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EVALUATION OF COLUMN EXTRACTION: A NEW PROCEDURE FOR THE ANALYSIS OF DRUGS IN BODY FLUIDS

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SUMMARY

A method is described for the extraction of drugs from body fluids, whereby liquid–liquid extraction is replaced by liquid–solid elution. The aqueous sample is absorbed on a column filled with dry supporting material. By elution with organic solvents lipophilic substances are extracted from the water phase which remains on the column. The eluate containing the drugs is free of emulsions. For optimal extraction any pH value and a variety of solvents can be used. Column extraction of urine, serum and blood is easily performed and gives high recovery rates, which are superior to conventional extraction. Thin layer chromatography of drugs following conventional XAD-2 and column extraction, demonstrates identical qualitative results, but shows higher purity of the column extracts. The sensitivity of detection is enhanced.

INTRODUCTION

Drugs and other pharmaceutical substances, as well as their metabolites, must be extracted from body fluids before they can be determined by chromatographic, photometric and immunological means. Extractions performed using organic solvents often lead to the formation of emulsions, which render phase separation difficult and cause substance losses. These difficulties are overcome by a new procedure in which liquid–liquid extraction in a separating funnel is replaced by liquid–solid elution on a chromatography column.

Principles of column extraction

An aqueous sample, such as urine, serum or blood, is applied to and absorbed by, a column packed with granular support material, and remains on the column as the stationary phase. The column is then eluted with organic, water-immiscible solvents (Fig. 1).

This causes lipophilic compounds such as drugs and their metabolites, to become extracted from the aqueous phase into the solvent, so that they are obtained in the eluate. The latter is free of emulsions. The eluate can either be tested directly or evaporated down to a residue in which the substances are then determined in concentrated form. It is not necessary to dry the solution prior to evaporation.

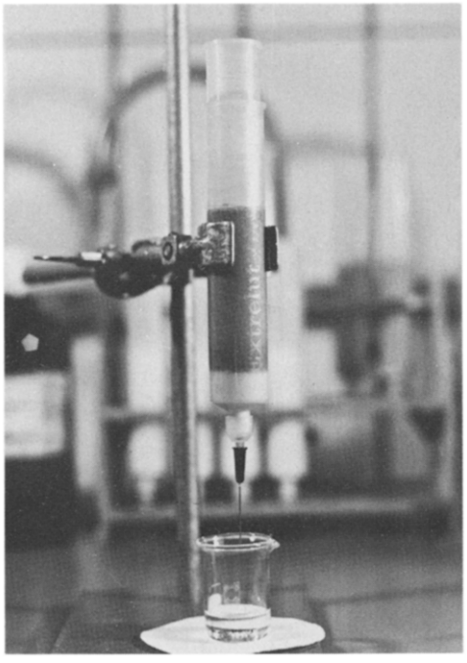


Fig. 1. Extraction column, loaded with blood.

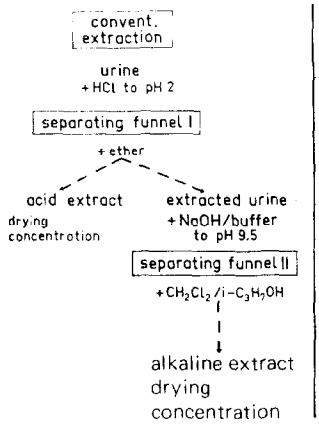
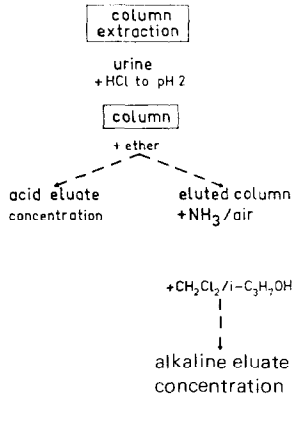
The *support* materials employed are wide-pore diatomaceous earth or silica gel types with granular structure and large pore volume. They are capable of being loaded to the limit of their water-absorption capacity without releasing any of the water upon elution with solvents. The supports are inactive and chemically inert; the pH value of the absorbed aqueous solution can range between pH 1 and pH 13. It is possible therefore, to elute substances at an optimum pH and to separate acid, neutral and alkaline compounds. Also the pH of a solution may be changed on the column. So the subsequent extraction of drugs from acid and alkaline media can be performed on the column (Table I). The neutralisation of the urine after acid elution is achieved by drawing ammonia through the column, rendering the pH of the absorbed solution from 2 to 10.

Basically, all *solvents* which are employed in liquid–liquid extractions may be used for column extraction; for instance ether, ethyl acetate, chloroform, or alcoholic mixtures such as a mixture of dichloromethane/isopropanol (85:15).

Typical *sample materials* are urine, blood, plasma, serum, gastric juice, liquor, and tissue extracts.

TABLE I

Comparison of separation procedures

Liquid–liquid extraction	Column extraction
	

EXPERIMENTAL

General procedure

Usually columns for the extraction of 20 ml of aqueous solution were used.

Columns are prepared from 50 ml plastic syringes, filled with 70 ml of granular diatomaceous earth (Extrelut®, E. Merck, Darmstadt). The solvent flow is regulated by steel needles connected with the syringe outlet.

20 ml of the aqueous solution is applied onto the column and absorbed by the support. After 10 minutes the column is eluted with organic solvent — for urine samples 40 ml of solvent yield a total of 25 ml of eluate within 5 to 15 minutes.

The eluate is concentrated without drying under a stream of nitrogen and the residue is used for thin layer chromatography (TLC) or gas–liquid chromatography (GLC). For photometric analysis the eluate is re-extracted with aqueous acids or bases.

Urine

Column extraction

Samples are applied without filtration after adjustment with HCl or concentrated NH_4Cl/NH_3 buffer (pH 9.5) or after any other pretreatment (*e.g.* hydrolysis).

For the separation of acidic and basic substances 20 ml of urine, acidified with HCl and NH_4Cl, are absorbed onto the column, and the first elution is performed with 40 ml of ether. For neutralisation a stream of ammonia is passed through the column; the ammonia is produced by drawing air through a bottle containing concentrated NH_3 solution. The second elution is then performed with 40 ml of dichloromethane/isopropanol (85:15) to extract the basic substances.

Recovery

Solutions of barbital in NH_4Cl/tartaric acid solution pH 2 (10 µg/ml) and of morphine and codeine in urine pH 9.3 (1 µg/ml) were simultaneously eluted via a column and extracted in a separating funnel. For barbiturate analysis the residues of the eluates or extracts respectively were determined by their optical density at 254 nm in acid and alkaline solution [1]. Morphine and codeine were determined by GLC (silylation with bis-(trimethylsilyl)-trifluoroacetamide/chlorotrimethylsilane (BSTFA/TMCS); glass columns packed with 5% silicone SE 30 on Chromosorb G AW-DMCS 100/120 mesh; FID).

Serum

Serum spiked with eupaverine and papaverine (200–700 ng/ml) was diluted 1 + 1 with 0.1 mol/l NaOH. 3 ml of these solutions were eluted on a column or were extracted in a roller apparatus [2] with 12.5 ml of di-isopropyl ether/methyl acetate (70:30). The column eluates or the extracts after centrifugation were washed with 0.1 mol/l H_2SO_4, the aqueous phase neutralised with NaOH and again extracted with di-isopropyl ether. The residues of the ether phase were subjected to GLC (glass column; 3% silicone SE 52 on Gaschrome Q 100/120 mesh; alcali FID).

Blood

EDTA or citrate blood was spiked with morphine, papaverine or levomepromazine (300 ng - 50 µg/ml). The blood was haemolyzed by addition of 2, 4 and 9 parts of 0.025% NH_3; after 15 minutes 20 ml of haemolysate was applied to an extraction column and eluted with 50 ml of ethyl acetate. The eluates were concentrated and re-extracted with 1 mol/l HCl. After washing with n-hexane the concentrations of the HCl-phase were determined photometrically at the substance-specific wavelengths [3].

Other extraction techniques

XAD-2 extraction

Commercially available columns filled with XAD-2 resin were stored overnight under distilled water, washed with 3 ml of water and used for extraction of urines according to producer instructions (Brinkmann

Instruments, Inc., Westbury, N.Y.). Solvents used were dichloroethane/ethyl acetate (4:6) or chloroform/propanol-2 (6/1).

Conventional extraction

For analysis of urines, to 20 ml of sample 40 ml of solvent were added in a ground glass stoppered centrifugation tube. This was rotated in a cylinder for 30 minutes at 30 rev/min. After centrifugation at 3000 rev/min the extracts were dried, evaporated and analyzed by TLC.

Thin layer chromatography (TLC)

TLC was performed with methods commonly used for drug detection [4–6].

RESULTS

Extraction of urine

Determination of drugs and pharmaceuticals is performed following selective extraction of urine in acid and alkaline media. Tables II and III compare the yields of barbiturates and opiates obtained using conventional extraction and column extraction. For identical yields, continuous extraction in the column uses maximal half as much solvent as in liquid–liquid extractions. For 20 ml of urine 40 ml of solvent are used, in which 80–100% of the extractable substances are eluted.

Using the technique of subsequent acid and basic elution, comparable results are obtained. The recovery of barbiturates and bromocarbamides at the acid stage is complete. Following neutralisation with ammonia, elution of basic substances with dichloromethane/isopropanol gives recoveries of 80% and more (Morphine, codeine, quinine, aminopyrine).

For screening purposes, acidic and alkaline pharmaceuticals are often eluted together at pH 8.5–9. Alkaline compounds are completely extracted, whereas for barbiturates it is necessary either to use more solvent or to perform several extractions. Column extraction of 20 ml of urine with 40 ml of dichloromethane/isopropanol yields 65% of barbital and 72% of phenobarbital.

Extraction of serum

Serum may be extracted via a column after being diluted with buffer. Using the determination of eupaverine and papaverine the conventional method of extraction was compared with the new method (see Table IV). In the conventional extraction method, emulsification made it necessary to centrifuge the extract, twice in some cases. The column produced clear solutions in every case. Both methods give roughly the same yields. Reproducibility of the conventional extraction is markedly inferior to that

TABLE II

Comparison of conventional extraction (E) and column extraction (CE): Recovery of barbital (200 μg/20 ml) from pH 2 buffer

Substance	Method	Volume of fractions (ml)	Recovery in fraction				Amount of solvent (ml)
			1	2	3	total	
Barbital	E	40	85%	13%	—	98%	80 ether
	CE	20	99%	—	—	99%	35[+)]
Barbital	E	40	69%	20%	5%	94%	120 chloro-
	CE	20	81%	16%	—	97%	55[+)] form

[+)] Dead volume of column = 15 ml solvent

TABLE III

Comparison of conventional extraction (E) and column extraction (CE): Recovery of morphine and codeine from urine at pH 9

Substance	Method	Volume of fractions (ml)	Recovery in fraction			Amount CH_2Cl_2/ i-C_3H_7OH (ml)
			1	2	total	
Morphine	E	40	combined:		100%	80
	CE	20	96%	6%	102%	55
Codeine	E	40	combined:		100%	80
	CE	20	98%	1%	99%	55

Concentrations: 20 μg/20 ml.

TABLE IV

Extraction of serum: Recovery rates of 200–700 ng/ml papaverine by serum extraction method*

Recovery	
Conventional extraction	Column extraction
83.4%	86.1%
s.d. = 13.6%	s.d. = 4.9%
N = 12	N = 12

*3ml serum (1 + 1 diluted) + 12.5 ml di-isopropyl ether/methyl acetate (70:30).

achieved with column extraction, as shown by the different standard deviations.

Extraction of blood

Extraction of whole blood by shaking with solvents frequently leads to agglutination of the blood and to substance losses. By contrast, the elution

TABLE V

Column extraction of whole blood: Yields as function of dilution

Substance	Dilution blood + H_2O/NH_3	Recovery	Time of elution [min]
Morphine	1 + 2	61.7%	30
	1 + 4	72%	12
	1 + 9	92.3%	9
Papaverine	1 + 2	93.7%	10
	1 + 4	96.8%	9
	1 + 9	92.9%	10
Levomepro-mazine	1 + 2	57%	14
	1 + 4	65%	10
	1 + 9	56%	9

Concentrations: 300 ng - 150 μg/ml.

of blood via a column following dilution and haemolysis is easily and rapidly carried out. The method was tested using morphine, papaverine and levomepromazine as examples (see Table V). With morphine the yield was dependent on the viscosity of the blood. The recovery was 62% with threefold and 92% with tenfold dilution. The yield of papaverine was 95% and of levomepromazine 60% in all cases. For practical purposes one part of blood and 4 parts of water or diluted alkali are used.

Comparison of column and XAD-2 extraction

Using spiked urines and urine samples from patients with intoxications, TLC was performed following extraction with the column and the XAD-2 resin method. When identical quantities of urine are used and all extractions are performed at the pH range prescribed for the XAD method, the yield with the column method is 20% higher for alkaline substances and 50% higher for barbiturates.

Figure 2 gives an example of the superior substance yield and the lower ballast loading in the case of column extraction. Owing to the higher ballast loading, TLC following XAD elution is impaired when large quantities of extract are applied. Table VI demonstrates that the effective detection limit with column extraction is 40 to 50% lower than with the XAD-2 extraction, considering maximum sample loading and recoveries. By column extraction at acid pH values it will be possible to increase further the sensitivity for barbiturates.

Comparison of column and conventional extraction in the detection of drugs

A test was performed on 40 urines containing known drugs. The samples were analyzed simultaneously in another institute using highly specific

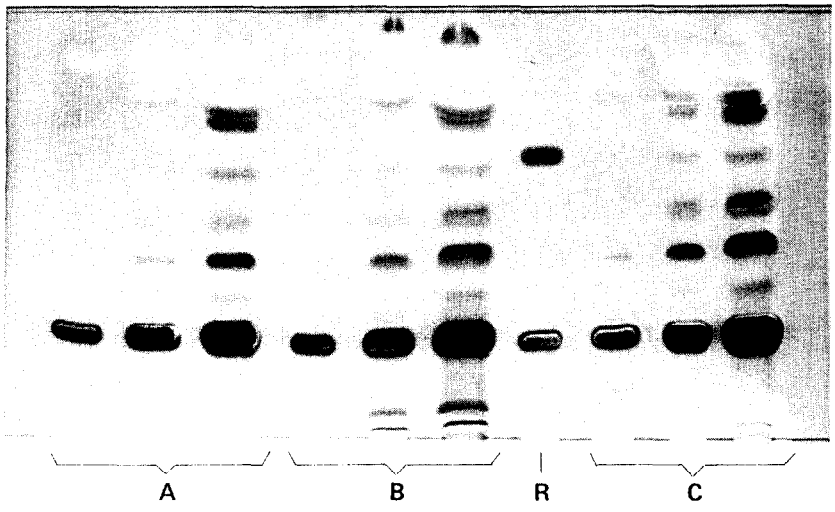


Fig. 2. TLC of residues from XAD-2 and column extraction. TLC of extracts corresponding to 1, 3 and 9 ml of urine. R ... Reference: morphine (bottom), codeine (top).
Extractant: A ... XAD-2, 1,2-dichloroethane/ethyl acetate (4:6). B ... XAD-2, chloroform/propanol-(2) (6:1). C ... Column extraction, dichloromethane/propanol-(2) (85:15).

TABLE VI

TLC detection limits in urine for XAD-2 and column extraction

Substance	Detection limit by TLC		Spray reagent
	XAD-2 pH 9.4	Column extraction pH 8.9	
Morphine	0.5 μg	0.3 μg	H_2SO_4/iodoplatinate
Codeine	1 μg	0.6 μg	
Phenobarbital	1 μg	0.5 μg	Diphenylcarbazone/ $HgSO_4$

methods (*e.g.* GLC, spectroscopy) or were obtained following the intake of known preparations. The samples were subjected in our laboratory to a TLC screening test [5, 6] following different ways of extraction:

(a) Differential elution

Barbiturates and neutral compounds were separated from basic compounds (amphetamines, phenothiazines, alkaloids, opiates, analgesics) by subsequent elution at pH 2 and pH 10. TLC was performed using 3 solvents and sequential spraying with detecting agents. Elution at pH 2 and pH 10 after adjustment with ammonia gave rise to no complications and proved itself suitable for serial determinations.

TABLE VII

Detection of drugs in 40 urine samples: Number of positive findings with TLC and reference methods

Substance	Reference methods	Differential elution (a)	Single extraction/ elution (b)	Hydrolysis + extraction/ elution (c)
Benzodiazepines	9			13
Methaqualone	12			13
Morphine	7	8	8	9
Codeine	2	2	2	2
Phenothiazines	5			5
4-aminophenazone	10	8	13	
Diphenhydramine	3	5	4	
Meprobamate	1	1	1	
Barbiturates	7	13	11	
Bromide	8	3	2	

Extraction techniques see text.

(b) Single extraction/elution

All unconjugated substances were eluted together at pH 9 through a column, or alternatively extracted in a roller apparatus [2].

(c) Hydrolysis + extraction/elution

For the detection of drugs excreted in conjugated form (benzodiazepines, methaqualone, morphine) the urine samples were acid hydrolyzed and eluted or extracted as mentioned in section (b).

Column and conventional extraction procedures produced identical results for all of the samples. The chromatograms obtained following elution were generally better to evaluate. The eluates are not so highly charged with pigments and other substances interfering with the chromatographic process. The detection sensitivity is greater, since following elution more concentrate can be applied to the plate.

Table VII gives an overall balance of the findings for the urine samples tested. The reference values are confirmed in every case — except for bromide, which is detected by TLC only in the form of bromocarbamides or bromobarbiturates. The higher number of positive findings compared with the reference values can be attributed to the high sensitivity of the TLC method. The extraction techniques (a) and (b) produce results which are essentially identical. The chromatograms obtained following two-fold elution are easier to evaluate however, especially in the case of barbiturates. In the analysis of conjugated drugs it is necessary to hydrolyse the sample in every case.

CONCLUSION

Following these initial investigations, more than 2000 columns have been tested in several laboratories with good success. Besides identical results the greatest advantage was the yield of purer concentrates and savings of time and effort.

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