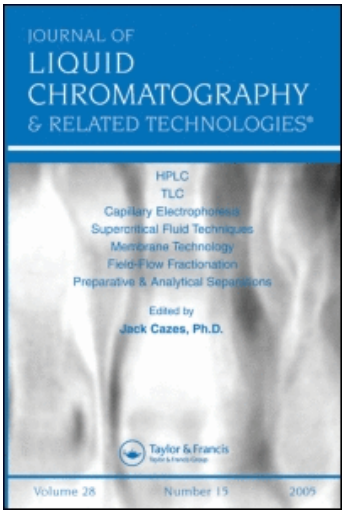

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Automatic Determination of Clomipramine, Imipramine and Their Demethylated Metabolites in Plasma by High Performance Liquid Chromatography

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AUTOMATIC DETERMINATION OF CLOMIPRAMINE, IMIPRAMINE AND THEIR DEMETHYLATED METABOLITES IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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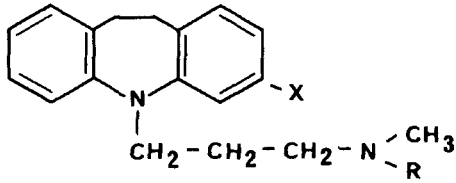
ABSTRACT

Determination of tricyclic antidepressant drugs (clomipramine, imipramine and their demethylated metabolites) in plasma can be carried out by extraction on a precolumn (n-octyl bonded silica gel) and washing with phosphate buffer (0.005 M, pH = 11.5). The elution is carried out on-line (with back-flush) on an alkylnitrile bonded silica gel column with the mobile phase : acetonitrile-methanol-phosphate buffer 0.005 M, pH = 7.2, 50 : 25 : 30 (v/v). The AASP (Advanced Automated Sample Processor) allows full automatization for this determination. The total analysis time is only 10 minutes The obtained reproducibility is 5.6 % for clomipramine and 4 % for demethylclomipramine while the detection limit (10 $\mu g L^{-1}$) is lower than the therapeutic concentrations of these antidepressants (and metabolites) in plasma. The correlation with the classical (but tedious) method involving liquid-liquid extraction is excellent.

INTRODUCTION

The past two decades have seen many advances in the use of tricyclic antidepressants. However, the treatment is inadequate for 30 to 50 % of patients, possibly due to non-compliance or some undesirable secondary effects. A tentative explanation may be found in the narrow therapeutic range [1] as well as by some interindividual pharmacokinetic variations [2]. The major metabolite of these drugs is generally a demethylated derivative which is also pharmacologically potent [3]. So, a simultaneous determination of both molecules is necessary. For imipramine (**Figure1**), the imipramine and desipramine (demethylated metabolite of imipramine) concentration in plasma varies between 150 to 250 μgL^{-1} [4]. In the case where desipramine has been prescribed its content must be in the concentration range 75-160 μgL^{-1} [5].

For clomipramine, the therapeutic range of clomipramine and demethylclomipramine (major metabolite) , which is somewhat different according to various authors, is between 150 and 300μgL^{-1}.



$R = CH_3$	X = H	Imipramine	-IMI
R = H	X = H	Desipramine	-DES
$R = CH_3$	X = Cl	Clomipramine	-CMI
R = H	X = Cl	Demethylclomipramine	-DCMI

Figure 1. Studied tricyclic antidepressant drugs and their demethyleted metabolites.

The methods used have been reviewed in [6]. Seveval are adequate for the monitoring of plasma levels during chronic therapy : gas chromatography with thermoionic detection [7] or coupled with mass spectrometry [8] ; adsorption [9] and reversed phase (with or without ion pairing) high performance liquid chromatographic methods [10,11]. Alkylphenyl [12] or alkylnitrile bonded silica gel have been used more recently. Immunochemical methods [13] do not allow a quantitative determination of the original molecule and of its major metabolite, because of their too much close chemical structures.

HPLC determination of endogenous and exogenous substances in biological samples rich in proteins, requires a preliminary step for their elimination. If the concentration of the analyte and/or the detector sensitivity do not require a concentration step, the protein elimination can be achieved by precipitation according to various methods [14].

In the case of tricyclic antidepressants, the low plasma concentrations following single oral doses require an enrichment step which can be obtained either by liquid-liquid extraction or by sorbent extraction. It is difficult to simplify and to automate a liquid-liquid extraction procedure. As an alternative, the automated sample preparation using sorbent extraction has thoroughly been studied [15] and applications have been described for some physiological substance [16] as well as for drug analysis [17]. In these systems, a precolumn is used seveval times, which constitutes a major drawback owing to the decrease of its capacity and, consequently, purification and enrichment are decreasing as the number of analysis increases.

The Varian AASP system allows automated on-line precolumn enrichment process. This paper describes the use of this new technology to the quantitative measurement of clomipramine, demethylclomipramine, imipramine and desipramine in plasma. As it will be seen, the benefit of this sample preparation system is quicker sample turn-round times, greater efficiency and better automatization, all leading to time and cost savings.

EXPERIMENTAL

1. Reagents and Standards

Acetonitrile (HPLC grade) was purchased from Chromoptic (Rathburn). Methanol (Normapur grade) and the other reagents (analytical grade) from Prolabo. The water was freshly distilled on quartz before use (Quartex apparatus).

The tricyclic antidepressants and their demethylated metabolites (clomipramine, demethylclomipramine, imipramine and desipramine hydrochlorides) were kindly provided by Ciba Geigy. Solutions were prepared in ethanol (1 gL^{-1}) and stored at 4°C. These solutions were then diluted with phosphate buffer 0.005 M, pH = 11.5 as necessary and used to prepare the appropriate concentrations (50-300 μgL^{-1}). Clomipramine (internal standard for imipramine and desipramine) and imipramine (internal standard for clomipramine and demethylclomipramine) were used as 2 mgL^{-1} standard solutions in the phosphate buffer (0.005 M, pH = 11.5).

2. Extraction and Injection System

In the proposed system (AASP), each cassette is composed of 10 sorbent extraction cartridges, each of which is packed with 40-50 mg of bonded silica sorbent .

Each sample (or standard solution) is applied on a cartridge, and this is followed by a washing solvent that can remove interferences without eluting the analytes. The cassette is then loaded in the AASP system for automated on-line chromatographic elution (**Figure 2**). Up to 10 cassettes (i.e 100 samples) can be loaded at one time. Sample number and analysis time can be programmed as well as valve reset time ; the latter is limited to complete elution of compounds of interest in order to keep other strong retained impurities in the cartridge.

Each cartridge is conditioned first with 2 mL of methanol and then with 2 mL of water ; 0.5 mL of spiked plasma or of a considered patient serum, 0.05 mL of internal standard and 0.5 mL of phosphate buffer (0.005 M, pH = 11.5) are successively introduced into the reservoir. Homogenization is obtained with an automatic pipette by aspiration and back flow. This mixture is pushed onto the cartridge using a pneumatic manifold. Phosphate buffer (6 mL) is used to clean up the cartridge (not automatic).

3. Liquid-liquid Extraction Procedure

The internal standard solution 0.05 mL is added to 1 mL of plasma, followed by 0.5 mL of 2 M sodium carbonate solution. The alcalized plasma is then extracted with 9 mL of hexane by stirring

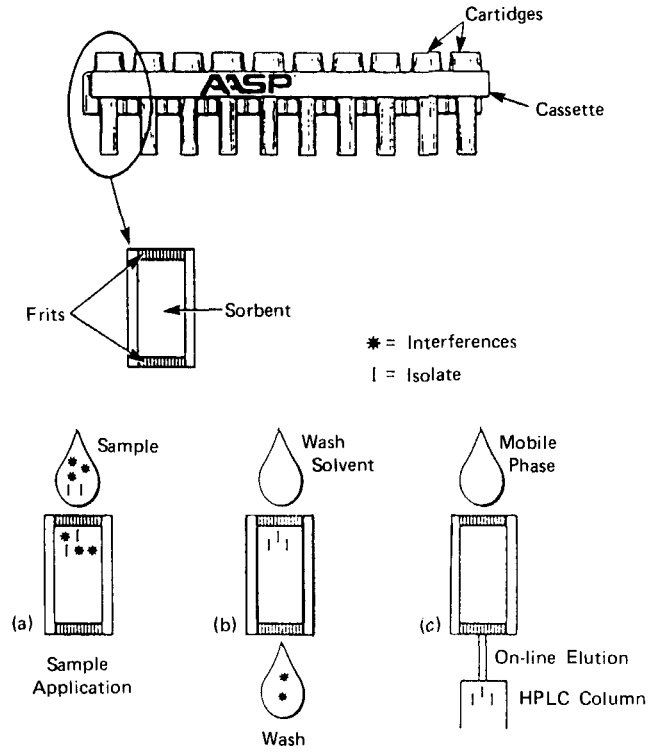


Figure 2. AASP sorbent extraction and on-line elution.

for 15 minutes. After separation of the phases by centrifugation, the organic layer is transferred into an evaporation tube and then evaporated at 60°C under a stream of nitrogen. The residue is re-dissolved in 0.1 mL of mobile phase, of which 0.05 mL is injected into the liquid chromatograph.

4. Chromatographic Separation

The chromatograph consisted of a Varian 2010 pump and a Varian 2050 spectrophotometer, set at 252 nm. chromatogram

recording and peak integrations were obtained through a Shimadzu CR-3A integrator.

The column (15 cm x 0.46 cm I.D.) was packed with alkylnitrile bonded silica gel (Spherisorb CN, 5 µm). The mobile phase consisted of acetonitrile - methanol - phosphate buffer (0.005 M, pH = 7.2), 50 : 25 : 30 (v/v). Flow rate used was 1.5 $mL.min^{-1}$.

RESULTS AND DISCUSSION

1.Choice of Sorbent Extraction Conditions

This choice is very important because it must allow the quantitative retention of antidepressant drugs and their metabolites in plasma as well as their complete elution into the analytical column by the mobile phase.

1.1 Stationary Phase

The studied molecules have an aliphatic amine structure (pK_A = 9.5) and can be denoted as **B** . Their basic character suggests that the extraction should be carried out either under protonated form HB^+ on cation exchanger precolumn in acidic medium, or under molecular form on an alkyl bonded silica gel precolumn in alkaline medium.

The considered molecules are very hydrophobic, as it can be seen on **Table 1** where calculated values of partition coefficient according to Rekker for a water-n-octanol system have been listed. We have chosen extraction on alkyl bonded silica gel precolumn rather

Table 1

Calculated Values According to Rekker of Logarithm of Partition Coefficients of the Concerning Molecules in Water-octanol System

	CMI	DCMI	IMI	DES
log P	6.40	5.90	5.69	5.19

than on cation exchange precolumn : the strong hydrophobicity of molecules leads to a stronger retention on alkyl bonded precolumn than on a cation exchanger.

1.2 Mobile Phase and pH

Figure 3 shows the retention variation of clomipramine and demethylclomipramine versus methanol content in the mobile phase (water-methanol) on n-octyl bonded silica gel 20 µm (obtained from the AASP cassettes) for various pH (Same retention behaviours were observed with imipramine and desipramine). The variation curves log k'= f(x) (x is the methanol percentage in the mobile phase) are linear **[18]**. By extrapolation of these curves for x = 0, the capacity factor values k' in pure water can be obtained.

The capacity factor k' of antidepressant drugs increases with pH value (**Figure 4**); as a matter of fact, the molecular character of base **B** is more pronounced with increasing pH. In an alkaline medium (pH=11.5), when compounds exist as molecular form, a good fit between capacity factors and their hydrophobic characters (measured by their partition coefficient values) was observed.

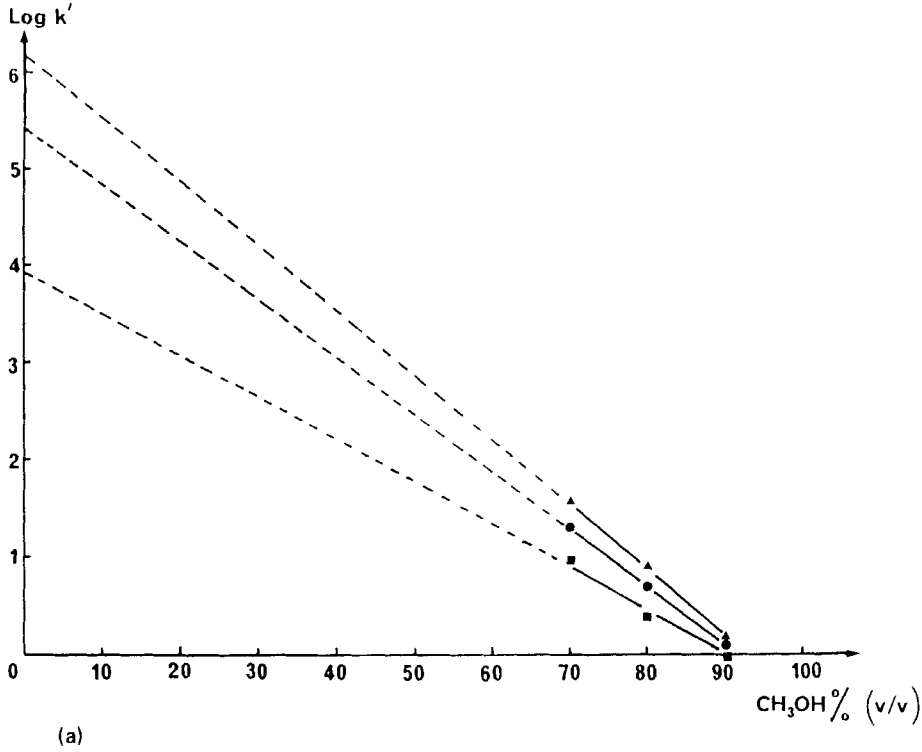


Figure 3. Variation of capacity factor logarithm versus methanol content in mobile phase on a n-octyl bonded silica gel for several pH.
Column : length : 10 cm ; I.D. : 0.2 cm.
Stationary phase : octyl bonded silica gel, particle size : 20µm (from AASP cartridge).
Flow rate : 1.5 mL.min^{-1}. Detection : UV at 252 nm.
a : clomipramine ; **b** : demethylclomipramine.
▲ pH = 11.5, ● pH = 9, ■ pH = 7.

(continued)

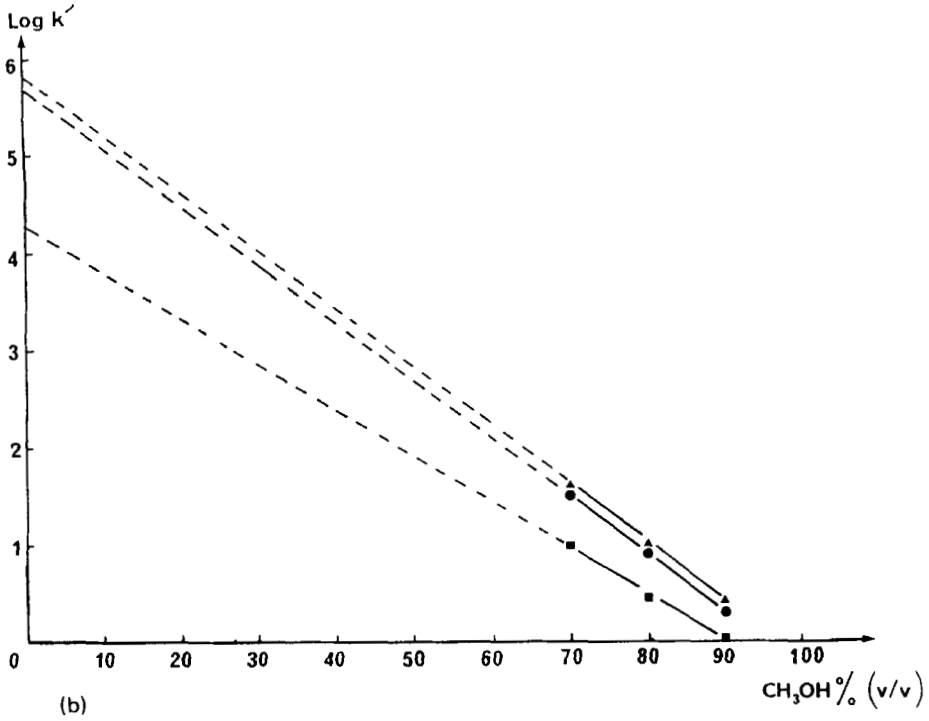


Figure 3b

Consequently, we have chosen phosphate buffer (0.005 M ; pH=11.5) for drug sorbent extraction. With n-octyl bonded silica gel as precolumn stationary phase, capacity factors are greater than 10^5 when the pH is above 9 (**Figure 4**). This guarantees a quantitative extraction.

As it will be shown later, this solution is compatible with the chromatographic separation system. Nevertheless, silica gel solubility increases greatly in basic medium, and the chosen pH (11.5) is not suitable for prolonged use for cartridges. But, as cartridges are

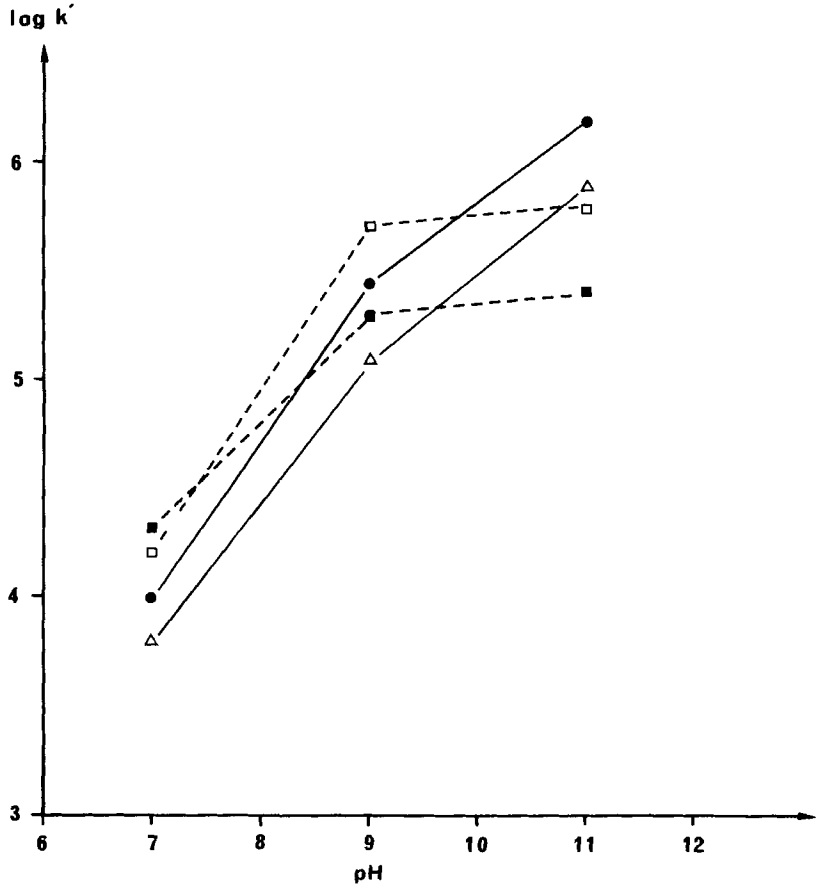


Figure 4. Variation of capacity factor logarithm (extrapoled for pure water) for antidepressants and their metabolites versus pH.
Same chromatographic conditions as in **Figure 3**.
● clomipramine, △ imipramine, □ demethylclomipramine, ■ desipramine.

only used once and then discarded, and the contact time with the alkaline buffer is short, this phenomenon can be neglected ; moreover, alkyl bonded silica gel is less soluble than bare silica gel in basic medium.

1.3 Cartridge Washing and "Back-Flush"

Cartridge washing after plasma extraction is also important. Proteins and interferences coming from endogenous substances present in plasma can be eliminated by this washing step and , consequently, the chromatographic resolution can be improved. **Figure 5** shows the influence of the precolumn washing volume (phosphate buffer 0.005 M ; pH = 11.5) on the chromatogram baseline of blank plasma sample. A 6 mL washing volume is enough to suppress nearly all interferences.

Due to their high capacity factor values, the studied tricyclic antidepressants are retained on the top of the cartridge. The AASP system and the chromatographic circuit are connected in such a way that the mobile phase circulates through the cartridge in the opposite direction from the one used during percolation (back-flush). Thus, we can neglect the influence of the cartridge void volume on efficiency and the elution volume of the compounds is very small.

2. Choice of Analytical Separation Conditions

2.1 Stationary Phase

Tricyclic antidepressant drugs are often separated by adsorption chromatography on silica gel and partition chromatography

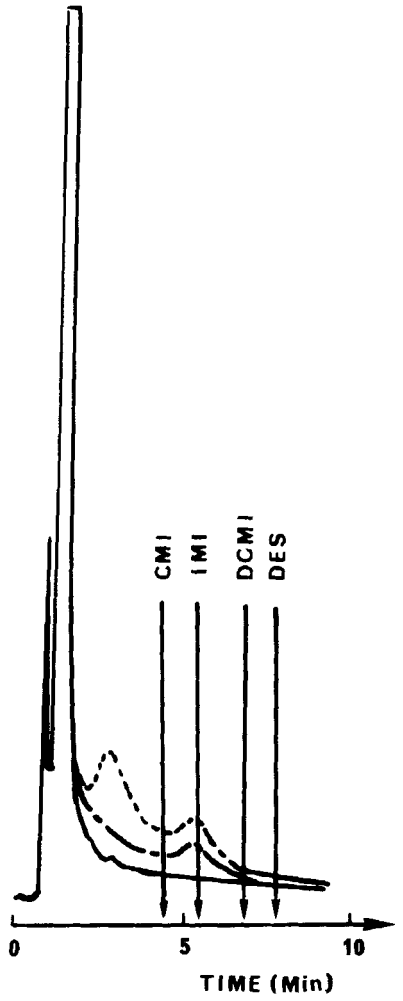


Figure 5. Influence of washing volume on chromatogram baseline obtained by percolation of 0.5 mL blank plasma + 0.5 mL phosphate buffer 0.005 M, pH = 11.5 mixture.
Column : length : 15 cm ; I.D. : 0.46 cm.
Stationary phase : Spherisorb CN 5 µm.
Mobile phase : acetonitrile-methanol-phosphate buffer 0.005 M, pH = 7.2, 50 : 25 : 30 (v/v).
Flow rate : 1.5 mL.min^{-1}. Detection : UV at 252 nm.
Sensitivity : 0.01 AUFS.
- - - - - - washing with 2 mL phosphate buffer 0.005 M, pH = 11.5.
——— — — washing with 4 mL phosphate buffer 0.005 M, pH = 11.5.
——————— washing with 6 mL phosphate buffer 0.005 M, pH = 11.5.

on bonded silica gel. The disadvantage of the first technique is the short life time of columns and the interferences coming from the adsorption of polar compounds present in biological samples. Moreover, the reproducibility of separations (an important factor in quantitative analysis) requires a strict control of the water content in the mobile phase **[19,20]**. Finally, classical eluting mobile phases are not compatible with the aqueous medium chosen for the sorbent extraction (the eluting solution is not miscible with the aqueous volume contained in cartridge bed). Thus, partition chromatography on alkylnitrile bonded silica gel have been chosen. This method is compatible with the sorbent extraction procedure as described above.

2.2 Mobile Phase

Optimization of the separation leads to the use of the following ternary mixture as the mobile phase : acetonitrile-methanol- phosphate buffer (0.005 M ; pH = 7.2), 50 : 25 : 30 (v/v). This eluting phase allows a very rapid elution of the antidepressant drugs extracted on precolumn.

In fact, at this pH (7.2), the antidepressant drugs are in an ionized form and thus, are very soluble in the aqueous-organic mobile phase. This ensures their quantitative elution from precolumn.

With this chromatographic system, a satisfactory separation can be observed. The resolution values are as follow : R_S(CMI-IMI) = 1.6 ; R_S(DCMI-DES) = 1.3 (**Figure 6**).

3. Extraction Recovery on Precolumn

Table 2 shows the values of different extraction recoveries for the considered substances. The recovery is about 100%

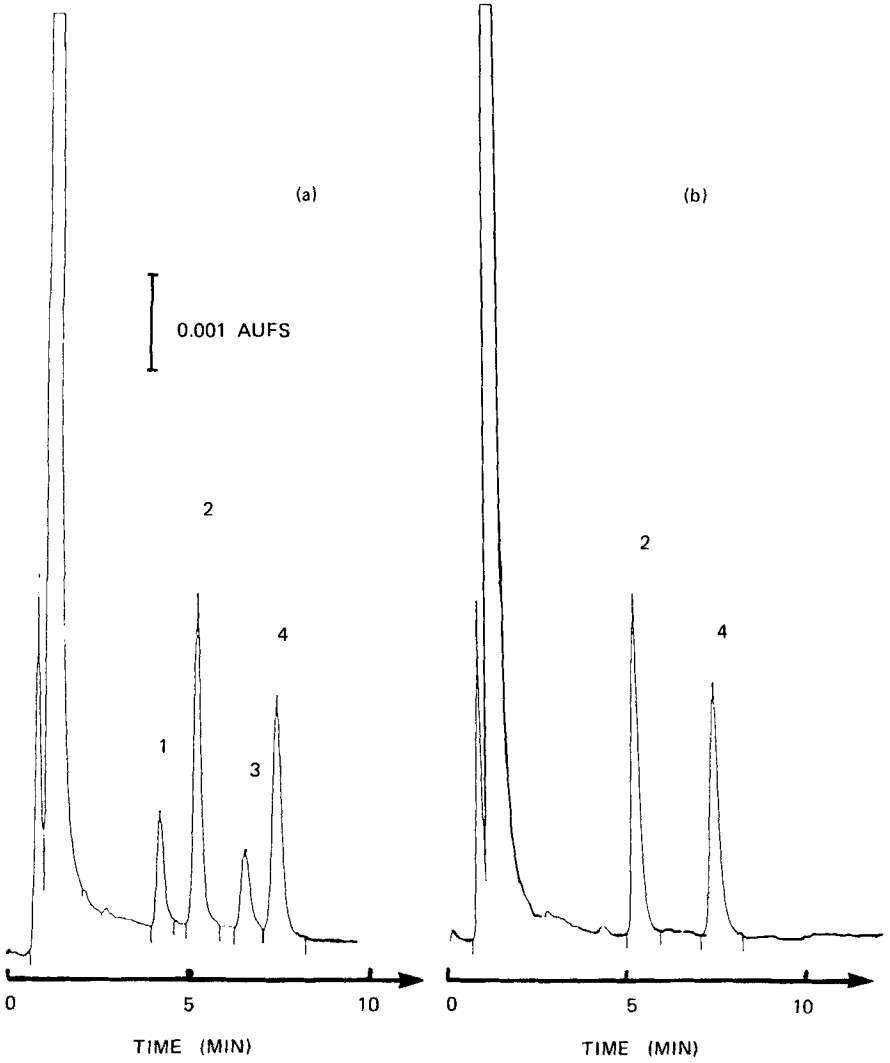


Figure 6. Separation by partition chromatography on alkylnitrile bonded silica gel of tricyclic antidepressant and metabolite mixture.
Same chromatographic conditions as in **Figure 5**.
a : chromatogram of 0.5 mL of a plasma of patient after clomipramine treatment. Internal standard : imipramine.
b : chromatogram of 0.5 mL of a plasma without clomipramine.
1 : clomipramine ; **2** : imipramine ; **3** : demethyl-clomipramine ; **4** : desipramine.

Table 2

Extraction Recoveries % [Mean (C.V.), n=5] of the Studied Tricyclic Antidepressant Drugs and Their Metabolites.

	CMI	DCMI	IMI	DES
Sorbent extraction of a synthetic mixture	98 (4.5)	95 (3.0)	102(3.6)	94 (3.4)
Sorbent extraction of a spiked plasma	85 (5.7)	72 (2.0)	95 (3.3)	85 (4.0)
Liquid-liquid extraction of a spiked plasma	80 (5.4)	44 (4.2)	76 (4.9)	48 (4.6)

for synthetic mixture but is lower with spiked plasma, probably due to the interactions between the drug molecules with proteins, a part of drug molecules could be carried away with proteins during precolumn washing. Nevertheless, the extraction recoveries are always superior to those obtained in liquid-liquid extraction (with hexane in basic medium).

4. Linearity, Reproducibility, Detection limit, Correlation with Liquid-liquid Extraction.

Calibration curves are linear, from 10 μgL^{-1} to 300 μgL^{-1}, with correlation coefficients greater than 0.99. Results of reproducibility and accuracy studies (within-day run) with plasma spiked with 150 μgL^{-1} clomipramine and demethylclomipramine (imipramine as internal standard) are summarized in **Table 3**.

Table 3

Reproducibility and Accuracy Measured with Clomipramine and Demethylclomipramine Spiked Plasma at 150 μgL^{-1}.
Internal Standard : Imipramine.

	CMI	DCMI
Sample number	8	8
Average (μgL^{-1})	149.3	146.8
Standard deviation (μgL^{-1})	8.5	5.8
Coefficient of variation (%)	5.6	4

The reproducibility of the method is characterized by a relative standard deviation of 5.6% while it is approximately to 15% by external standardization. This shows the necessity of an internal standardization because of the presence of proteins in plasma which influences extraction recovery.

The detection limit, under described conditions, is $10\mu gL^{-1}$ This is sufficient for the therapeutic monitoring of these antidepressant drugs .

The comparison of the results obtained by the AASP system and those obtained by liquid-liquid extraction has been done for the determination of the clomipramine in 16 patient samples (**Figure 7**). The correlation coefficient is equal to 0.97 which can be considered as quite satisfactory.

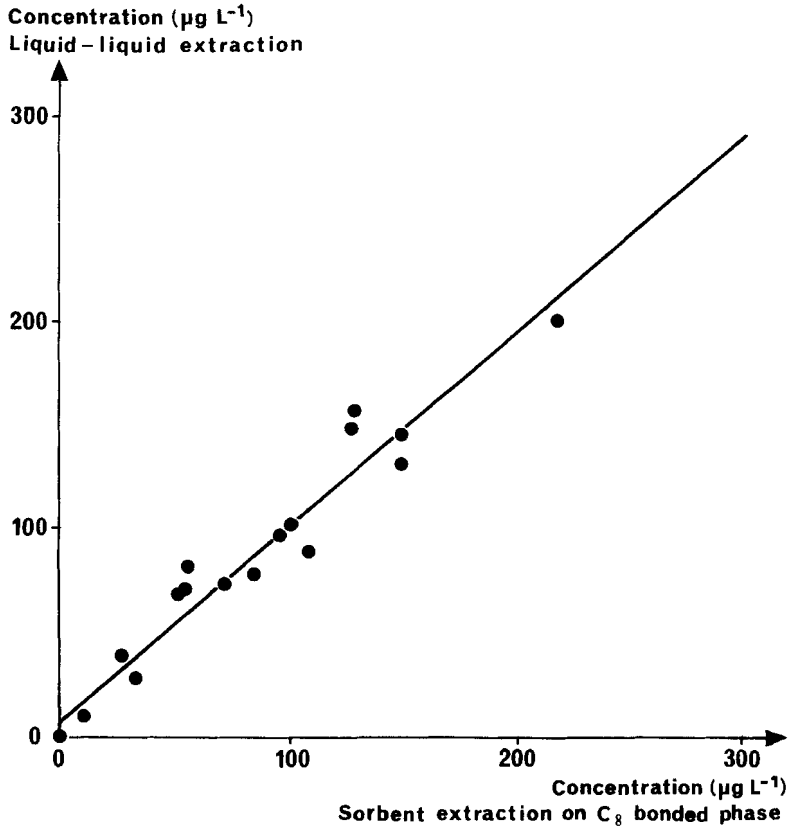


Figure 7. Comparison of obtained results in the case of clomipramine determination with AASP and liquid-liquid extraction (n = 16 ; r = 0.97 and slope = 0.946).

CONCLUSION

The AASP system allows a rapid determination of tricyclic antidepressant drugs (clomipramine, imipramine and their demethylated metabolites) in plasma, by HPLC.

The preparation of biological samples, which is a very tedious step, has been largely simplified by the use of the AASP

system. Its automation and its good reproducibility are two assets for routine analysis. As series of samples must be treated, which is generally the case in clinical analysis, this method is much more suitable than liquid-liquid extraction. The total analysis time is 10 minutes and, once the cassette is charged, determination is fully automatic.

Generally speaking, this kind of analysis setting requires a careful study of extraction conditions on precolumn as well as the study of its compatibility with the analytical separation system.

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