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LIQUID–SOLID EXTRACTION OF LYOPHILIZED BIOLOGICAL MATERIALS FOR FORENSIC ANALYSIS

II. APPLICATION TO BILE SAMPLES FOR THE DETECTION OF DRUGS

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SUMMARY

A method is described for the detection of pharmaceuticals and drugs of abuse in bile fluid based on the principles of lyophilization and liquid–solid extraction with subsequent thin-layer chromatography and fluorometry followed by thin-layer chromatography with independent confirmation of all drugs.

An acidified bile extract is centrifuged at high speed, the supernatant lyophilized and the drug material eluted from the residue by a double organic extraction procedure, and then subjected to thin-layer chromatography. Fluorometric confirmation of morphine and methadone is achieved after elution of drug from the visualized silica gel plate.

INTRODUCTION

Recently, we reported on the application of liquid–solid extraction of previously acidified and lyophilized urine samples as a novel method of urinalysis for drugs of abuse[1]. We now wish to describe the extension of this method for the analysis of both the acidic/neutral (A/N) and basic (B) drugs in bile samples.

The analysis of bile fluid provides a useful corollary to the chemical examination of other fluids and tissues, especially urine and liver. However, the viscous nature of bile fluid and the presence of large quantities of metabolic degradation products render the standard, direct extraction procedures used for the analysis of serum or urine inapplicable. Hence, the procedure employed must provide a solution from which most such material has been eliminated, but with retention of drugs.

The method described below provides a rapid, efficient procedure for the quantitative extraction of all classes of compounds of pharmaceutical or forensic interest.

MATERIALS

Spinco Model L ultracentrifuge with No. 21 Head. Aminco Bowman spectrophotofluorometer. Brinkmann silica gel plate, 250 μ. Virtis freeze-drier, No. 10, condenser capacity 40 lbs. water. Beakers, 150 ml, 20 ml, Kimax. All chemicals used are of reagent grade quality.

METHODS

Preparation of sample and lyophilization

To 5.0 ml of bile fluid placed directly in a plastic centrifuge tube (No. 2ı Head) is added an equal volume of conc. hydrochloric acid (35–37 %), and 70 m water. The tube is securely capped and the contents briefly but thoroughly shaken The tubes are then centrifuged at 15,000 r.p.m. for 25 min. The supernatants ar poured directly into 150-ml beakers and completely frozen at $-50°$ in the Virti freeze-drier. The chamber is then evacuated to a residual pressure of $1 \cdot 10^{-4}$ atm at which time the shelf temperature is adjusted to 80° to facilitate the lyophilizatio process. After all samples are completely lyophilized, they are cooled to room tem perature for chromatography.

Extraction and chromatography

The residues from the lyophilization step contain the neutral drugs, the basi drugs as their hydrochloride salts, the acid drugs in their unionized state, and varyin amounts of water-soluble bile material. The following procedure is then applied.

The residues are thoroughly mixed with 3 ml of methanol and allowed to stan for about one half hour. 9 ml of acetone are added, the contents mixed and th suspensions filtered, the filtrate being evaporated to dryness. Morphine glucuronid present in the original sample will be precipitated out in this step. Subsequen papers in this series will deal specifically with the isolation and chromatography of th glucuronide. The evaporated residues are now extracted with 10-ml volumes o "basic" chloroform–methanol(9:1). This solution is prepared by equilibrating approx imately 500 ml of chloroform–methanol (9:1) with 10 ml of conc. ammonium hydrox ide. The bottom layer, which is cloudy in appearance, is removed and used (withou filtration) for the extraction step. The beakers are allowed to stand with occasiona swirling for several minutes, the suspensions are filtered, and the filtrates are evap orated to dryness.

It should be noted that the alkalinity of the "basic" extracting solution i not sufficient to effect significant ionization of intermediate acids of the barbiturat type. Hence, the final residue will contain not only basic and neutral compound but most of the barbiturates that may be present as well. Although these procedure have been used by the authors for the detection of opiates, it is clear that this metho allows for screening of both the A/N and B classes of compounds.

Description of the systems for the chromatography of the combined A/ and B fractions or the B fraction alone have already been given[1–3]. It should b pointed out that where analysis of only the B fraction is desired it is not necessar to remove the A/N drugs since the latter do not interfere in any way with the chrom tography or visualization of the former as described in the references cited. Silic gel plates, 250 μ, should be used and best results have been obtained with spots diameters of 1 cm.

Confirmation of drugs

The presence of morphine may be confirmed by a procedure utilizing t chromophorically visualized areas of the silica gel plate. Following application the spray reagents, the silica gel from those areas of the visualized plate believe

to contain morphine are carefully scraped off the plate and placed in 20-ml beakers. 10 ml of conc. hydrochloric acid–water (1:1) are added, and the beaker placed on a steam bath for 10 min with occasional swirling. The suspension is then adjusted to pH ~9 by the addition of conc. ammonium hydroxide and sufficient sodium bicarbonate to produce a saturated solution, and then extracted with 20 ml of chloroform–methanol (9:1). The organic layer is filtered into a 30-ml beaker and evaporated to complete dryness.

The fluorometric procedure described by BROICH[4] is now applied. To each dried beaker is added 0.5 ml of conc. sulfuric acid. The beakers are allowed to stand at room temperature for ½ h, and then 5.0 ml of water and 6.0 ml of conc. ammonium hydroxide are carefully added in succession. The beakers are briefly swirled and then autoclaved for ½ h at 260° and a pressure of 21 lbs. After cooling to room temperature, the resulting solution is examined fluorometrically with excitation set at 390 mμ and emission scanned from 400–450 mμ. Solutions containing the morphine fluorophore will yield a sharp, distinct maximum at 430–435 mμ. Negative solutions will show varying amounts of broad, non-specific emission in the indicated range.

Recently, MCGONIGLE[5] has described a fluorometric assay for methadone based on the principles outlined by BROICH[4]. This procedure may now be applied to the confirmation of methadone, whose presence is indicated by thin-layer chromatography. The appropriate areas of silica gel are extracted essentially as described above (in the present case, however, the aqueous phase need only be brought to any pH above 7 prior to the organic extraction) and the dried residues are treated accordingly.

As has been emphasized and discussed in previous publications by the present authors, confirmation by independent analysis of all drugs whose presence is indicated by TLC is essential for both the clinical and forensic purposes[1,2].

RESULTS AND DISCUSSION

The procedures described in the present paper provide a convenient and reliable method for the detection of drugs of abuse and pharmaceuticals in bile fluid. The high speed centrifugation effects removal of much of the viscous material that would otherwise render extraction procedures impractical. The lyophilization assures complete retention of all compounds present. The use of both a methanol–acetone extraction, followed by a "basic" chloroform–methanol extraction, effects removal of most interfering material that would otherwise obscure the chromatography. Although a certain amount of brownish impurity may be present in the final residue, it migrates considerably slower than drug material in the solvent system used, and hence, does not interfere with the visualization of compounds. The use of a "basic" organic extraction solution which, nevertheless, lacks any significant aqueous phase provides several distinct and unique advantages. It effects a one-step conversion of the basic hydrochloride salts to the free base with passage into the organic phase essentially by liquid–solid extraction. In addition, the moderate basicity, insufficient to convert barbituric acids to their salts, permits extraction of the barbiturate drugs as well as the neutral drugs. Furthermore, it removes additional pigmented impurities from the final residue.

The general applicability to material eluted directly from the visualized

silica gel of such techniques as microcrystallography, GC, spectrophotometry and, particularly, the highly sensitive (μg/ml) fluorescence assay for morphine and methadone makes possible convenient, accurate drug confirmation.

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