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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF EMETINE AFTER OXIDATIVE ACTIVATION TO A FLUORESCENT PRODUCT

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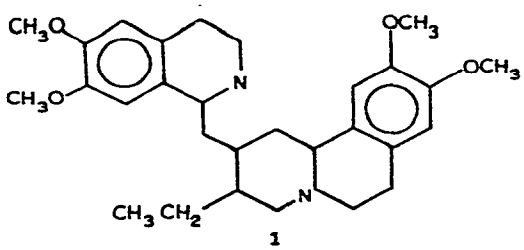
SUMMARY

A clinically useful analytical method is described for monitoring plasma levels of emetine. The drug is initially extracted from plasma with dichloromethane (0.3 volumes). The extract can be analyzed directly by paired-ion reversed-phase high-performance liquid chromatography to levels of 500 ng/ml of plasma by spectrophotometric monitoring of column effluent. For analysis of emetine at lower concentrations, the dichloromethane extracts are subjected to mild mercuric acetate oxidation prior to separation, thereby converting emetine to a fluorescent product. Spectrofluorometric monitoring of the column effluent readily extends the sensitivity of the assay to 10 ng of emetine/ml of plasma. At these levels measurements can be made with a precision of $\pm 4\%$.

INTRODUCTION

Emetine (1), an alkaloid of *Cephaelis ipercacuanha*, has been used in the therapy of a wide variety of disorders since the seventeenth century. Its usefulness in amebiasis has been well established[1]. At the cellular level emetine inhibits protein biosynthesis[2]



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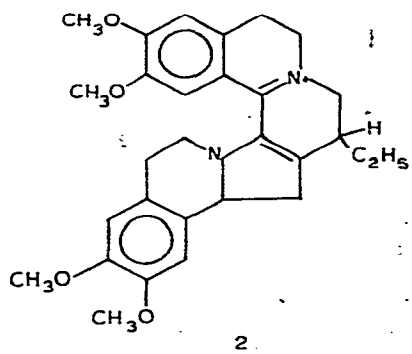

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and interferes with oxidative phosphorylation[3], suggesting its possible role in cancer chemotherapy. Lewisohn[4] and Van Hoosen[5] described clinical tumor regressions in a series of patients with a variety of malignancies after emitine therapy. These findings, reported prior to 1920, have more recently been corroborated by several clinical studies[6–8]. Emetine's important clinical advantage as an antineoplastic agent is its non-myelosuppressive behavior[6] and the disadvantages are the severe myopathy and cardiac arrhythmias[6–10] which occur in association with the dosage schedules used to date.

In an effort to utilize more effectively the chemotherapeutic properties of the drug while minimizing its toxic effects, the pharmacokinetics of emetine will be studied in man.

A variety of methods have been described for the analysis of emetine in crude plant material and pharmaceutical formulations. These techniques include aqueous[11] and nonaqueous[12] titrimetry, spectrophotometry[13–15], thin-layer chromatography (TLC)[16], and normal-phase high-performance liquid chromatography (HPLC) of the underivatized drug[17] and its Dns derivative[18]. In previous studies carried out in biological matrices the method of choice has been the oxidation of emetine to produce fluorescence and the measurement of total solution fluorescence. Mercuric acetate has been used as the oxidant under strenuous conditions[19] to insure the production of a single fluorescent product, rubremetine (2).

This report describes alternative conditions for the molecular modification of emetine to a fluorescent product and subsequent ion-pair reversed-phase HPLC analysis. Emetine may be quantitated in plasma to levels of 10 ng/ml.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 A pump, Model U6K injector, and Model 440 absorbance detector operated at 280 nm. Fluorescence detection was done with a Schoeffel Instr. (Westwood, N.J., U.S.A.) FS 970 L.C. fluorometer. Mobile phase optimization was accomplished with the addition of a second pump and a Waters 660 solvent programmer. The column used was a Waters μBondapak C_{18} (30 cm $\times$ 3.9 mm I.D.).

UV-Vis spectra were recorded using a Cary (Palo Alto, Calif., U.S.A.) Model 118 spectrophotometer and 1-cm quartz cells. Fluorescent emission and excitation spectra were recorded with a Hitachi–Perkin-Elmer (Norwalk, Conn., U.S.A.) Model MPF-2As spectrophotofluorimeter.

Materials

Emetine hydrochloride was obtained from Sigma (St. Louis, Mo., U.S.A.). Sodium pentane, hexane, heptane, and octane sulfonates were purchased from Eastman (Rochester, N.Y., U.S.A.), and were used as received. Naphthalene was purchased from Aldrich (Milwaukee, Wisc., U.S.A.) and mercuric acetate was purchased from Fisher Chemical (Fair Lawn, N.J., U.S.A.). Methanol and dichloromethane were Fisher HPLC grade. Chloroform was Fisher spectral grade and 1,2-dichloroethane was ACS reagent grade from Matheson, Coleman and Bell Manu-

facturing Chemists (Norwood, Ohio, U.S.A.). Ethanol, USP (95%) was used and all water was distilled in glass following mixed bed deionization.

Plasma was either purchased (Community Blood Center, Kansas City, Mo., U.S.A.) as recovered human plasma containing citrate-phosphate–dextrose anticoagulant, or was obtained from blood freshly drawn from healthy human volunteers using heparinized collection tubes. Plasma was stored at 5° and was used within one week of collection.

Extraction

Emetine was extracted from plasma (9 ml) or phosphate buffer (pH 7.4, 0.1 *M*) with 0.3 volumes of dichloromethane by shaking for 3 min in sealed conical centrifuge tubes. Phase separation was accomplished by centrifugation at 1200 *g* for 15 min.

Oxidation

A measured volume (7.5 ml) of dichloromethane extract was removed and evaporated to dryness in a conical centrifuge tube with a gentle stream of nitrogen. An ethanol solution (500 μl) of mercuric acetate [10 g $Hg(CH_3COO)_2$ per 100 ml of acetic acid–35% aqueous sodium hydroxide (73:27) diluted with 95% ethanol to 1 l] was added to the residue, vortex mixed and sealed for 1 h at room temperature (20–24°) prior to HPLC analysis.

Chromatography of underivatized emetine from plasma and buffer extracts

The mobile phase consisted of 2.5 m*M* octane sulfonate and 0.5% glacial acetic acid in methanol–water (56:44). The flow-rate was maintained at 2.0 ml/min. Injections of buffer were as large as 100 μl but no more than 25 μl of organic extracts were injected. Column effluent was monitored spectrofluorometrically at 280 nm.

Chromatography of mercuric acetate reaction mixtures

The mobile phase was as described above but the methanol concentration was increased to 60%. The flow-rate was 2 ml/min. Injections (5 μl) of the ethanol mixture were made on-column. Column effluent was monitored spectrophotometrically [$\lambda_{ex} = 225$ nm, cutoff filter $\lambda_{em} = 418$ nm].

RESULTS AND DISCUSSION

Initially a chromatographic system was developed to monitor unchanged emetine in buffer and plasma extracts. Emetine was chromatographed as an ion aggregate on an octadecylsilane bonded phase column using C_5–C_8 alkyl sulfonates as the hetaerons and UV absorbance detection at 280 nm. Fig. 1 shows the effect on retention of the addition of methanol to the mobile phase. In each case the alkyl sulfonate concentration was 2.5 m*M* and the concentration of acetic acid was 0.5%. Table I shows the system efficiency achieved with each of the alkyl sulfonates. In addition to greater efficiency the use of the more hydrophobic hetaeron and higher methanol concentration resulted in a simpler chromatogram in the vicinity of the emetine peak. This is probably due to the more rapid elution of neutral species relative to the cationic emetine. Sodium octane sulfonate was chosen as the counter ion at a methanol concentration of 56%. Under these conditions emetine was well

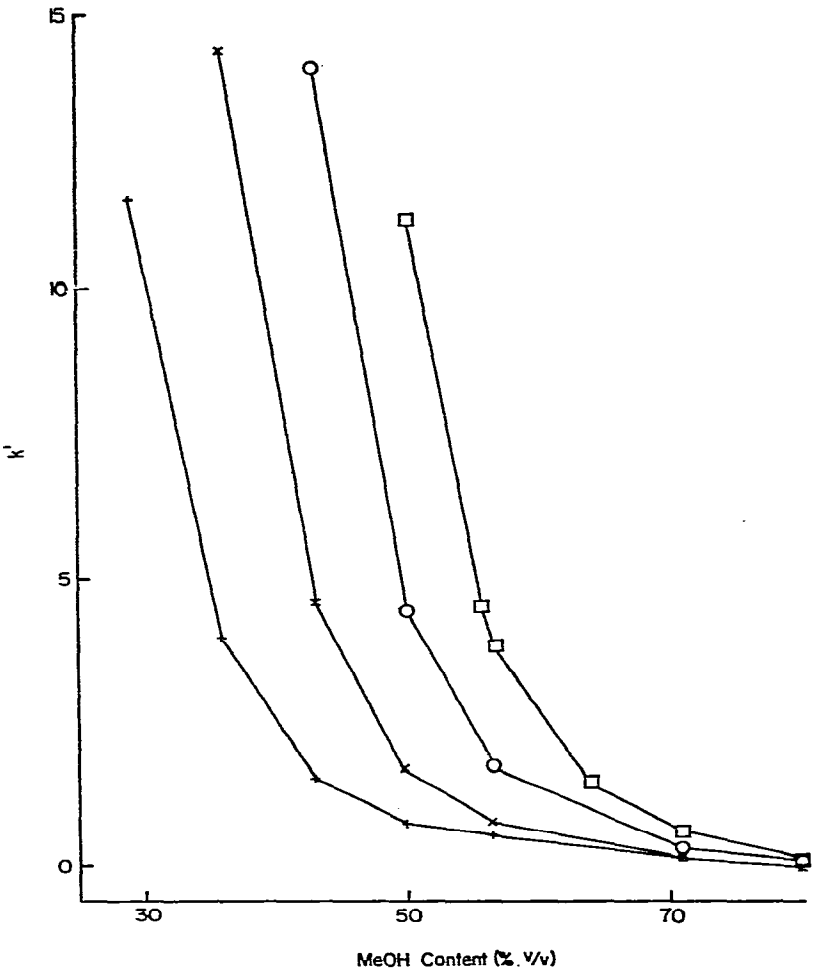


Fig. 1. Capacity factor (k') for emetine, *vs.* methanol concentration in mobile phase containing 0.5% (v/v) acetic acid and 2.5 m*M* sodium alkyl sulfonate. Pentane (+); hexane (×); heptane (○), octane (□) sulfonate.

TABLE I

NUMBER OF THEORETICAL PLATES (N) FOR INDIVIDUAL CHROMATOGRAPHIC SYSTEMS AT $k' = 4.2$ WITH $CH_3(CH_2)_nSO_3Na$ HETAERONS

$N = 16[t_r/t_w]^2$; t_r = retention time, t_w = peak width at baseline. $k' = (t_r - t_u)/t_u$; t_u = time of elution of unretained species (water). 25 μl of 10^{-4} *M* emetine hydrochloride were injected onto C_{18} column with mobile phase 2.5 m*M* alkyl sulfonate, 0.5% acetic acid in methanol–water of composition to yield k' (emetine) = 4.2.

n	N
4	1450
5	1600
6	1850
7	2200

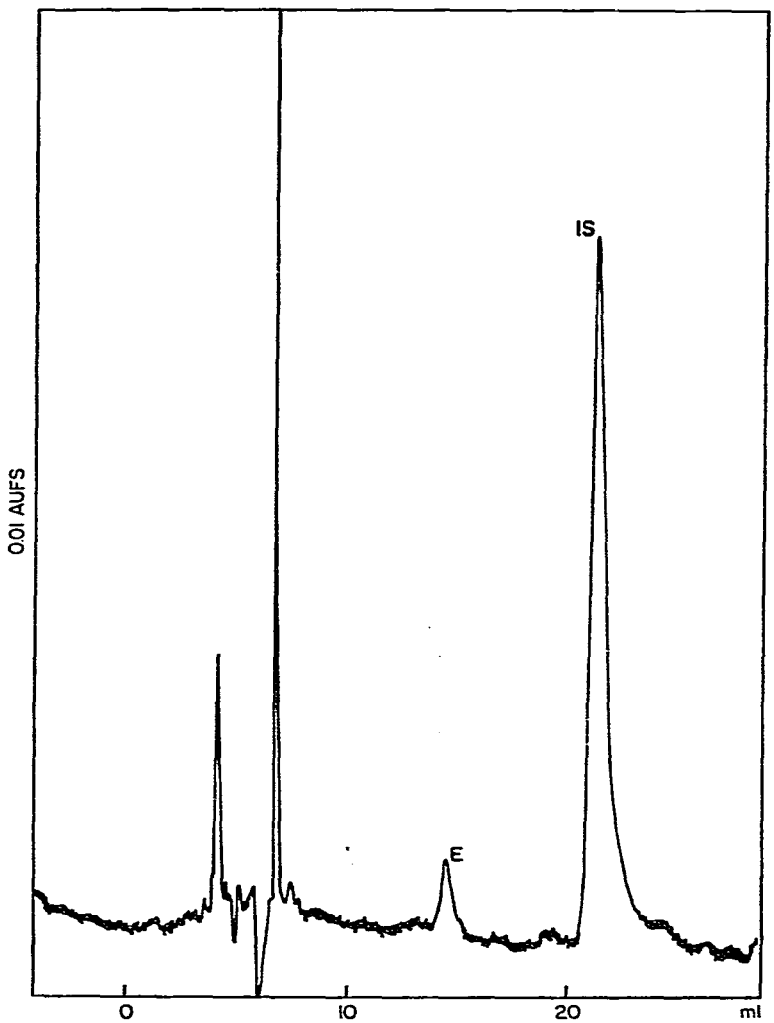


Fig. 2. Chromatogram of underivatized emetine extracted with dichloromethane from plasma. Emetine (E, $V_r = 14.5$); naphthalene (IS, $V_r = 21.2$). Octadecylsilane bonded phase column with 0.5% acetic acid and 2.5 mM sodium octane sulfonate in methanol–water (56:44) as the mobile phase.

resolved from coextracted plasma constituents as seen in Figs. 2 and 3, and rapidly eluted from the column (retention volume, $V_r = 14.5$ ml).

The efficiency of emetine extraction was determined with chloroform, dichloromethane, and 1,2-dichloroethane from phosphate buffer. The buffer containing emetine was analyzed before and after single-batch extraction. The extract was analyzed after extraction. Emetine was quantitatively extracted when the phase-volume ratio (org:aq) was 1:10 with all solvents used. Analyses were subsequently carried out by extraction with 0.1–0.3 volumes of dichloromethane.

Fresh plasma was spiked with emetine at concentrations of 1–100 μg/ml. Samples (3 ml) were extracted with 1 ml of dichloromethane containing 10^{-4} M naphthalene present as the internal standard. HPLC analysis and plotting of peak

height ratios (emetine to naphthalene) yielded a straight line of slope 0.101 $(\mu g/ml)^{-1}$, intercept of −0.056 with a correlation coefficient of 0.999. This system had a practical detection limit of 0.5 $\mu g/ml$, making it adequate for monitoring emetine administered as a bolus injection or from oral dosage forms, but inadequate for use in clinical investigations of slow i.v. infusions of the drug.

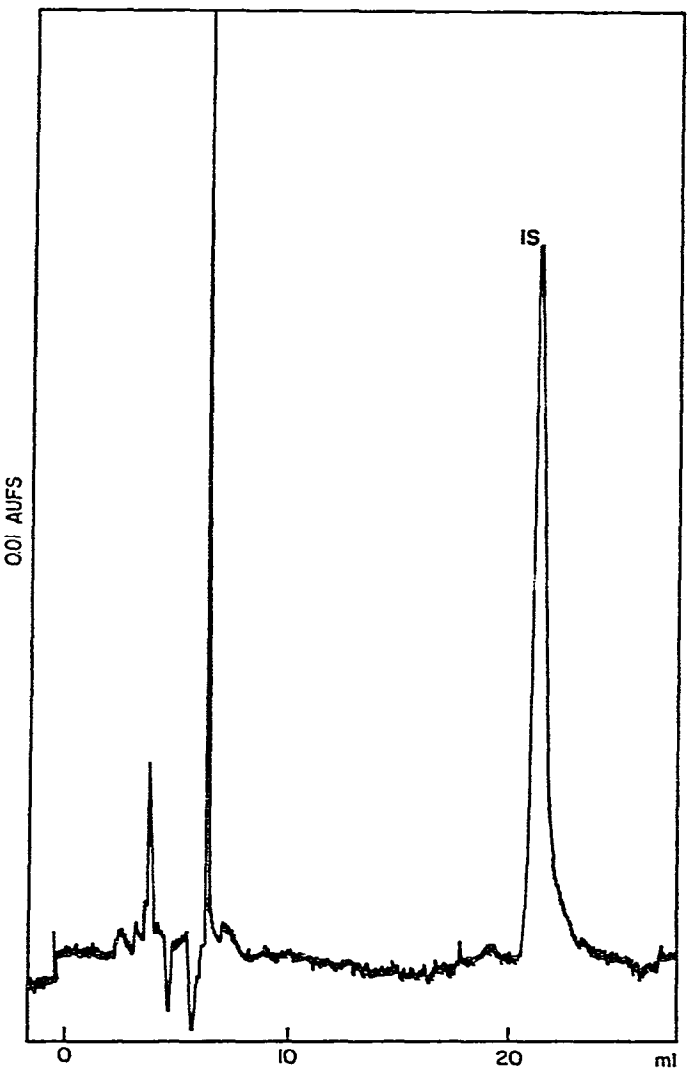


Fig. 3. Chromatogram of a dichloromethane extract of plasma in the absence of emetine. Chromatographic system as in Fig. 2.

To improve the sensitivity of the method, emetine was subjected to pre-column oxidative molecular modification to a fluorescent species which was then detected spectrofluorometrically in the column effluent. Using the chromatographic system just described, the oxidation of emetine was monitored under a variety of conditions.

Choosing only mercuric acetate as the oxidant, temperature and solvent were varied, seeking conditions that would yield a single product of enhanced detectability in a short period of time, under conditions convenient for large sample through-put.

When emetine (160 μg/ml) was refluxed in 5% (v/v) acetic acid containing 1% (w/v) mercuric acetate, the emetine peak (E) (capacity factor, $k' = 4.2$) disappeared with apparent first-order behavior ($t_{1/2} = 12$–14 min) and a new peak, B, ($k' = 4.6$) concomitantly appeared. This product was fluorescent ($\lambda_{max,ex} = 355$ nm; $\lambda_{max,em} = 450$ nm) but the peak corresponding to the product began to decrease in intensity after the reaction had proceeded for 40 min ($t_{1/2}$ 100–120 min). Two other peaks, C

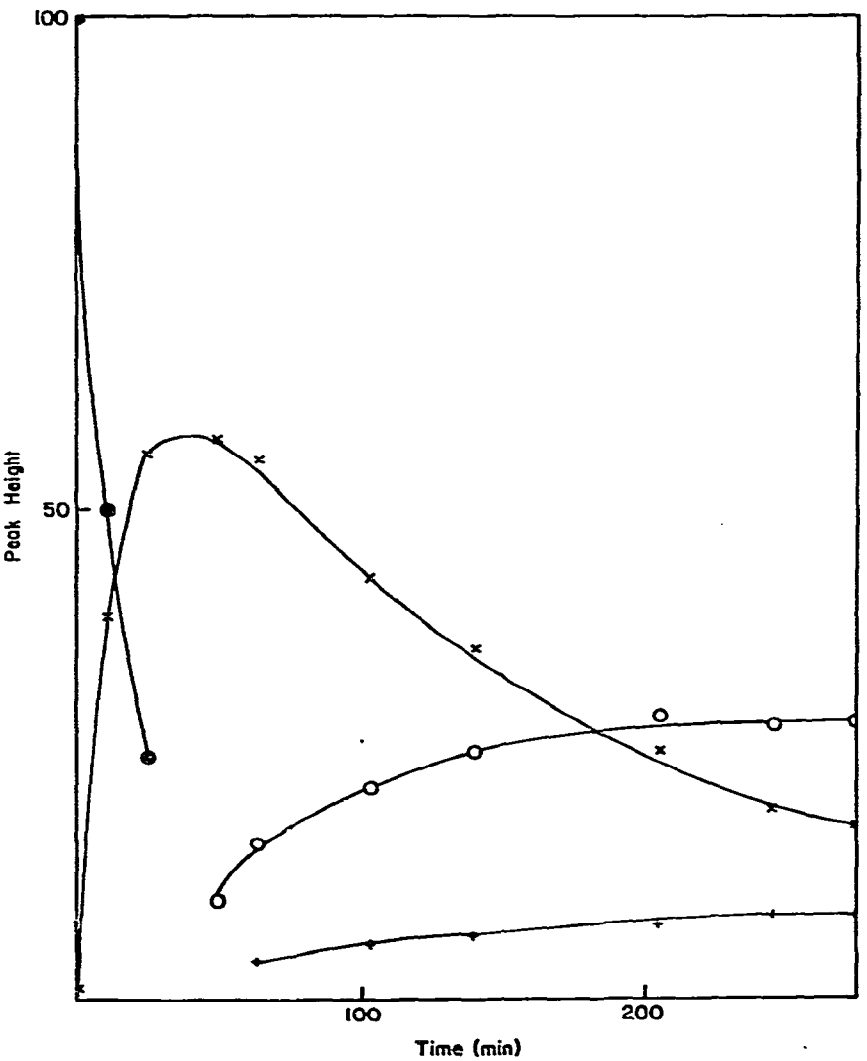
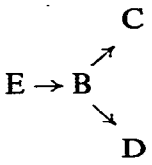


Fig. 4. Reaction course observed when emetine is refluxed in 5% aqueous acetic acid made 1% in mercuric acetate. Reaction followed by HPLC (octadecylsilane bonded phase column; 0.5% (v/v) acetic acid and 2.5 m*M* sodium octane sulfonate in methanol–water (56:44) mobile phase). E (●); B (×); C (○); D (+).

and D ($k' = 3.9$, $k' = 14.4$) appeared at a rate similar to the loss of peak B, suggesting:



as a possible reaction pathway. Fig. 4 shows the peak height *vs.* time data for this reaction scheme. Attempts were made to alter reaction conditions to preserve B, since both C and D seemed to be ultimate reaction products and therefore not suitable as analytical derivatives.

Reduction of the reaction temperature did little to improve the reaction course except to effectively displace the time axis of Fig. 4. At no point did it appear that the emetine was quantitatively converted to B.

The more strenuous conditions employed in the procedure of Schwartz and Rieder[19] (1 h at 130°) may produce a single product, or alternatively the fluorescence of a combination of oxidation products may be measured.

The reaction could be controlled by solvent modification. Addition of ethanol to the reaction medium facilitated oxidation of emetine and stabilized B. At an optimum level of 70% ethanol, reaction to form B was complete at room temperature in 60 min and B was stable for several hours. Under these reaction conditions formation of C and D was not observed.

Plasma extracts subjected to this treatment were then analyzed by paired-ion reversed-phase chromatography on a C_{18} column using methanol–water (60:40) containing 0.5% acetic acid made 2.5 m*M* in sodium octanesulfonate as a mobile phase. The derivatized product eluted with a retention volume of 8.6 ml [underivatized emetine, $V_r = 8.1$ ml] and was monitored spectrofluorometrically [$\lambda_{ex} = 225$ nm; $\lambda_{em,cutoff} = 418$ nm]. The resulting chromatogram is shown in Fig. 5a and a blank in Fig. 5b.

To validate the pre-column derivatization method a calibration curve was constructed by subjecting plasma sample containing emetine (20–1050 ng/ml) to the analysis scheme presented. Regression analysis of the data for this curve generates the line $y = 9.7 \cdot 10^{-3}\, x + 3.7 \cdot 10^{-2}$ with a correlation coefficient of 0.998. Chromatographic analysis must be carried out within 2 h after completion of oxidative derivatization to eliminate interfering peaks corresponding to slowly formed fluorescent derivatives of plasma constituents. The methods, as described, have a practical detection limit of 10 ng emetine/ml of plasma.

Thus, two methods are presented for the analysis of emetine in human plasma. After extraction, emetine can be analyzed directly by paired-ion reversed-phase chromatography when levels are $\geqslant$ 500 ng/ml; by introducing an oxidation step between extraction and chromatography, emetine can be detected spectrofluorometrically to levels of 10 ng/ml of plasma.

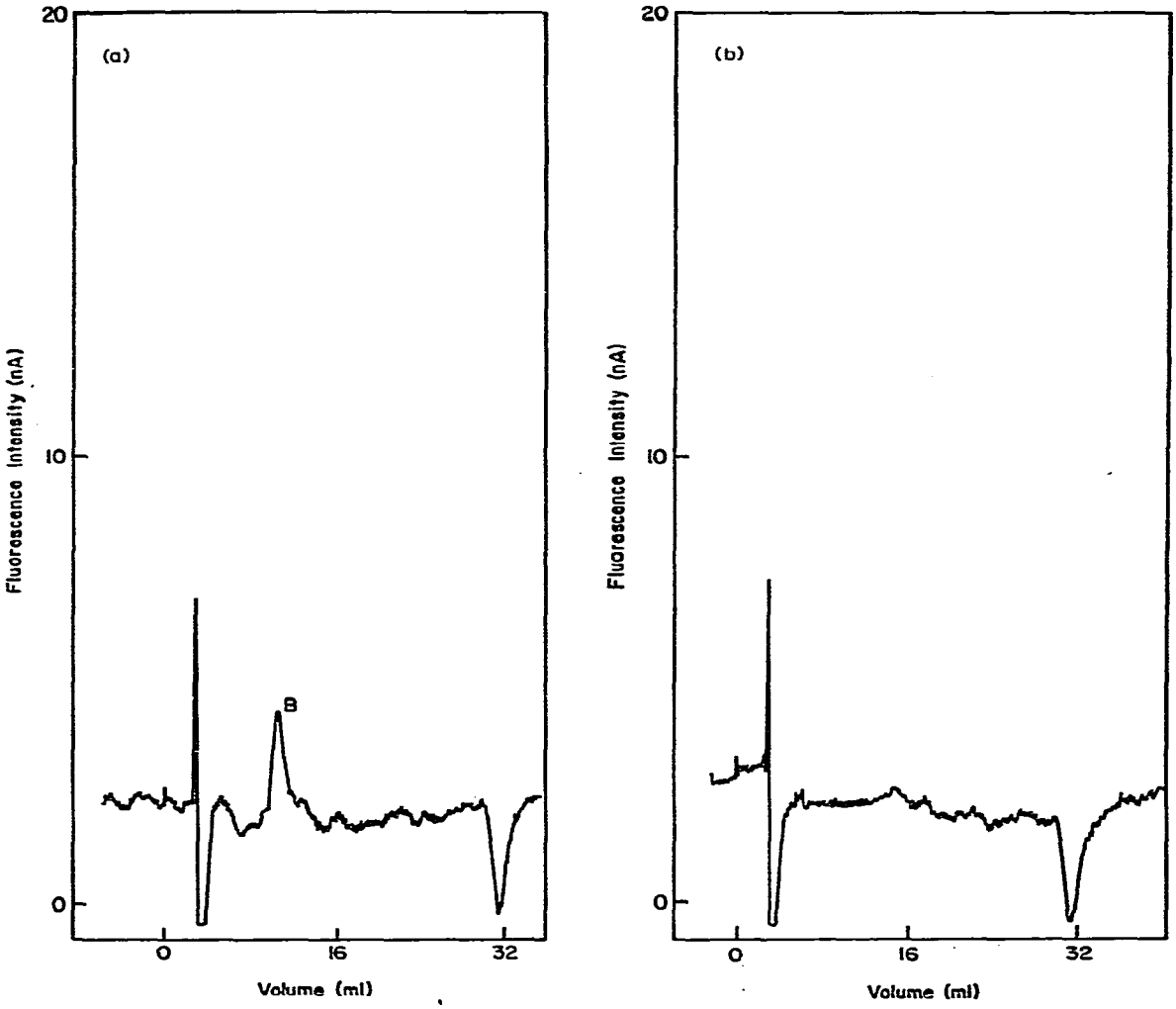


Fig. 5. (a), Chromatogram of oxidation product (B, $V_r = 8.6$) formed as described in text from emetine (20 ng/ml) extracted with dichloromethane from plasma. (Octadecylsilane bonded phase column; 0.5% (v/v) acetic acid and 2.5 m*M* sodium octane sulfonate in methanol–water (60:40) mobile phase). (b), Chromatogram of reaction mixture of dichloromethane extract of plasma not containing emetine. Reaction and chromatographic conditions as in (a).

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