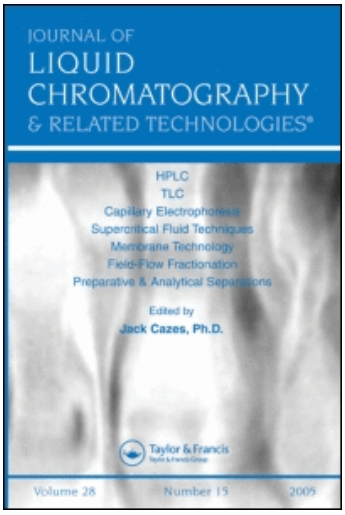

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A Rapid Procedure for the Purification of Biological Samples to be Analyzed by High Performance Liquid Chromatography

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A RAPID PROCEDURE FOR THE PURIFICATION OF BIOLOGICAL SAMPLES TO BE ANALYZED BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

One of the major problems in the analysis of drugs in biological fluids such as plasma by high performance liquid chromatography on reverse-phase columns is to obtain insoluble extracts in the mobile phase, depending on the method of sample preparation, plus the irreversible retention of apolar compounds on the chromatography column.

The present report proposes a rapid procedure for the purification of samples obtained by liquid-liquid extraction using small volumes of n-hexane. The method is based on the differential partition of matrix components between the n-hexane phase and the mobile phase consisting of water and of another organic solvent such as methanol or acetonitrile.

INTRODUCTION

Analysis by high performance liquid chromatography necessarily involves one or more steps of biological sample preparation for the extraction, concentration

and purification of the substances to be analyzed. Liquid-liquid extraction of plasma samples generally involves the use of low-polarity organic solvents, resulting in the extraction of drugs of interest and of all soluble components of the matrix. The next step consists of evaporation of the organic solvent and reconstitution of the residue with an appropriate volume of the mobile phase to be employed. When this mobile phase consists of an organic solvent plus water, a common fact in reversed-phase chromatography, the apolar components extracted by the organic solvent are not solubilized, resulting in suspensions which may damage the injector and the chromatography column. Even when the mobile phase is sufficiently apolar to solubilize these interferents, the latter may be retained at the top of the chromatography column, with a consequent reduction of its useful life.

This problem may be solved using several extraction steps, liquid-solid extraction or filtration of the sample dissolved in the mobile phase, before chromatography. These methods, however, have the disadvantages of requiring longer times for analysis, high-cost adsorption columns and small sample volumes (25 μL in some cases), respectively.

In view of these considerations, a method based on differential partition between the mobile phase and n-

hexane was developed. The apolar compounds are preferentially solubilized by n-hexane, while the compounds of interest - which are normally more polar - are solubilized in the water-organic mobile phase. This partition is performed immediately before analysis using small volumes of the two phases, thus reducing the cost of solvent use and the time for sample preparation.

The present communication reports on the results obtained by applying this method to the analysis of certain drugs in plasma for therapeutic control purposes or for pharmacokinetic studies. The major advantages of the method are its simplicity, reproducibility and the increased useful life of the chromatography columns.

APPLICATIONS

Carbamazepine and its Metabolites

Carbamazepine (CBZ), a drug administered for the treatment of epilepsy, is metabolized in the organism to carbamazepine-10,11-epoxide (CBZ-E), which also has antiepileptic activity. CBZ-E, in turn, forms carbamazepine-10,11-dihydroxide (CBZ-diOH). The method initially developed was based on the extraction of the drugs (CBZ and CBZ-E) and of the internal standard (methaqualone) with 2 mL dichloromethane using 200 µL

TABLE 1
Reproducibility of CBZ and CBZ-E Analysis.

Drug	Concentration (μg/mL)	Intra-assay		Interassay	
		n	CV(%)	n	CV(%)
CBZ	4.0	10	2.8	5	4.4
CBZ-E	2.0	10	3.8	5	4.7

n = number of samples analyzed
CV = coefficient of variation

of plasma alkalinized with 200 μL of 1.5 N NaOH. The organic phase was separated and evaporated dry under an air flow and the residue was reconstituted with 100 μL of the mobile phase and 100 μL of n-hexane. The preparation was left to stand for a few minutes and 10 μL of the lower phase was chromatographed on an RP-18 column (Merck) using acetonitrile:H_2O (1:1, v/v) as the mobile phase, with detection by absorbance measurements at 220 nm (1).

Table 1 shows the results obtained in reproducibility studies. The intra-assay precision was determined by analyzing plasma aliquots (n = 10) enriched with CBZ and CBZ-E on the basis of a single calibration curve. The interassay precision was determined by analyzing aliquots of the same samples over 5 consecutive days. The coefficients of variation obtained in the intra-assay and interassay tests were less than 5%.

TABLE 2
Reproducibility of CBZ, CBZ-E and CBZ-diOH Analysis.

Drug	Concentration	Intra-assay		Interassay	
	(μg/mL)	n	CV(%)	n	CV(%)
CBZ-diOH	0.80	8	6.1	5	8.8
	6.40	8	2.0	5	3.2
CBZ-E	0.80	8	5.8	5	5.2
	6.40	8	1.3	5	4.2
CBZ	1.60	8	2.9	5	6.9
	12.80	8	1.9	5	6.7

n = number of samples analyzed
CV = coefficient of variation

The method was later modified to permit the simultaneous quantitation of CBZ, CBZ-E and CBZ-diOH (2). The modification involved the use of carbamazepine 10-hydroxide as internal standard and of ethyl acetate:chloroform (1:1, v/v) as the extraction solvent. The three drugs were separated on an RP-18 column (Merck) using acetonitrile:H_2O (3:7, v/v) as the mobile phase. When the residue resulting from the extraction step was reconstituted with the mobile phase and n-hexane, phase separation was performed by centrifugation at 1800 g for 10 min.

Again, quite satisfactory results were obtained in the reproducibility study (Table 2).

Clonazepam

Clonazepam, another drug indicated for the treatment of epilepsy, is essentially eliminated by biotransformation, with the formation of 7-amino and 7-acetamide clonazepam, which are devoid of biological activity.

The method developed for the analysis of clonazepam is based on the use of 1 ml plasma with an internal standard added (desalkylflurazepam) extracted with ethyl ether in a basic medium. The organic phase was separated, evaporated dry and reconstituted with 50 µL of the mobile phase (acetonitrile:water, 1:1, v/v) and 25 µL n-hexane. After shaking in a Vortex-type shaker for 10 seconds, 10 µL of the lower phase was analyzed on an RP-18 column packed with 3 µm particles (Varian), with detection by absorbance measurements at 313 nm (3).

Studies of intra-assay precision (n = 10) and of interassay precision (n = 7) carried out on plasma samples enriched with 20 and 60 ng/mL clonazepam demonstrated coefficients of variation of less than 5%, as shown in Table 3.

Albendazole Metabolites

Albendazole, an anti-helminthic agent currently used in the treatment of neurocysticercosis, is rapidly

TABLE 3
Reproducibility of Clonazepam Analysis.

Concentration	Intra-assay		Interassay	
(ng/mL)	n	CV(%)	n	CV(%)
20.0	10	1.7	7	2.9
60.0	10	0.9	7	4.1

n = number of samples analyzed
CV = coefficient of variation

metabolized to albendazole sulfoxide (ASOX) and albendazole sulfone (ASON) and is not detected in plasma after oral administration.

The method proposed here (4) consists of the extraction of metabolites and internal standard (mebendazole) from 500 µL of plasma with chloroform:isopropanol (9:1, v/v) after acidification with 200 µL 0.01 M HCl containing sodium metabisulfite at the concentration of 4 g/L. The last agent is added to prevent oxidation of the drugs under analysis.

The organic phase was separated and evaporated dry under an air flow and reconstituted in 100 µL of the mobile phase and 100 µL n-hexane. After phase separation by centrifugation, 20 µL of the lower phase was chromatographed.

Table 4 shows that the method is quite reproducible.

TABLE 4
Reproducibility of ASOX and ASON Analysis.

Drug	Concentration (μg/mL)	Intra-assay n	Intra-assay CV(%)	Interassay n	Interassay CV(%)
ASOX	0.50	10	2.9	5	7.0
	1.50	10	1.7	5	3.5
ASON	0.10	10	3.4	5	6.4
	0.30	10	1.6	5	3.5

n = number of samples analyzed
CV = coefficient of variation

FINAL CONSIDERATIONS

The method described here, although previously unreported in the literature, is extremely simple and permits a second purification step of the sample to be analyzed. n-Hexane does not reduce the interfering peaks in the chromatogram, especially those that elute rapidly, since these compounds have greater polarity and therefore are preferentially solubilized by the mobile phase. However, apolar compounds which are retained at the top of the column, thus reducing the useful life of the latter, remain in the n-hexane phase. This can be easily observed since the n-hexane phase often presents a yellow color due to pigments present in plasma. Chromatographic analysis of this phase on GF_{254} silica gel plates (Merck) with the mobile phase consisting of ethyl acetate:methanol: ammonia (85:10:5, v/v) demonstrated the presence of

three spots (Rf = 0, 0.3 and 0.8) after spraying with 0.1% bromothymol blue (w/v) in ethanol:water (9:1, v/v), a lipid indicator (5).

The estimate of the useful life of a chromatography column is impaired by the effect of several parameters such as type and origin of the stationary phase, particle diameter and diameter of the column itself, mobile phase, type and preparation of the sample analyzed, and, last but not least, the care provided by the operator for column use and storage. Despite these difficulties in evaluation, the proposed method resulted in an increase of the useful life of the chromatography columns. To illustrate, we may mention that a Merck column was used for at least two years, with approximately 1000 injections of extracts obtained from biological materials purified by the method described, with no loss of efficiency and/or increased pressure, and with no need to change the pre-column.

Other low-polarity organic solvents such as n-heptane, chloroform, and ethyl acetate can also be utilized as long as this does not involve the appearance of interfering peaks in the chromatogram due to impurities present in these solvents or due to the solvent itself partially solubilized in the mobile phase. The best results for the experiments described here were obtained with n-hexane.

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