Journal of Chromatography, 474 (1989) 59–68
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 378

APPLICABILITY OF CONTINUOUS-FLOW FAST ATOM BOMBARDMENT LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY IN BIOANALYSIS

DEXTROMETHORPHAN IN PLASMA

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SUMMARY

Continuous-flow fast atom bombardment (CF-FAB) is an interface for combined liquid chromatography–mass spectrometry using FAB as the ionization method. The applicability of CF-FAB for quantitative bioanalysis was studied for a model compound, dextromethorphan, in plasma samples using conventional high-performance liquid chromatography. The flow-rate reduction was achieved either by splitting or by the phase-system switching approach. The features of both systems are discussed.

INTRODUCTION

In recent years various liquid chromatography–mass spectrometry (LC–MS) interfaces have been developed and successfully applied in bioanalysis[1]. Despite the fast progress, a number of difficulties still exist in daily practice strongly depending on the interface type used. Many of the interfaces have limitations in the handling of very polar and thermally labile compounds frequently encountered in bioanalysis. In this respect the recently introduced method of continuous-flow fast atom bombardment (CF-FAB)[2,3] has opened up new possibilities. The CF-FAB technique has been used for the analysis of peptides[3–11], dansylated amino acids[6], antibiotics[7,12], bile acids[2,13] and oligosaccharides[14].

CF-FAB has so far mainly been applied in qualitative analysis using either flow injection analysis[2–4,6–9,12–14] or micro high-performance liquid chromatography

0021-9673/89/$03.50

(HPLC)[2,5–14]. Advantages of CF-FAB over conventional FAB are the fact that ion-suppression effects are reduced considerably and the determination limits are improved[3,4]. Therefore, it is interesting to explore the potential of CF-FAB in quantitative analysis.

Dextromethorphan, (+)-3-methoxy-17-methylmorphinan, was selected as the model compound, because of its solubility and high stability in water and its good FAB sensitivity, especially in acidic solutions. Methods for the determination of dextromethorphan and its metabolites in human plasma[15,16] and urine[15] have been reported using conventional HPLC with fluorescence detection. Typical concentration levels of dextromethorphan in plasma are less than 10 ng/ml after a single dose of 60 mg of dextromethorphan hydrobromide[15,17]. The applicability of the CF-FAB method for quantitative bioanalysis was studied by analysing dextromethorphan in plasma. Because conventional HPLC is used in most bioanalytical studies, the possibilities of conventional HPLC coupled with CF-FAB were investigated. Flow-rate reduction is achieved by using a splitter between the HPLC apparatus and the CF-FAB interface.

EXPERIMENTAL

Equipment

The two different experimental set-ups that have been used are referred in the text as system I and system II. The most important differences between those two systems are summarized in Table I.

The HPLC system consisted of either a Pharmacia P-3500 pump (Uppsala, Sweden) or an LKB 2150 pump (Bromma, Sweden), a Rheodyne 7125 injector (20 μl, Berkeley, CA, U.S.A.) and a Techopak reversed-phase C_{18} column (15 cm × 3.9 mm I.D., packed with 10 μm material, HPLC Technology, Cheshire, U.K.). The temperature of the column and the stainless steel capillaries was kept at 56°C in order to reduce the viscosity of the mobile phase. The column effluent was directed to a splitter (see below), which was connected to a Finnigan MAT prototype CF-FAB

TABLE I

SYSTEM DESCRIPTIONS

	System I	*System II*
Pump	Pharmacia	LKB
Mobile phase[a]	TFA–Gly–ACN–H_2O	TFA–Gly–ACN–H_2O
Composition (w/w%)	0.25-10-30-59.75	0.25-9.9-29.8-60.05
HPLC flow-rate	1.0 ml/min	1.2 ml/min
Split capillary	19 cm × 200 μm I.D.	44 cm × 150 μm I.D.
CF-FAB capillary	85 cm × 75 μm I.D.	75 cm × 75 μm I.D.
Split ratio	1:200	1:170
Split flow-rate	5 μl/min	7 μl/min
Mass spectrometer	MAT 8200	MAT 90
Data system	SS300	ICIS
Source temperature	40°C	60°C
Source pressure	*ca.* 0.1 Pa	*ca.* 0.01 Pa

[a] TFA = trifluoroacetic acid; Gly = glycerol; ACN = acetonitrile.

probe (Bremen, F.R.G.) with a stainless steel target. The CF-FAB probe was fitted on to either a Finnigan MAT 8200 or Finnigan MAT 90 double-focusing mass spectrometer, operated at 3 kV and 5 kV, respectively. Both instruments were equipped with a FAB gun (Ion Tech, Teddington, U.K.) using xenon and producing a beam of neutral atoms of 6 kV energy.

In the Finnigan MAT 90 instrument (system II) an exchangeable ion volume with a wick was applied. The wick was prepared from compressed paper and positioned at the bottom of the ion volume. Additional vacuum pumping at the ion source housing was obtained by a liquid nitrogen trap. In the Finnigan MAT 8200 instrument (system I) there was no possibility to use an exchangeable ion volume, a wick or a cold trap.

A schematic diagram of the laboratory-made splitter is given in Fig. 1. The HPLC column was connected to a zero dead volume stainless-steel union-T (TEE.020″, Upchurch Scientific, Washington, U.S.A.) by means of a short stainless-steel capillary (1/16 in. O.D., 0.5 mm I.D.) in which the 75 μm I.D. fused-silica capillary (SGE, Melbourne, Australia) going to the CF-FAB probe is inserted to a position as close as possible to the column end. The split ratio was determined by the length and internal diameter of the fused-silica capillaries, the actual dimensions of which are given in Table I. The flow-rates stated in Table I were measured by weighing the effluents.

Reagents

Dextromethorphan hydrobromide was obtained from the Research Center of Orion Pharmaceutica (Espoo, Finland). Trifluoroacetic acid and hexane were purchased from E, Merck (Darmstadt, F.R.G.). Glycerol (98% chem. pure) was supplied by Lamers & Pleuger ('s Hertogenbosch, The Netherlands), acetonitrile (ChromAR) by Promochem (Wesel, F.R.G.), sodium carbonate (AnalaR) by BDH (Poole, U.K.) and triethylamine by Pierce (Rockford, IL, U.S.A.). Water was distilled before use.

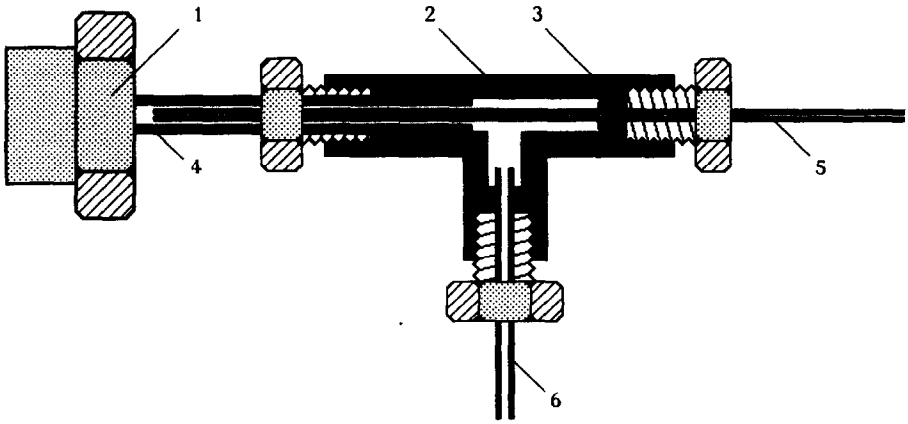

Fig. 1. Schematic diagram of the CF-FAB splitter. 1 = LC column; 2 = union-T; 3 = vespel ferrule; 4 = stainless-steel capillary; 5 = CF-FAB capillary (75 μm I.D.); 6 = split capillary (200 μm I.D.).

The mobile phase was prepared daily in order to avoid bacterial growth and mould formation. Before use it was filtered and degassed ultrasonically. The mobile phase reservoir was flushed with helium during the experiments.

Sample preparation

Stock solutions containing 15 mg of dextromethorphan hydrobromide were prepared in 10 ml of water. Plasma samples containing 0.11, 0.22, 0.44, 0.88, 1.75, 3.5, 7.0, 14.0 and 28.0 μg/ml of dextromethorphan were prepared by adding the appropriate amount of dextromethorphan in 50 μl of water to 2.0 ml of blank human plasma. A mixture of 0.5 ml of saturated sodium carbonate in water and 5 ml of 0.1% (v/v) of triethylamine in hexane was added to the plasma. The tubes were gently shaken on a horizontal shaker for 20 min. After centrifugation for 5 min at 1000 *g*, 4.0 ml of the organic layer were separated and evaporated to dryness in a gentle stream of helium at 40°C. The residue was dissolved in 50 μl of the mobile phase. An aliquot of 20 μl was injected on to the HPLC column. This procedure is a modification of the method described by East and Dye[15]. Recoveries of *ca.* 96% have been reported for the original procedure[15].

RESULTS AND DISCUSSION

System description

In most bioanalytical applications conventional bore columns are used. With several LC–MS interfaces, miniaturized HPLC columns are applied to achieve flow-rate reduction in order to avoid large split ratios. It is important to realize that no significant improvements in concentration detection limits are to be expected from the use of microbore columns since the advantage of reducing the split ratio is balanced by the necessary reduction in injection volume[18].

In the case of CF-FAB the optimum flow-rate is *ca.* 5 μl/min. For coupling with conventional HPLC columns with typical flow-rates of 1 ml/min a considerable split of 1:200 is necessary. For microbore columns the typical flow-rates are about an order of magnitude lower, resulting in a split of 1:10, but also the maximum injection volume is diminished by at least a factor of 10. Therefore, similar concentration detection limits will be obtained in both cases. Only if the sample size is limited does miniaturization become attractive. In most cases conventional HPLC columns are also preferred in bioanalysis for their high sample loadability. Moreover, miniaturization of sample pretreatment procedures is rather difficult. Because of these arguments, coupling of conventional HPLC with CF-FAB was investigated for bioanalysis and a splitter was designed for this purpose.

Several types of flow splitter for use in CF-FAB experiments have been described: a commercially available pneumatic splitter[19] and a splitter with a needle valve[11]. Fig. 1 gives a schematic diagram of a low-cost laboratory-made splitting device, used in the experiments described here. With certain precautions this splitter is very easy to handle. The split ratio is determined by the length and the internal diameter of the fused-silica capillaries. It is important to wash the splitter with water daily after use to prevent bacterial growth and mould formation. No additional peak broadening due to the splitter has been observed. Day-to-day variation of the split ratio is smaller than 10%. When one of the fused-silica capillaries accidentally becomes clogged it can be easily replaced.

In FAB, glycerol is generally used as the matrix. In CF-FAB glycerol can be added to the mobile phase of the HPLC system either in pre-column[1–10,12–14] or post-column[11] mode. In the pre-column mode glycerol is mixed with the mobile phase. Unfortunately, a mobile phase containing 10% of glycerol has a much higher viscosity than the corresponding conventional mobile phase, resulting in an increased pressure drop over the column. A higher temperature of the mobile phase is needed to decrease the viscosity. In that case it must be taken into account that the increased temperature and the polarity of glycerol influences the chromatography. When glycerol is added in post-column mode, the chromatography is not affected. However, adequate post-column mixing of pure glycerol with the column effluent is rather difficult. Post-column addition of glycerol can only be achieved by adding a solvent mixture containing glycerol, resulting in dilution and peak broadening. In this study pre-column addition of glycerol was used. The increased temperature (56°C) not only decreased the viscosity of the mobile phase but also improved the chromatography of dextromethorphan.

Analysis of plasma samples

The applicability of the CF-FAB method for quantitative bioanalysis was studied by determining dextromethorphan in plasma samples. Total ion current chromatograms of a blank plasma sample and a spiked plasma sample are shown in Fig. 2. The sample has been spiked with 28 μg/ml of dextromethorphan, which corresponds to 85 ng introduced into the mass spectrometer. No interfering background peaks were observed in the chromatogram of the blank plasma sample. The CF-FAB LC–MS spectrum of dextromethorphan taken from the chromatogram in Fig. 2B is given in Fig. 3 without background subtraction. Compared with conventional FAB the background of glycerol clusters is considerably reduced. Only the protonated glycerol clusters at m/z 93 and 185 can be seen. The protonated molecule of dextromethorphan at m/z 272 and the protonated glycerol cluster at m/z 277, although of very low abundance, are used in selected-ion monitoring.

Analytical data

The within-run precision of the method was studied by analysing spiked plasma samples containing 1.8 or 28 μg/ml of dextromethorphan using a stainless steel target. Table II summarizes the coefficients of variation (C.V.) of the peak areas and peak heights. The data in Table II were acquired under various conditions. With system I an untreated stainless steel target was used, whereas with system II results are given for an acid-treated stainless steel target. No significant differences in the precision of the method were observed at the two plasma levels investigated. The acid treatment roughens the surface of the target and hence enhances the wettability[20]. The use of the wick together with the acid-treated target improves the liquid film properties of the target, resulting in an improved stability of the baseline[20] and precision of the method. The cold trap, which is used to increase the pumping capacity of the mass spectrometer at the ion source housing, also improves the baseline stability, partly because pressure fluctuations influence the stability of the FAB gun. Application of the wick, the acid-treated stainless-steel target and cold trap improves the C.V. of the peak heights and areas. A typical series of peaks for plasma samples containing 1.75 μg/ml of dextromethorphan as obtained with the latter system is given in Fig. 4. The glycerol

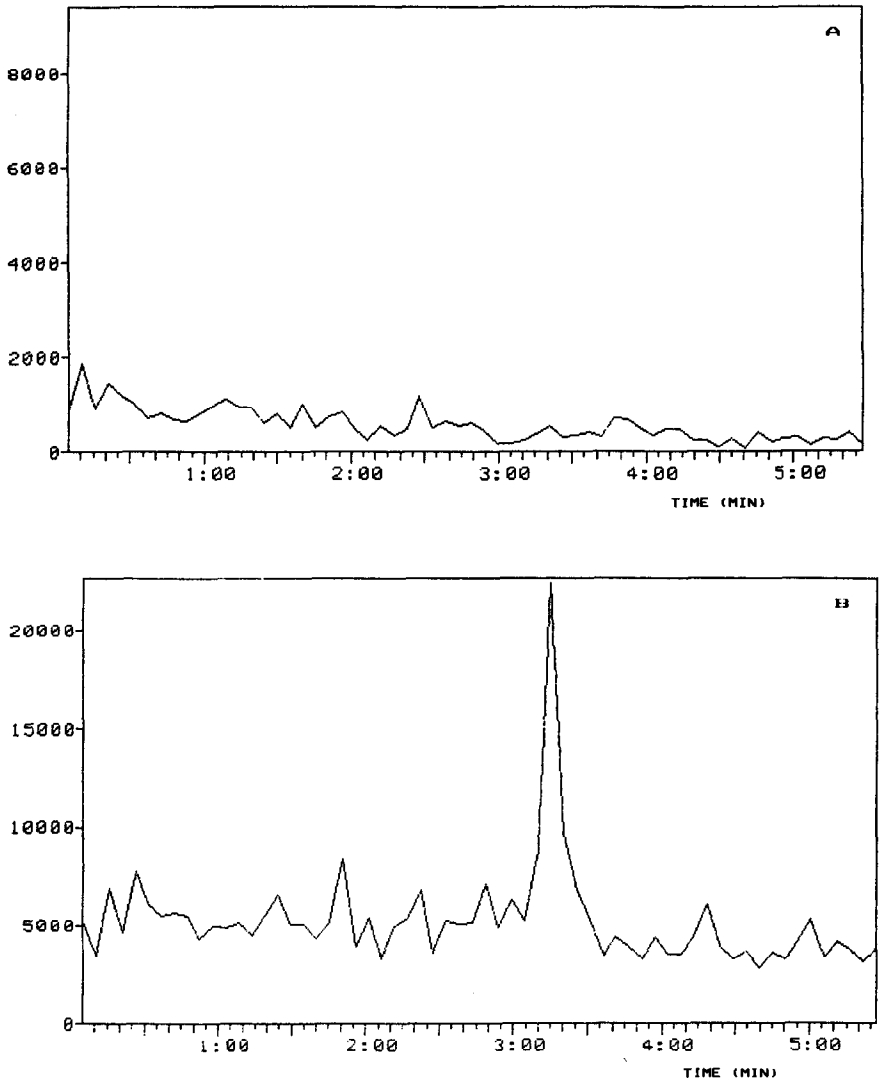

Fig. 2. Total ion current chromatograms of human plasma extracts. (A) Blank plasma sample. (B) Spiked plasma sample containing 28 μg/ml of dextromethorphan (retention time 3 min 15 s). System I (see Table I) with an untreated stainless-steel target.

background was monitored to check the conditions of the system during the run. By using a labelled internal standard the precision of the method can be improved further, and the effect of occasional instabilities as well as effects of the changes in the conditions (*e.g.* properties of the liquid film, pressure and temperature of the ion source) are avoided.

The recovery of the extraction method was found to be *ca.* 80% at the 1.76 μg/ml level by comparing the peak areas as obtained from extracted spiked plasma samples and extracted blank samples, to which dextromethorphan was added after extraction.

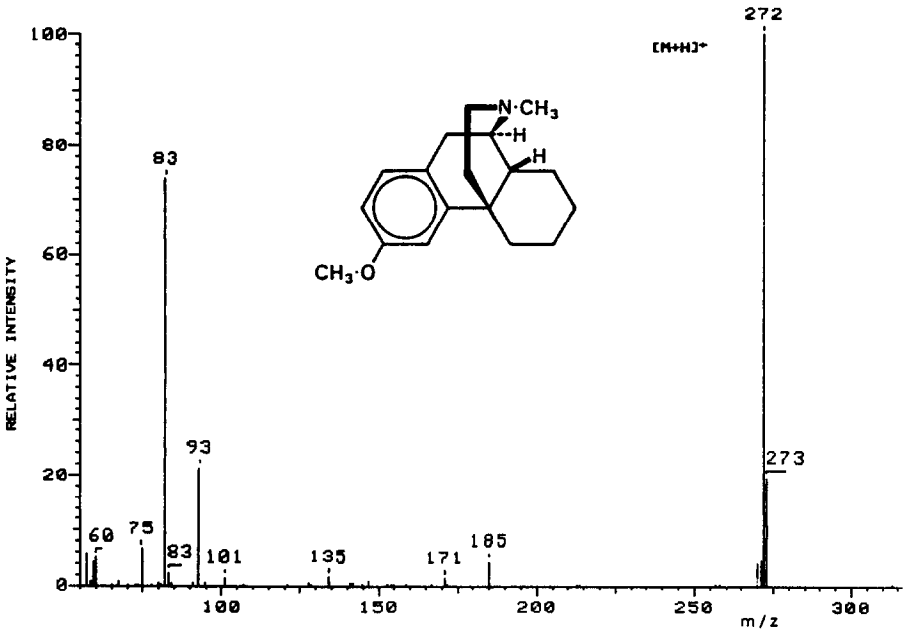

Fig. 3. The LC-FAB mass spectrum of dextromethorphan (MW 271) taken from the analysis shown in Fig. 2B. System I (see Table I) with an untreated stainless-steel target.

The linearity (peak area *vs.* concentration) of the method was checked in the range 0.11–28.0 μg/ml dextromethorphan in plasma. The calibration curve is linear (correlation coefficient, $r = 0.995$) over the range 1.75–14.0 μg/ml corresponding to 7–53 ng of dextromethorphan introduced into the mass spectrometer. The calibration samples containing 0.11–0.88 μg/ml were measured separately. Day-to-day reproducibility of the absolute peak areas is not very good: peak-area ratios up to 10 were found between two successive days. Daily calibration is therefore necessary, and an isotopically labelled internal standard would certainly improve the overall performance.

The determination limit with a signal-to-noise ratio of 10 in selected-ion monitoring is *ca.* 1 μg/ml, when system I is used. With system II the determination limit is 110 ng/ml, corresponding to 400 pg introduced into the spectrometer.

TABLE II

WITHIN-RUN PRECISION OF THE PEAK AREAS AND PEAK HEIGHTS FOR DETERMINATION OF DEXTROMETHORPHAN IN HUMAN PLASMA BY MEANS OF CF-FAB LC–MS WITH A STAINLESS-STEEL TARGET

Target	*Conc. (μg/ml)*	*N*	*C.V. (%)*		*Conditions*
			Area	*Height*	
Untreated	28	4	22	29	System I
	1.8	5	24	28	System I
Acid-treated	1.8	6	15	16	System II

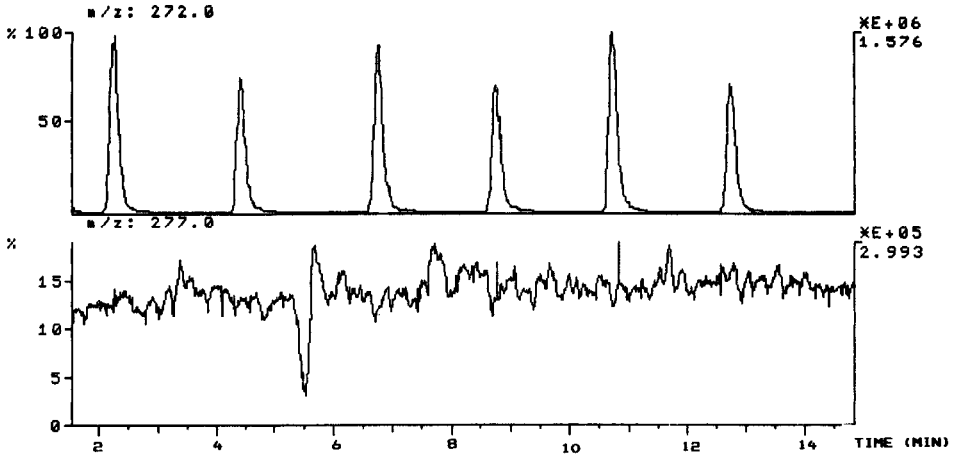

Fig. 4. Multiple ion monitoring chromatograms of the protonated dextromethorphan (upper trace, *m/z* 272) and the $[3M + H]^+$ of glycerol (lower trace, *m/z* 277). Plasma spiked with 1.75 μg/ml of dextromethorphan, corresponding to 7 ng into the spectrometer. System II (see Table I) with an acid-treated stainless-steel target.

Phase-system switching

The mandatory splitting ratio of 1 to 200 considerably restricts the determination limits of the method. Plasma levels of dextromethorphan below 10 ng/ml, which are found in practice, cannot be determined. The use of microbore LC columns in stead of conventional columns gives no significant advantages in this respect, because the lower injection volume balances the reduced splitting ratio. Preliminary experiments indicate that splitting can be avoided completely by the use of the phase-system switching (PSS) approach[21,22]. This approach, which is based on valve-switching techniques, was originally designed for solving mobile phase incompatibilities in target compound analysis with LC–MS, but it has several additional features, such as the

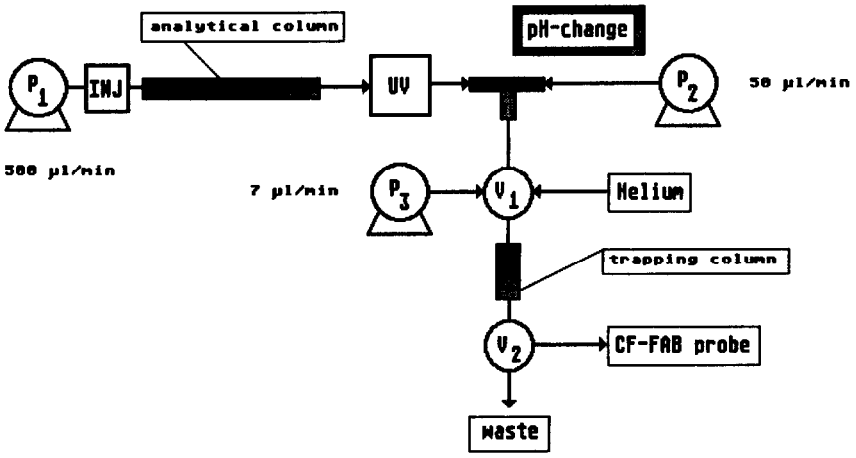

Fig. 5. Schematic diagram of the phase-system switching.

possibility of peak compression and of changing the flow-rate into the spectrometer. In PSS the compound of interest is trapped after the analytical separation on to a short trapping column, from which after washing and drying of the column it is eluted in an appropriate solvent and at a flow-rate favourable to the LC–MS interface applied. The applicability of this approach has been demonstrated for both the moving-belt interface and the thermospray interface[21–23]. If the appropriate trapping column dimensions are chosen, the PSS approach can also be used for the flow-rate reduction necessary in CF-FAB and for the post-column addition of glycerol. In the PSS system illustrated schematically in Fig. 5, an RP-2 analytical column (100 mm × 3.0 mm I.D.) is used at a flow-rate of 1.0 ml/min in combination with a XAD-2 trapping column of 50 mm × 1.0 mm I.D. In order to make trapping of dextromethorphan possible, a pH change after the analytical column is necessary. The optimization of the conditions (the trapping column dimensions, the washing and drying steps and the minimization of the dead volume of the system) is presently under investigation and will be reported together with quantitative analysis of dextromethorphan with the PSS system.

CONCLUSION

The CF-FAB method can be used in quantitative bioanalysis of very polar and thermally labile compounds. The analytical performance of the method is sufficient for most bioanalytical applications concerning determination of plasma levels. A system equipped with a wick, a cold trap and surface-treated targets shows a better stability, which is reflected in the precision of the method. At present the quantitation levels are restricted by the mandatory splitting. However, preliminary results from the use of the phase-system switching approach, in which the necessary flow-rate reduction is realized without splitting, indicate that these problems can probably be solved as well.

ACKNOWLEDGEMENT

One of the authors (P.K.) thanks Orion Pharmaceutica and the Finnish Academy of Sciences for the support of this project.

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