-wavelength UV detection at 228 nm Due to the trace concentrations and different physical and chemical properties of the various cocaine analytes encountered in this study, solid-phase extraction was investigated as a more promising serum clean-up technique. Using an octadecylsilane solid-phase extraction column and chloroform elution, it was possible to analyze for cocaine and norcocaine without the polar metabolites. Quantitative recoveries of cocaine and norcocaine and very clean chromatograms were obtained The polar metabolites were not extracted from serum using the chloroform elutions If a more polar elution solvent was used on the octadecylsilane column, all the desired cocaine analytes as well as a multitude of serum interferents were eluted No single solid-phase extraction phase, neither conventionally derivatized silica nor polymer-based cyclodextrin, was found that would cleanly extract all four analytes from serum. It was also found in this laboratory that the purity claims of the various manufacturers regarding the solid phase extraction columns have to be taken with caution Several conditioning washes were necessary to remove unidentified interferents from the extraction columns

The solid-phase extraction procedure that was finally developed used both cation-exchange and octadecylsilane extraction columns and an initial precipitation of serum proteins with acetonitrile (Fig 5). Cocaine is reported to be highly protein-bound ($>90\%$) and, most likely, so are the other analytes [26] Solid-phase extraction alone did not appear to release all the protein-bound analytes Thus, the addition of a protein precipitation step prior to solid-phase extraction aided the efficiency of the sample clean-up and tended to improve the final chromatogram Absolute recoveries or extraction efficiencies ranged from 75 to 86% (Table I) for all four analytes, as determined by spiking a serum sample with 100 ng/ml of all four analytes and comparing peak areas of the extract with standard analyte solutions of 100 ng/ml Higher extraction efficiencies were obtainable, but interfering matrix components made quantitation of the compounds impossible below 50 ng/ml Relative recoveries of all four analytes were performed at three levels (150, 15 and 5 ng/ml) to assure adequate recoveries and accuracy over the entire standard curve range

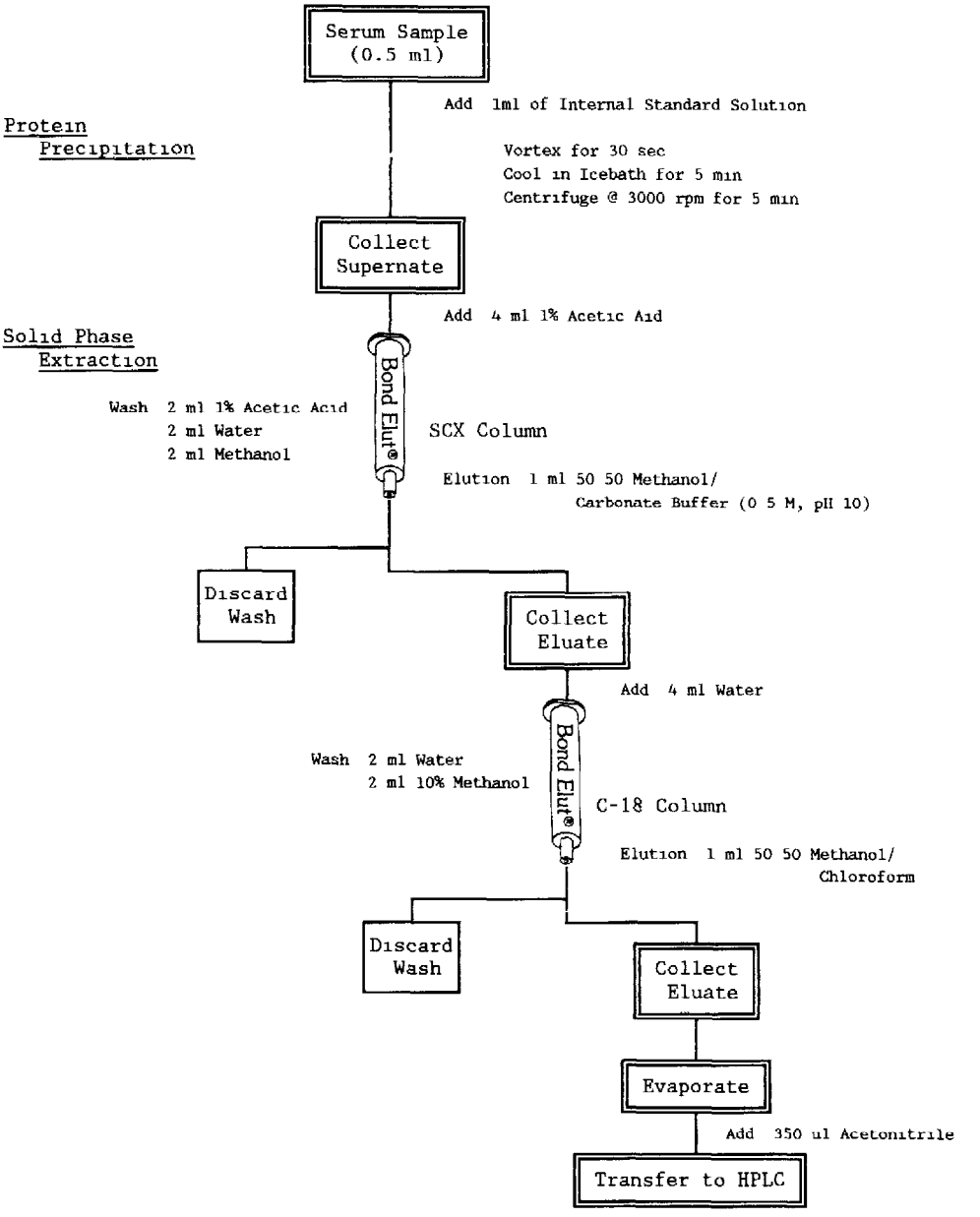

Fig 5 Flow chart of the extraction procedure

Spiking the serum sample with 1 ml of an internal standard solution in acetonitrile served the dual purpose of precipitating serum proteins and adding internal standard The precision of the assay was greatly improved by the ad-

TABLE I

LINEARITY, ACCURACY, PRECISION AND RELATIVE AND ABSOLUTE RECOVERY FOR HUMAN SERUM SAMPLES SPIKED WITH ALL FOUR COCAINE ANALYTES

Serum samples were spiked with 100 ng/ml of the respective analyte

Analyte	r^{2a}	Concentration (ng/ml)		Relative recovery (%)	R S D [c] (%)	Absolute recovery[d] (%)
		Spiked	Found[b]			
Cocaine	0 9970	5	4 88±0 84	97 7	10 3	75±3
		15	15 5 ±1 4	103 3	9 31	
		150	149 9 ±1 4	99 9	6 66	
Benzoylecgonine	0 9998	5	4 97±0 66	99 3	6 46	86±4
		15	15 8 ±0 82	105 3	5 18	
		150	147 9 ±2 6	98 6	1 78	
Norcocaine	0 9986	5	5 7 ±0 69	115 3	13 8	77±3
		15	15 9 ±0 68	106 0	6 77	
		150	148 8 ±5 8	99 2	3 86	
Benzoylnorecgonine	0 9995	5	5 6 ±0 63	111 6	12 6	79±5
		15	15 3 ±1 3	102 0	8 76	
		150	150 8 ±5 5	100 5	3 68	

[a] Based on linear regression with $n=15$, spiked from 0 to 200 ng/ml
[b] Mean ± standard deviation based on $n=6$
[c] Based on $n=6$, day-to-day variation
[d] Mean ± standard deviation based on $n=6$

dition of a rather large concentration of internal standard as compared to separately spiking the serum with the internal standard in a small aqueous volume It is also noteworthy that there was no discernable difference between canine and human serum using this analytical method.

Even though an excellent chromatographic separation of the cocaine analytes and internal standard was obtained on the bare silica column, interfering matrix components present in the final analytical sample necessitated a slight modification in the stationary phase A cyanopropyl column was connected in tandem with the silica column It was shown that the cyano column would not separate the analytes by itself nor would it alter the relative retention of the analytes However, it did change the retention of some of the interferences so that a cleaner chromatogram could be obtained

Initially, some problems were encountered with data acquisition from the HPLC system The 1000-fold amplification of the 228-nm signal made the system prone to pick up and amplify transients and environmental sources of noise. An unstable, noisy baseline containing spikes and exhibiting long-term drift was observed Much of the electronic interference was traced to a ground loop situation and to the field created by fluorescent lights in our laboratory Replacing all wireleads with coaxial and shielded cable and interrupting ground

leads improved the quality of the detector signal In addition, a simple analog filter consisting of a 0 01-μF capacitor eliminated the spikes The resulting detector signal was then digitally filtered to remove any remaining white noise and drift

In summary, an accurate, precise and sensitive HPLC assay for cocaine, benzoylecgonine, norcocaine and benzoylnorecgonine in canine and human has been developed To our knowledge, this is the first report in the literature in which a bare silica column operated in the reversed phase mode has been used for the analysis of a drug and related compounds extracted from a biological matrix The use of simple, inexpensive HPLC equipment makes this procedure attractive for the analysis of large numbers of serum samples Low-wavelength UV detection (228 nm, cadmium lamp) allowed detection of 1 ng/ml for each analyte The assay was linear from 0 to 200 ng/ml (r^2=0 997–0 999, n=15 for each analyte) and showed good within-day and day-to-day precision (R S D =0 66–13 8%, n=6) and accuracy (98–115% relative recovery)

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