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High-performance liquid chromatography determination of flunitrazepam and its metabolites in plasma by use of column-switching technique: comparison of two extraction columns

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Abstract

A study, using on-line column-switching high-performance liquid chromatography, evaluated two different extraction columns for the determination of flunitrazepam and its major metabolites: 7-aminoflunitrazepam, 7-acetamidoflunitrazepam and desmethylflunitrazepam. The procedure was based on the enrichment of benzodiazepines on the extraction column, followed by transfer of the compounds to the analytical column. The two extraction columns were compared: the first column was a BioTrap 500 MS (hydrophobic polymer), 20×4 mm I.D., and the second was a LiChrospher RP-18 ADS, 25×4 mm I.D. The analytical column used was a LiChrospher select B RP-8, 125×3 mm I.D. with 5 μm particle size. The extraction conditions for the two pre-concentration columns, such as extraction temperature, buffer concentration, buffer pH, acetonitrile percentage and flow-rate, were studied for the extraction from plasma of flunitrazepam and its metabolites mentioned above. The mobile phase of the analytical column was isocratic and composed of acetonitrile–20 m*M* phosphate buffer at pH 2.1 (35:65, v/v) and at a flow-rate of 0.3 ml/min.

Keywords: Flunitrazepam

1. Introduction

Flunitrazepam (FNZ), Rohypnol, [5-(2-fluorophenyl)-2,3-dihydro-1-methyl-7 nitro-1H-1,4-benzodiazepin-2-one] is a benzodiazepine derivative whose hypnotic effect predominates over the sedative, anxiolytic, muscle relaxing and anticonvulsant properties, which are characteristics of benzodiazepines. It is used in many hospitals, but its abuse and toxic effects have been mentioned by several authors [1–4].

Flunitrazepam undergoes biotransformation via *N*-desmethylation, 3-hydroxylation and glucuronidation, and reduction of the nitro group to an amine with acetylation (Fig. 1) [5].

In blood, serum or plasma, flunitrazepam and 7-aminoflunitrazepam (7-NH_2-F) are the main components. Desmethylflunitrazepam (DMF) can be detected in blood but is a minor metabolite. 7-Aminoflunitrazepam and desmethylflunitrazepam are active metabolites.

In urine the following metabolites are present:

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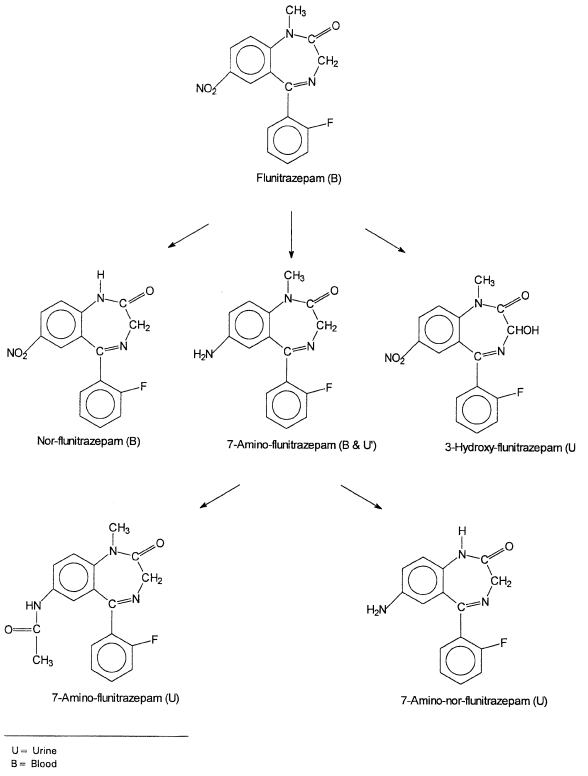

Fig. 1. Flunitrazepam metabolism [5].

7-acetamidoflunitrazepam (7-NHCOCH$_3$-F), 3-hydroxyflunitrazepam (3-OH-F) and 7-aminodesmethylflunitrazepam (NH$_2$-DMF). DMF can be found in urine but at a very low concentration [5].

A variety of analytical methods have been described for the determination of flunitrazepam and other benzodiazepines in biological matrices.

Numerous detection methods exist including gas chromatography (GC) and high-performance liquid chromatography (HPLC) [6,7,18–28].

GC has been traditionally recommended for the analysis of drugs in biological samples because of its high sensitivity and GC–mass spectrometry (MS) methods have often been reported [7–11].

The chromatographic theory and method development for column-switching or on-line pre-concentration technique has been well reviewed and described [12,13]. Recently, a paper was published [14] describing a HPLC method development of some benzodiazepines in plasma. This recent work showed excellent linearity and the recoveries values obtained were around 98% and the relative standard deviations (RSDs) for between- and within-day assays were <20% for low concentration close to the values of the limit of quantification (LOQ) and <4% for high concentration for all benzodiazepines studied.

In the present study, we compare two columns after optimization of conditions for each: a BioTrap 500 MS (hydrophobic polymer), 20×4 mm I.D. and a LiChrospher RP-18 ADS, 25×4 mm I.D. The aim of this work was to optimize extraction conditions for the two columns by using on-line column-switching HPLC technique and to compare column performances such as chromatogram trace, recovery, repeatability and reproducibility for flunitrazepam and its major metabolites.

2. Experimental

2.1. Chemicals

Flunitrazepam (RF 907), 7-aminoflunitrazepam (RA 912) and desmethylflunitrazepam (RD 919) were purchased from Promochem Sarl (Molsheim, France). Human plasma was obtained from the University Hospital of Geneva (Geneva, Switzerland). Monobasic and dibasic potassium phosphate and phosphoric acid were purchased from Merck (Darmstadt, Germany), acetonitrile of HPLC grade was obtained from Romil (Cambridge, UK).

2.2. Columns and mobile phases

The first on-line extraction column tested for column switching was a LiChrospher RP-18 ADS (Merck), which allows direct injection of untreated biofluids into the HPLC system without any clean-up procedure (except a simple centrifugation).

This extraction column is pH stable (between pH 2 and pH 7.5) with an external surface composed of hydrophilic electroneutral diol groups and a hydrophobic internal surface composed of C_{18} alkyl chains (Fig. 10).

The surface within the pore is an alkyl chain and the pore diameter is approximately 60 Å.

The second on-line extraction column studied was a BioTrap 500 MS (Chromtec, Hägersten, Sweden), a new biocompatible simple extraction column al-

lowing repeated direct injection of serum, plasma, fermentation broth, supernatant of cell culture and other complex matrices, into the HPLC system without any clean-up procedure (except a simple centrifugation). This simple extraction column is pH stable (pH 2 to 11) with a biocompatible external surface (α_1-acid glycoprotein) and a hydrophobic internal surface (hydrophobic polymer) (Fig. 10). The biocompatible surface is an extremely stable protein which tolerates the organic solvents used in reversed-phase HPLC.

The surface within the pore is a hydrophobic polymer and the matrix pores are small enough to exclude plasma protein and other macromolecular compounds.

The separation was performed using a C_8 reversed-phase column: LiChrospher select B 125×3 mm I.D. with 5 μm particle size and a guard column Nucleosil NH_2 8×4 mm I.D. with 5 μm particle size (Macherey-Nagel, Switzerland).

The analytical mobile phase was a mixture of 20 m*M* phosphate buffer (pH 2.1)–acetonitrile (65:35, v/v). The buffer solution was filtered through a 0.45-μm filter (Supelco, Bellefonte, PA, USA) before use. The separation method was validated recently [15].

The first on-line column-switching mobile phase consisted of 20 m*M* phosphate buffer (pH 7.2)–acetonitrile (94:6, v/v).

The second on-line column-switching mobile phase consisted of 30 m*M* phosphate buffer (pH 7.5)–acetonitrile (94:6, v/v). The program optimized for the column-switching procedure is given in Table 1. The flow-rates were 0.3 ml/min and 0.6 ml/min, for the analytical column and the extraction column, respectively.

2.3. Instrumentation and description of the column-switching system

A schematic column-switching HPLC technique set-up is given in Fig. 2. The on-line system consisted of two quaternary pumps, Model HP 1100, two columns, an extraction column, which acted as a trapping column, and an analytical column which were connected by a HP 1100 high-pressure six-port valve in back-flush configuration. The chromatography system was equipped with a diode-array detector, an automatic injector and an autosampler.

In the extraction position, the mobile phase delivered by pump 1 flushes the sample matrix via valve positions 4–5 directly to waste. Simultaneously, the analytes are retained on the hydrophobic phase.

In the transfer position, following the complete elution of the sample matrix, the six-port valve is rotated by 60°. In this position, the extraction column is coupled to the analytical column. The stronger

Table 1
Optimized conditions for the procedure

Extraction column	Biotrap MS 500 20×4 mm I.D. Thermostated at 35°C
Analytical column	LiChrospher select-B 125×3 mm I.D., 5 μm Thermostated at 25°C
Loading LiChrospher RP-18 ADS	K_2HPO_4 (20 m*M*, pH 7.2)
Loading BioTrap 500MS	K_2HPO_4 (30 m*M*, pH 7.2)
Flow and loading time	5 min at 0.6 ml/min
Transfer and analytical seperation	A=Acetonitrile (ACN) B=KH_2PO_4 (0.2 *M*, pH 2.1) Linear gradient 0 min: A–B (30:70) at 0.5 ml/min 25 min: A–B (35:65) at 0.3 ml/min
Detection	245 nm
Volume of plasma injected	50 μl

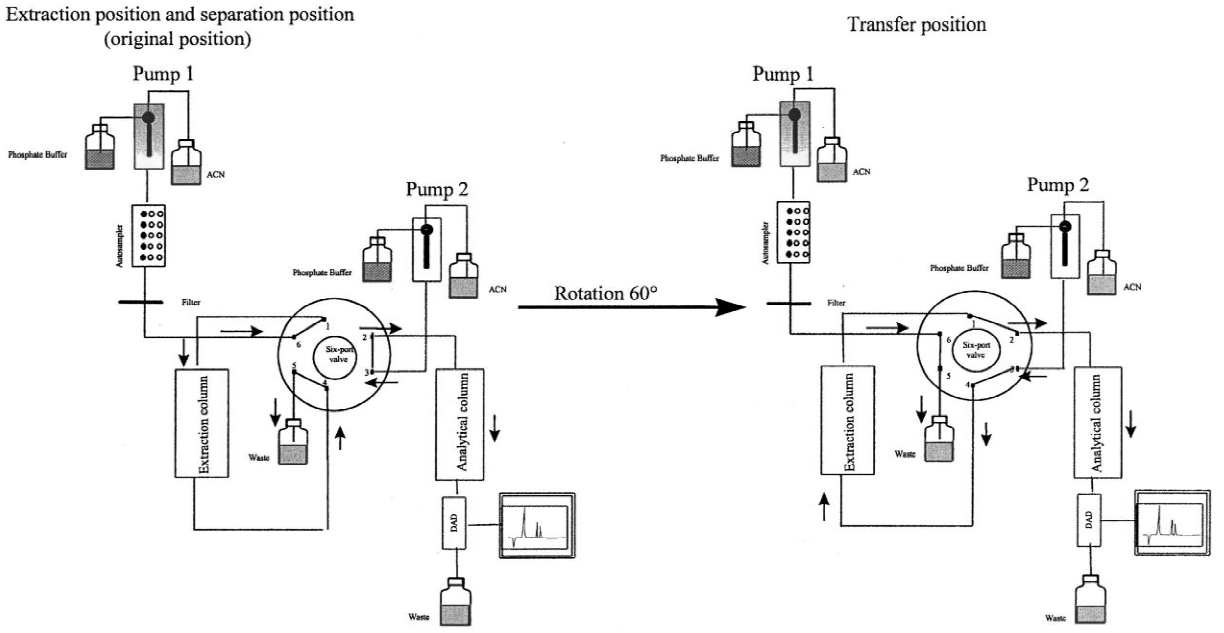

Fig. 2. Schematic representation of the column-switching set-up. Adapted from Refs. [15,16].

elution power of the second mobile phase delivered by pump 2 causes desorption of the analytes from the extraction column. The change in flow direction (backflush) causes additional concentration of analytes.

In the separation position, subsequent to transfer of analyte fraction into the analytical column, the valve is switched back to its original position (extraction position). Separation of the analytes now takes place in a conventional manner. The absorbance of the eluent was monitored at 254 nm. Chemstation software G2170AA installed on a Vectra personal computer (Model VL4; Hewlett-Packard) was used for instrument control, data acquisition and data handling.

2.4. Sample preparation

2.4.1. Standard solutions

Stock standard solutions of both flunitrazepam and its major metabolites: 7-aminoflunitrazepam, 7-acetamidoflunitrazepam, desmethylflunitrazepam were prepared by dissolution of each compound in methanol to obtain a concentration of 1 mg/ml. These solutions were stored at −20°C and remained stable for at least 12 months.

Comparison of peak area and resolution of the two metabolites of flunitrazepam (7-aminoflunitrazepam and 7-acetamidoflunitrazepam) was done by injecting 50 μl of a mixture of flunitrazepam and its metabolites in presence of 2.0 μg/ml of methylclonazepam as internal standard.

2.4.2. Preparation of phosphate buffers

The extraction phosphate buffer for LiChrospher RP-18 ADS, was prepared by transferring 3.6 ml of 1 *M* KH_2PO_4 and 5.5 ml of 1 *M* K_2HPO_4 into a 1000-ml volumetric flask, and made up to volume with distilled water.

The extraction phosphate buffer for BioTrap 500 MS was prepared by transferring 2.7 ml of 1 *M* KH_2PO_4 and 9.9 ml of 1 *M* K_2HPO_4 into a 1000-ml volumetric flask, and made up to volume with distilled water.

The analytical phosphate buffer was prepared by transferring 12.7 ml of 1 *M* KH_2PO_4 and 22.3 ml of 1 *M* H_3PO_4 into a 1000-ml volumetric flask, and made up to volume with distilled water. Buffer

solutions were always freshly prepared and filtered immediately before use to remove particles.

2.4.3. Determination of plasma samples

Untreated plasma (1 ml) was spiked with flunitrazepam and the metabolites mentioned above and 20 μl of appropriate internal standard (methylclonazepam at 10 μg/ml). After centrifugation, the spiked samples were placed into a glass injection vial and 50 μl was processed on-line as described above. Five injections were performed for each parameter.

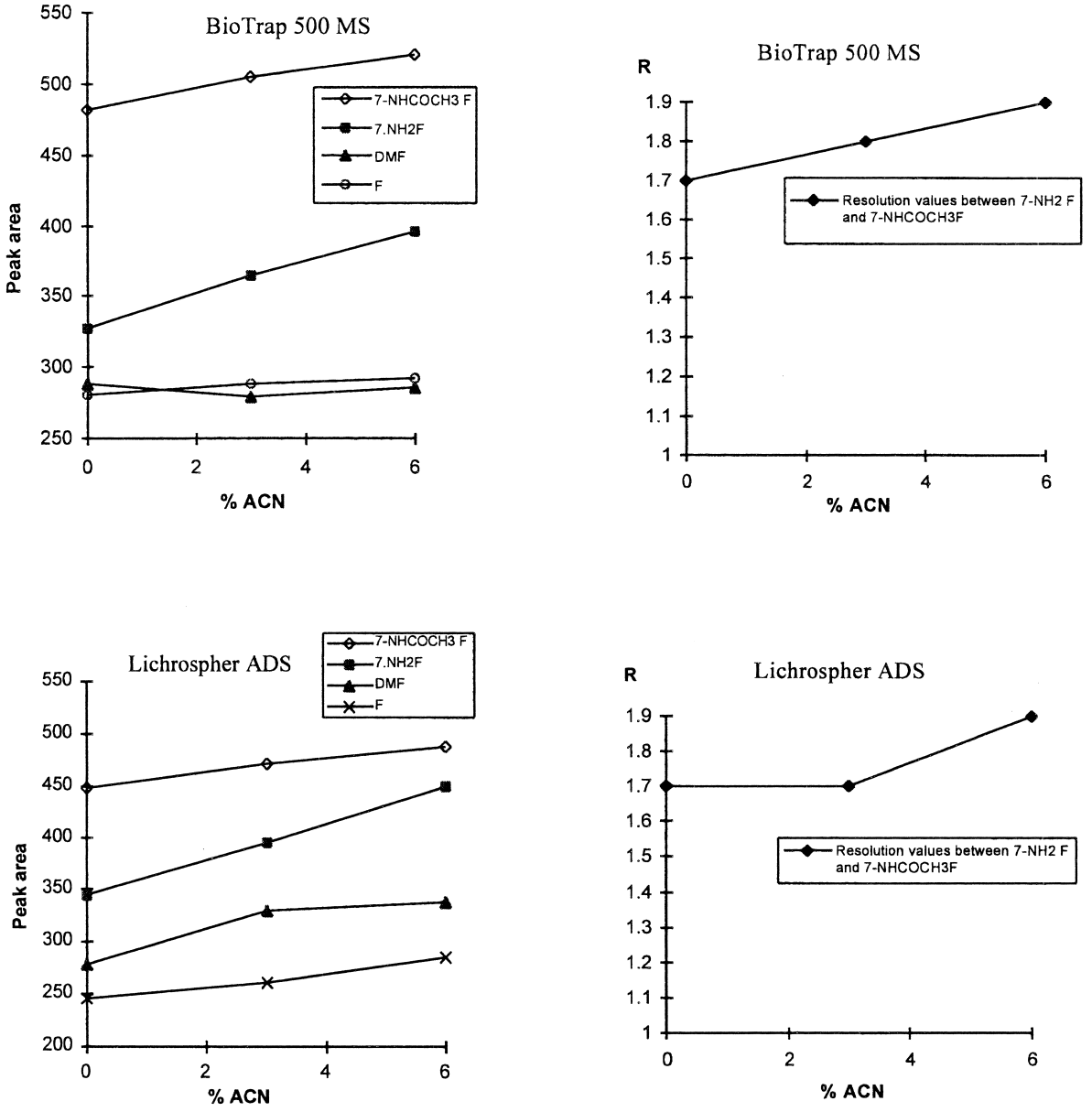

Fig. 3. Influence of acetonitrile percentage on chromatographic parameters.

2.4.4. Recovery

Recovery was calculated ($n=6$) by comparing the peak areas of flunitrazepam and 7-aminoflunitrazepam in spiked plasma samples at concentrations of 0.1 and 0.5 μg/ml, respectively, to the peak areas of a series of unextracted reference standards.

3. Results and discussion

The two automated methods proposed were optimized for flunitrazepam and its major metabolites: 7-aminoflunitrazepam, 7-acetamidoflunitrazepam, desmethylflunitrazepam in human plasma, using

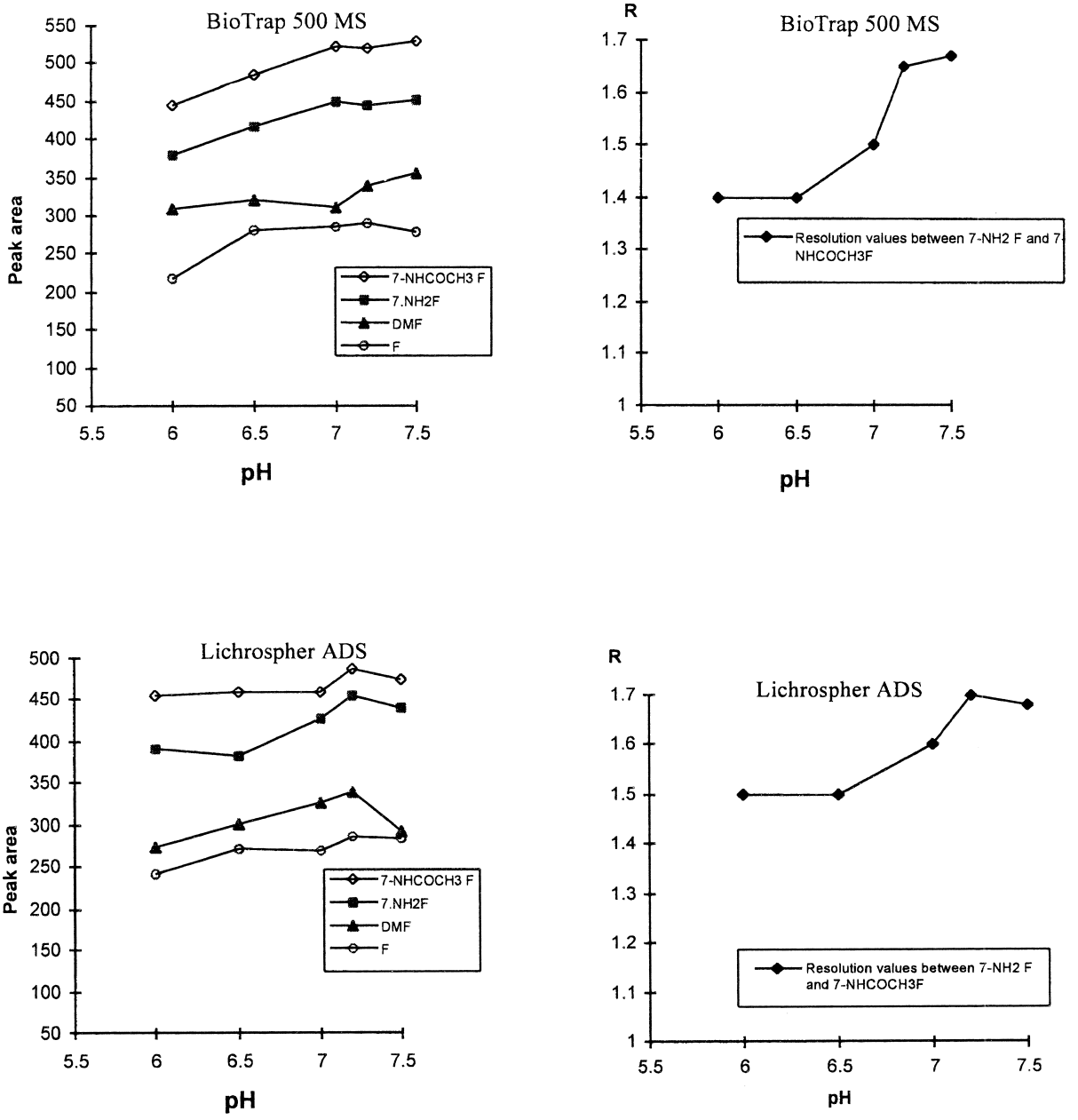

Fig. 4. Influence of buffer pH on chromatographic parameters.

methylclonazepam as internal standard. The structures of these compounds are given in Fig. 1.

We studied the influence of experimental conditions such as buffer pH, buffer concentration, acetonitrile (ACN) percentage, temperature and flow-rate on the following chromatography parameters: peak area values and the resolution values obtained for two flunitrazepam metabolites (7-NH_2F and 7-$NHCOCH_3$).

3.1. Acetonitrile percentage

Varying the acetonitrile percentage of the extraction mobile phase from 0 to 6% altered the peak area

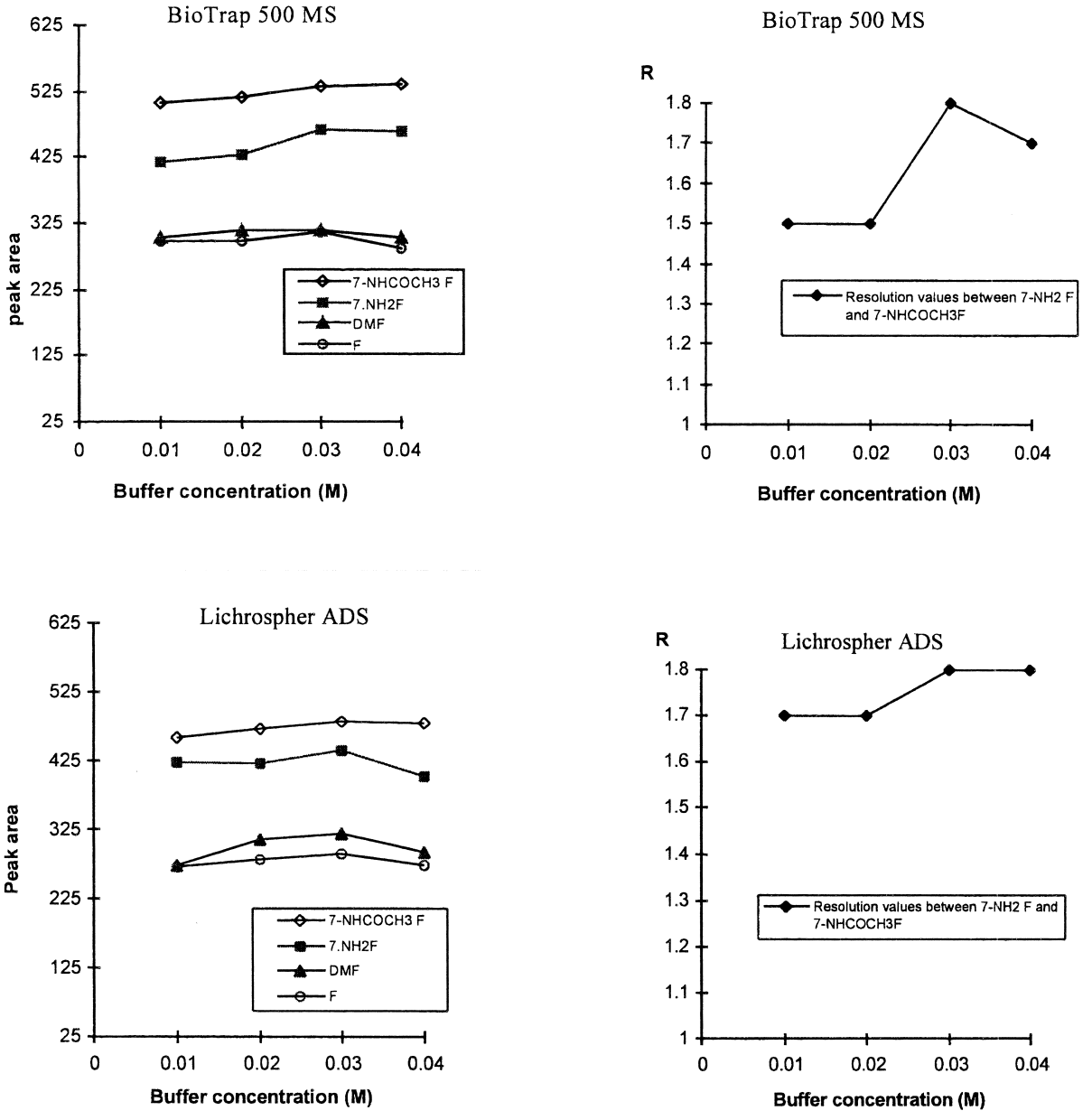

Fig. 5. Influence of buffer concentration on chromatographic parameters.

and the resolution of 7-NH_2-F and 7-$NHCOCH_3$-F. Increasing the percentage of organic solvent had a positive influence on the chromatographic parameters studied for the two extraction columns used (Fig. 3).

The preliminary tests concerning the compatibility between the extraction column and the extraction mobile phase showed that, with 8% ACN in the mobile phase, precipitation of plasma protein could occur in the column.

3.2. Buffer pH

Varying the pH of the extraction mobile phase between 6 and 7.5 significantly altered the peak area and the resolution values between the flunitrazepam metabolites. With the BioTrap 500 MS column, we observed a positive effect for the two chromatographic parameters studied. But the assays done with the LiChrospher ADS R18 column showed an increase followed by a decrease, at pH 7.5 for the peak area and resolution values (Fig. 4).

3.3. Buffer concentration

We examined the effect of varying the buffer concentration from 20 to 40 m*M*.

Fig. 5 showed that varying this parameter did not significantly alter peak area values, but that the resolution value between 7-NH_2-F and 7-$NHCOCH_3$-F was modified. The resolution value was altered to a greater extent by using the BioTrap 500 MS extraction column, but remained sufficient (R_s>1.6) at optimum conditions. The best chromatogram was achieved for the BioTrap 500 MS extraction column with 30 m*M* buffer concentration (Fig. 6).

3.4. Temperature

Varying the temperature between 10 and 60°C showed that the peak area was not significantly altered between 10 and 45°C. But we observed a decrease at 50°C for the two extraction columns. The resolution value was altered with the two extraction columns, but was significantly altered to a greater extent with the BioTrap 500 MS column, since we observed a stabilization between 35 and 45°C followed by a decrease at 50°C (Fig. 7). However, a stabilization was observed with LiChrospher RP-18 ADS between 20 and 35°C.

In order to avoid destruction of the external surface (protein, α_1-acid glycoprotein, for BioTrap 500 MS and of the hydrophilic electroneutral diol groups for LiChrospher RP-18 ADS) we chose to work at a temperature of 35°C.

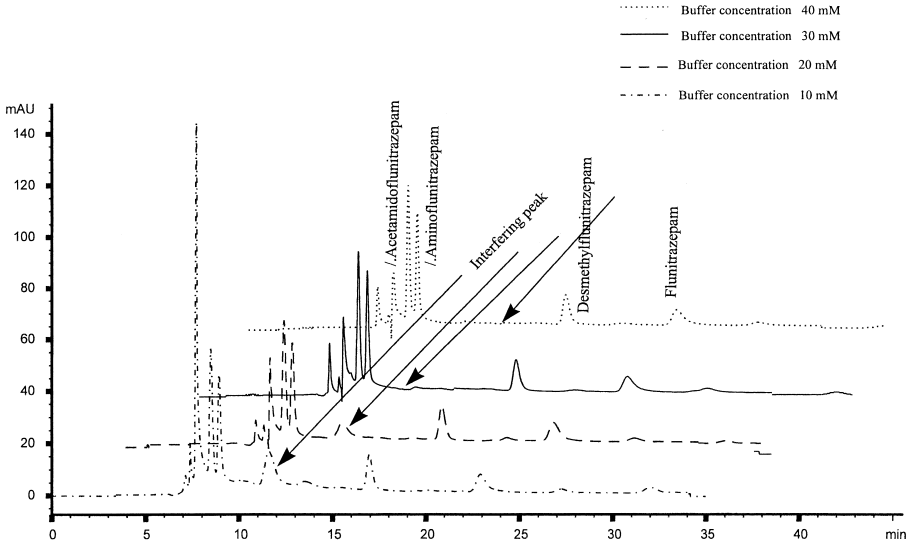

Fig. 6. Influence of buffer concentration on chromatographic trace of spiked plasma.

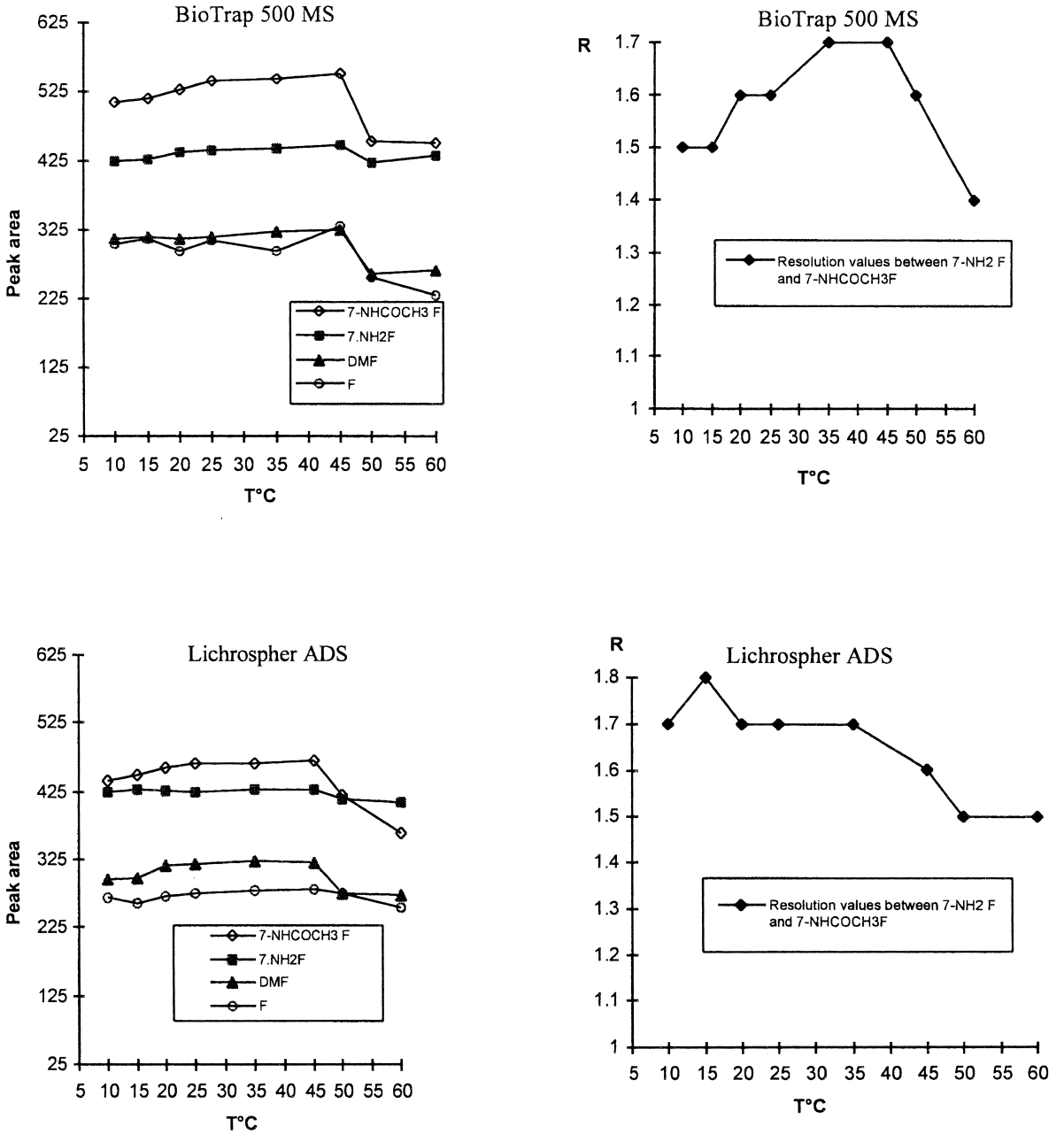

Fig. 7. Influence of temperature on chromatographic parameters.

3.5. Flow-rate

Variation of the flow-rate from 0.6 to 2 ml/min showed that the peak area and resolution values of 7-NH_2-F and 7-$NHCOCH_3$-F were significantly altered for the two extraction columns (Fig. 8). 7-NH_2-Fs' peak area was altered the most by using the BioTrap 500 MS extraction column, but resolution was not influenced with the LiChrospher ADS RP18 column. We observed a decrease at

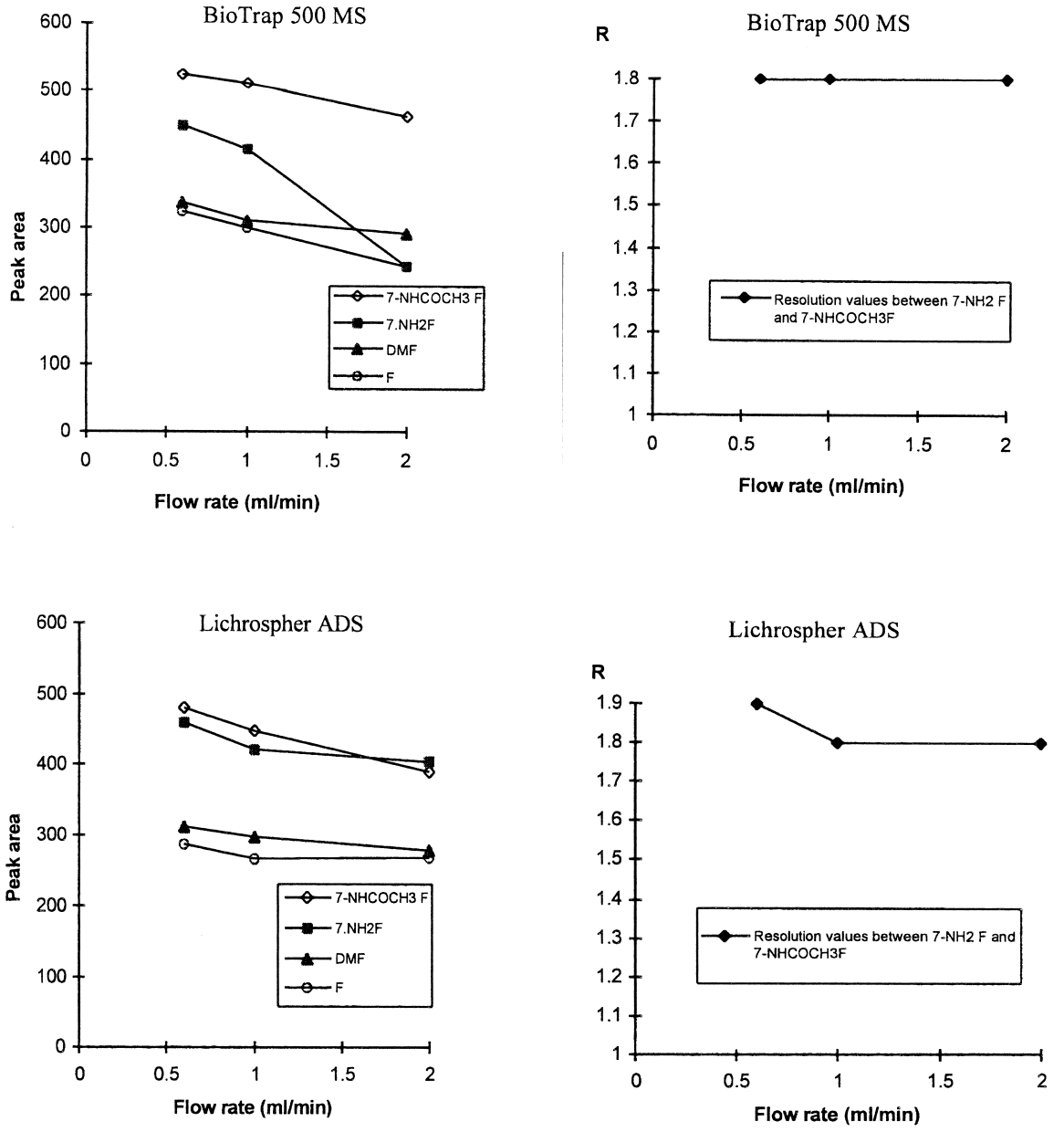

Fig. 8. Influence of flow-rate on chromatographic parameters.

1 ml/min followed by stabilization between 1 and 2 ml/min. This could be explained probably by the fact that at a high flow-rate the compound had no time to penetrate into the external surface pore in order to be retained and eluted later by the analytical mobile phase. This parameter seems less critical with the BioTrap 500 MS column than the LiChrospher ADS RP18 column.

Table 2
Data validation [1]

Regression data	Range (mg/l)	BioTrap 500MS				LiChrospher ADS RP18			
		Line	r	LOD (ng/ml)	LOQ (ng/ml)	Line	r	LOD (ng/ml)	LOQ (ng/ml)
Flunitrazepam	0.05–1.0	$y=0.2\cdot10^{-2}-0.42\cdot10^{-1}$	0.997	12	40	$y=1.9\cdot10^{-3}-10.5\cdot10^{-3}$	0.997	15	50
7-Aminoflunitrazepam	0.05–1.0	$y=0.14\cdot10^{-2}-0.71\cdot10^{-2}$	0.997	14	45	$y=1.4\cdot10^{-3}-9.4\cdot10^{-3}$	0.997	20	50

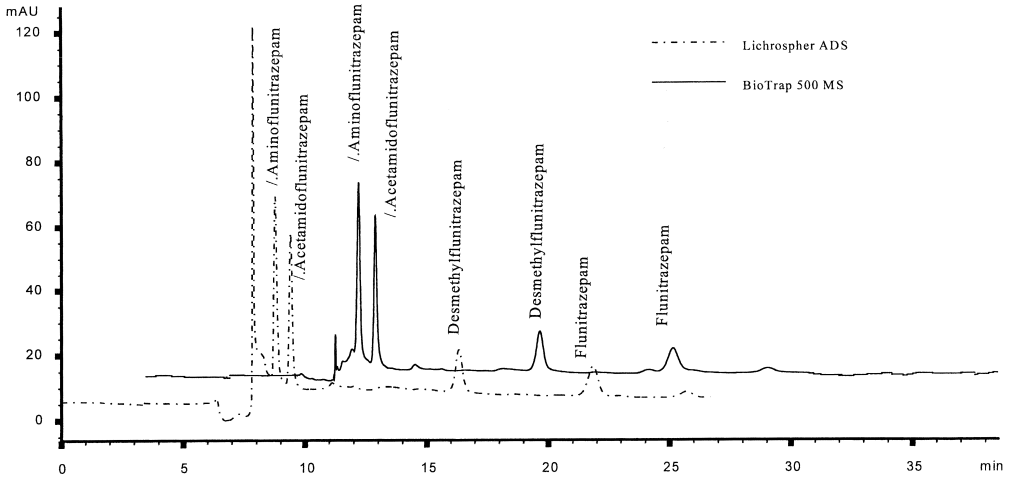

Fig. 9. Chromatogram trace of spiked plasma under optimized conditions for the two extraction columns.

3.6. Linearity, limits of detection and quantification

The standard curves for flunitrazepam and its major metabolite, 7-aminoflunitrazepam, were linear over the concentration range (0.05 to 1.0 μg/ml).

Coefficient of correlation was better than $r=0.997$ for the two extraction columns (Table 2). The LOQs were determined for concentrations with RSDs higher than 20%. As shown in Table 2, LOQ values obtained with BioTrap 500 MS, 40 ng/ml for flunitrazepam and 45 ng/ml for 7-aminoflunit-

Table 3
Data validation [11]

Regression data	Concentration (mg/l)	BioTrap 500MS			Lichrospher ADS R18		
		Repeatability (RSD, %)	Reproducibility (RSD, %)	Recovery (%)	Repeatability (RSD, %)	Reproducibility (RSD, %)	Recovery (%)
Flunitrazepam	0.05	12.3	20.2	91.0	–	15.6	90.0
	0.5	1.1	2.3	112.0	4.6	2.2	96.0
7-Aminoflunitrazepam	0.05	15.2	13.9	92.0	22.0	17.4	90.0
	0.5	0.9	1.5	101.0	3.5	2.9	94.0

razepam, were lower than the values obtained with LiChrospher ADS RP18 50 ng/ml for flunitrazepam and 50 ng/ml for 7-aminoflunitrazepam.

Fig. 9 shows a chromatogram trace of spiked plasma under optimized conditions.

3.7. Repeatability, reproducibility and recovery

Precision and reproducibility are expressed as RSDs. As shown in Table 3, the repeatability values obtained with BioTrap 500 MS were better than the values obtained with LiChrospher ADS RP18. The reproducibility values obtained with BioTrap 500 MS were lower than the values obtained with LiChrospher ADS RP18, except for flunitrazepam at a low concentration. Results are detailed in Table 3.

The recoveries obtained with BioTrap 500 MS were better than the recoveries obtained with LiChrospher ADS RP18. Table 3 summarizes the recoveries of flunitrazepam and its major metabolite, 7-aminoflunitrazepam.

4. Conclusion

The difference between the two extraction columns tested (see Figs. 9 and 10) can probably be explained by the difference in the composition of the external surface (Fig. 10). The external surface of LiChrospher ADS RP18 is made of hydrophilic electroneutral diol groups and the external surface of BioTrap 500 MS is made of a stable protein, α_1-acid

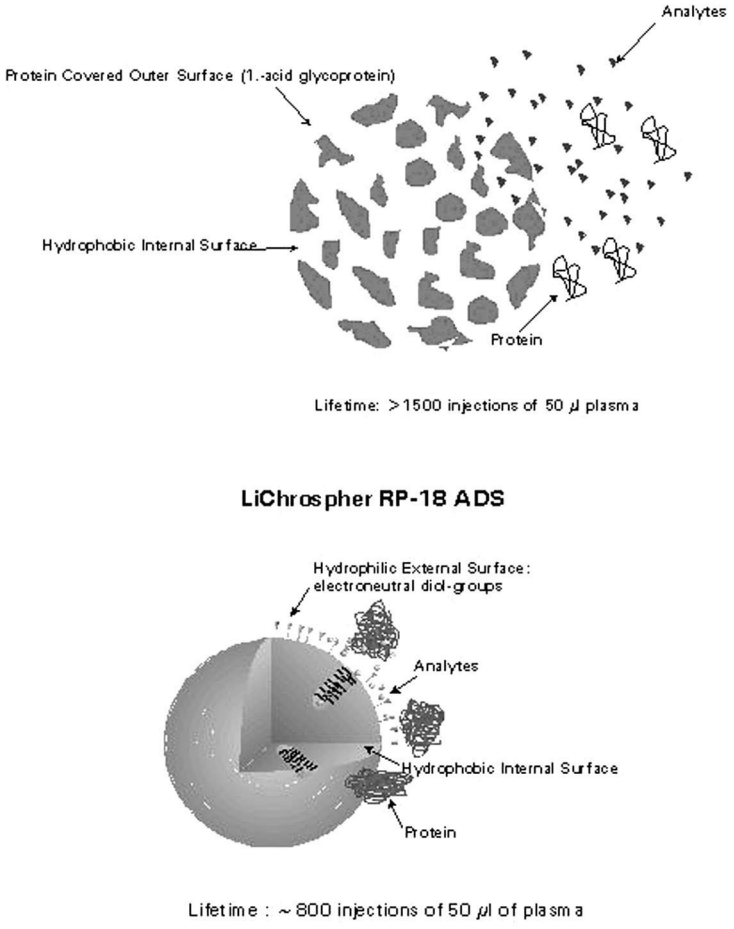

Fig. 10. Structural illustration of a BioTrap 500 MS and LiChrospher RP-18 ADS. Adapted from Refs. [16,17].

glycoprotein. Our experiments showed that the lifetime of BioTrap 500 MS is >1500 injections of 50 μl of plasma. However, the lifetime of LiChrospher ADS RP18 is still about 800 injections of 50 μl of plasma.

BioTrap 500 MS is pH stable from 2 to 11 and allows the extraction of basic, neutral and acidic drugs, but LiChrospher RP18 ADS, which is pH stable from 2 to 7.5, only affords the extraction of neutral and acidic drugs.

Therefore, the BioTrap 500 MS column seems more suitable for a general screening.

HPLC column-switching offers significant advantages in the determination of flunitrazepam and its major metabolites in plasma.

The technique is rapid, sensitive, and the measured LOQ allows one to determine concentrations in toxicological cases.

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