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Determination of tacrine metabolites in microsomal incubate by high performance liquid chromatography–nuclear magnetic resonance/mass spectrometry with a column trapping system

Donghui Bao, Venkataraman Thanabal, William F. Pool *

PGRD, Ann Arbor Laboratories, 2800 Plymouth Rd, Ann Arbor, MI 48105, USA

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Abstract

A column trapping system has been incorporated into high performance liquid chromatography–nuclear magnetic resonance–mass spectrometry (HPLC–NMR–MS) to reduce data acquisition time of NMR experiments. The system uses a trapping column to capture analytes after the HPLC column and back flush trapped analyte to the flow cell of the NMR probe for detection. A dilution solvent is mixed with eluent from HPLC column to reduce the influence of the organic content in the mobile phase before column trapping. The trapping column is also coupled with a mass spectrometer (MS) to get complementary MS data on the same peak. Studies on 1-hydroxylated 9-amino-1,2,3,4-tetrahydro-acridine (1-OH tacrine), indomethacin and testosterone with the column trapping system showed good recovery of analytes and over 3-fold mean increase in UV–VIS signal intensity. The time saving on NMR experiments with the column trapping system was demonstrated by the analysis of dog microsomal incubate with tacrine. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Column trapping; Column switching; Drug metabolism; HPLC–NMR–MS; NMR; MS

1. Introduction

Directly coupled high performance liquid chromatography (HPLC)–nuclear magnetic resonance (NMR) [1–4] has been increasingly used on studies of drug metabolism [5–10], natural products [11,12]and environmental samples [13,14] in recent years. Although, it combines the separation power of HPLC and rich structural information from NMR, liquid chromatography (LC)–NMR suffers from the low sensitivity of NMR compared with that of other detectors, like UV, fluorescence and mass spectrometry (MS). Stop-flow NMR acquisition mode is widely used, as more scans can be acquired on the analytes of interest to improve signal to noise ratio and perform more time-consuming two-dimensional NMR experiments [15]. Whereas, continuous-flow detection mode is only used where large amount of samples

* Corresponding author. Fax: +1-734-622-1459.
E-mail address: william.pool@pfizer.com (W.F. Pool).

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are available [2,10]. However, low analyte concentrations in many biological samples require long acquisition time or off-line concentration [16]. For example, over 10 h instrument time was required to acquire a simple 1-D spectrum of a fosphenytoin metabolite isolated from serum samples [17].

This paper describes an on-line column trapping method to concentrate HPLC elution peak before transferring it to the NMR probe. The NMR flow cells have volumes between 60 and 200 μl to compromise chromatography and NMR detection and magnetic field homogeneity [1,2]. These volumes are smaller than the volumes of analytical or semi-preparative HPLC peaks, especially those late eluting analytes, so only the fraction at peak apex is used to generate NMR signals. Our system used two columns—an analytical column for separation of analytes and a trapping column for on-line concentration. After trapping, a high organic content solvent, e.g. 50% acetonitrile/50% water, was used to back flush the analyte to NMR probe. The back-flushed peaks had small peak volumes due to the lack of diffusion on the trapping column. Therefore, the amount of sample available for detection is maximized. Additionally, 1/20 split of eluent from trapping column was send to MS. The complementary structural information derived from NMR and MS at the same run removed the ambiguity of the data sets from separate HPLC runs. Similar approaches to our LC–NMR–MS method have been developed for traditional NMR [18] and LC–NMR [19]. Three drugs, 1-OH tacrine, indomethacin, testosterone (Fig. 1), and a dog microsomal incubate with tacrine were used to examine the sensitivity improvement on UV–VIS and NMR signals.

2. Experimental

2.1. Chemicals

Indomethacin and testosterone were purchased from Sigma Chemical Co. (St. Louis, MO). Trifluoroacetic acetic (TFA) was purchased from Pierce (Rockford, IL). Deuterium oxide (D, 99.8%) and acetonitrile-d3 (D, 97–97%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Tacrine and 1-OH-tacrine

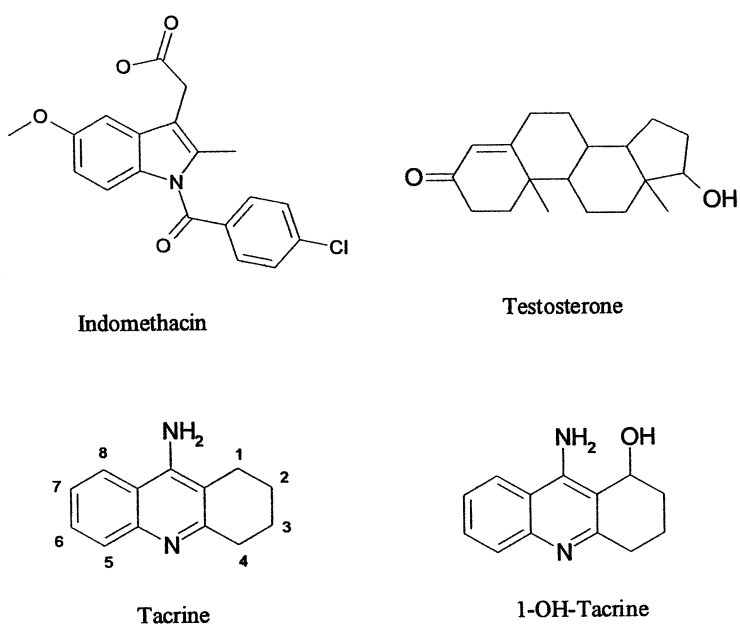

Fig. 1. Chemical structures of indomethacin, testosterone, tacrine and 1-OH-tacrine.

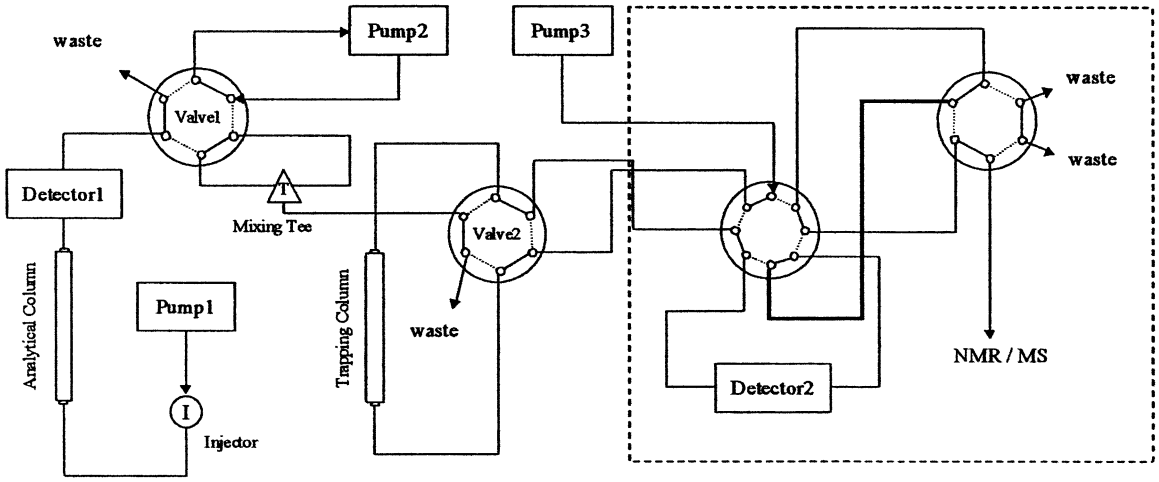

Fig. 2. Schematic representation of the column trapping system operated in the back-flush mode, resulting in transfer of the concentrate to NMR probe. The NMR spectrometer and analyte collector (Varian Inc.), surrounded by dashed lines, is in on-flow mode. See text for detailed description.

were obtained from Pfizer. All other solvents were of HPLC grade.

2.2. Microsomal incubation

Each microsomal incubate (6 ml) was prepared by mixing 1.0 mg protein per ml of dog liver microsomes D1000 (Xenotech, LLC, Kansas City, KS), 2.0 mM $MgCl_2$, 1.0 mM EDTA, 0.2 mM tacrine and 1.0 mM NADPH in 50 mM, potassium phosphate buffer, pH 7.4. The reaction mixture was incubated at 37 °C for 2 h. The reaction was stopped by addition of 1.2 ml of acetonitrile and centrifuged at $16\,000 \times g$ for 30 min. The supernatant was lyophilized and re-dissolved in 300 μl D_2O, then centrifuged at $16\,000 \times g$ for 15 min to remove insoluble particles. The concentrated microsomal incubate was adjusted to a final volume of 300 μl and stored at 4 °C before analysis.

2.3. Apparatus

The HPLC system (Fig. 2) consisted of three high pressure pumps (pump1, Varian 9012, Varian Inc., pump 2, model 590 and pump 3, model 6000A, Waters Inc, Milford, MA), two variable wavelength UV–VIS detectors (Varian 9050, Varian Inc., Palo Alto, CA) and two six-port switching valves (PR 700-100-02, Rheodyne, L.P., Rohnert Park, CA) connected to the peak sense relay port of detector 1. The system was controlled by Star Chromatography Workstation and Cascade software (Varian Inc). The HPLC columns included a Zorbax Rx-C18 (150×4.6 mm I.D.) as the analytical column, and a 5μ 100A Magic C18 Bullet column (Michrom BioResources, Inc. Auburn, CA) as the trapping column. Analytes eluted from the trapping column were analyzed by NMR and MS as described below.

2.4. HPLC conditions

The mobile phase for the analytical column consisted of acetonitrile and 0.05 M ammonium formate (pH 3.5) for 1-OH-tacrine, indomethacin and testosterone, while for the microsomal incubate acetonitrile and 0.1% TFA in D_2O was employed for the separation of metabolites of tacrine. Components were eluted with a linear gradient of 5–20% acetonitrile over 40 min for 1-OH-tacrine standard, 40–55% acetonitrile over 40 min for indomethacin, 30–43% acetonitrile over 40 min for testosterone and 4–15% acetonitrile over 60 min for microsomal incubate, at a

flow rate of 0.5 ml/min. The dilution solvent (from pump 2) for the trapping column was D_2O at flow rates of 0.5 ml/min. The back flush solvent (from pump 3) consisted of 50% acetonitrile-d3 and 50% D_2O at a flow rate of 0.5 ml/min. The injection volume was 100 μl of 10 μg/ml 1-OH-tacrine, indomethacin or testosterone in 50 mM ammonium formate 20% acetonitrile, or 100 μl of reconstituted microsomal incubate extraction (Section 2.2). The analytes were monitored at wavelengths of 320, 266 and 260 nm for 1-OH-tacrine, indomethacin and testosterone, respectively. Operations were carried out at ambient temperature.

2.5. NMR conditions

^{1}H-NMR spectra were obtained on a Varian Inova-600 spectrometer equipped with a 120 μl microflow probe at 25 °C. The spectrum width was 10 kHz, 18 K complex data points were acquired with a total repetition time of 2.9 s per scan. WET pulse sequence [20] was used to suppress solvent signals. Sixty four to 512 scans were acquired depending on sample concentration. A line broadening window function (LB = 2.0 Hz) was applied before Fourier transformation.

2.6. MS conditions

Waters ZMD single quadrupole mass spectrometer (Milford, MA) with an electrospray ionization source was used for the study. A flow splitter (LC Packings, San Francisco, CA) was placed after the trap column, permitting about 1/20 of the HPLC eluent to flow into the mass spectrometer (MS). The operating parameters of the MS were set as follows: capillary voltage 3.25 kV, RF lens 0.27 V, cone voltage 15 V, resolution 17 for both LM and HM, and source temperature 120 °C. The MS was operated in positive-ion mode with a scan dwell time of 1 s and range of 50–450 m/z.

3. Results and discussion

The column trapping system consisted of two HPLC columns coupled to the flow probe of the NMR spectrometer. The first column was run in reversed phase mode to separate analytes. Upon detection of analyte of interest by detector 1, valve 1 and valve 2 were switched to introduce the analyte fraction onto the trapping column, which was then switched back to back-flush elution solvent (50% acetonitrile-d3) to introduce concentrated peak into NMR probe. The LC–NMR was operated in stop-flow mode, and NMR data acquisition was started after analyte peak was parked in the flow cell of NMR probe. In order to retain target peak on the trapping column, 1:1 ratio of dilution solvent (D_2O) was mixed with eluent from analytical column to reduce the influence of acetonitrile in the mobile phase.

Fig. 3 shows the UV–VIS chromatograms of HPLC and the trapping system for indomethacin.

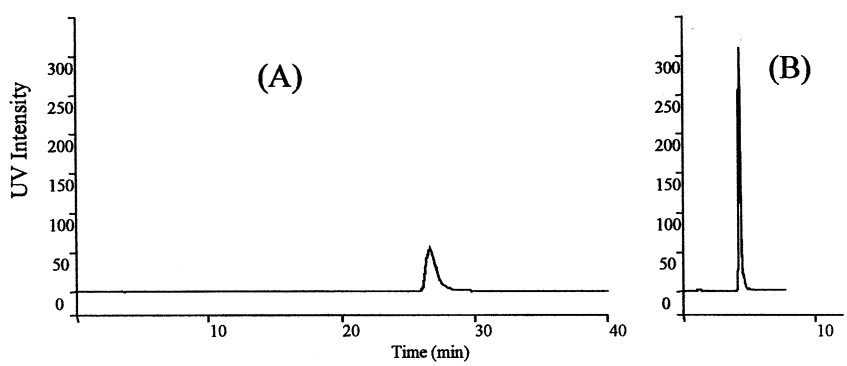

Fig. 3. Representative chromatograms of indomethacin on (A) HPLC column without column trapping and (B) back flushed from trapping column.

Table 1
Peak intensity ratios and recoveries, determined by comparison of peak areas, of three representative compounds by HPLC with and without column trapping

Compound	Intensity ratio	S.D.[a]	Recovery (%)	S.D.
1-OH Tacrine	3.40	0.26	88.4	3.9
Indomethacin	5.39	0.38	112	5.8
Testosterone	6.73	0.26	98.7	1.3

[a] S.D.: standard deviation, $n = 5$.

The peak width on HPLC column (150 × 4.6 mm I.D., 5 μm) was 51 s at a flow rate of 0.5 ml/min, Fig. 3A. After column trapping, peak width was compressed to 10 s at the same flow rate, Fig. 3B. Meanwhile, the peak intensity of trapped peak was over five times that of HPLC peak, which should correspond to the concentration of analyte at peak apex. Table 1 shows peak intensity ratios with/without column trapping system and recoveries, which were determined by comparison of peak areas, for 1-OH tacrine, indomethacin and testosterone. Among the three compounds tested, 1-OH tacrine had the lowest recovery and signal intensity improvement. Since, 1-OH tacrine is more hydrophilic than the other two compounds, the organic content from the analytical column eluent might have more impact on the trapping of 1-OH-tacrine. By reducing the organic content that passed the trapping column (elution solvent/water ratio 1:3), the recovery of 1-OH tacrine was improved (data not shown). However, the total HPLC run time was double compared with that at 1:1 mixing ratio. As the maximum flow rate on trapping column was 1 ml/min, increasing the flow rate of dilution solvent came at the expense of reducing that of the analytical column. The 1:1 ratio was a compromise between analyte recovery and HPLC run time and was used for the rest of the analyses. Overall, more than three fold UV–VIS signal intensity improvement and good analyte recovery were obtained on model compounds. This should increase analyte concentration in the NMR flow cell and significantly reduce NMR data acquisition time, since the NMR signal to noise ratio (S/N) is improved by signal averaging and the S/N improves as the square root of the number of scans.

In order to test the improvement on NMR spectra, the method was applied for the analysis of the metabolites in post reaction incubates of tacrine with dog liver microsomes. In previous study of dog urinary metabolites after oral administration of tacrine, 1-OH tacrine had been identified as the major metabolite by MS and ^{1}H NMR analysis on a metabolite fraction collected from semi-preparative HPLC [19]. Fig. 4 shows the chromatogram tacrine metabolites following analytical separation of dog microsomal incubate. The major metabolite eluted at 42.5 min consistent with 1-OH-tacrine standard. The major tacrine metabolite, after trapping column, was back flushed into NMR flow cell and MS (1/20 of the flow) with 50% acetonitrile-d_3, 50% D_2O.

MS analysis (Fig. 5) showed a $[M + D]^+$ at m/z of 219 corresponding to monohydroxylated tacrine (four exchangeable protons by ^{2}H), which verified that the trapped peak had expected molecular mass. The parallel MS design reduces the chance of trapping wrong peaks and wasting expensive NMR spectrometer time, especially for crowded chromatograms obtained from complex metabolite such as bile and urine. The NMR spectra of the major tacrine metabolite are shown in Fig. 6. Spectrum (A) and (B) were acquired when the 1-OH-tacrine peak was eluted from the analytical column and directly transferred into the flow cell of NMR probe without column trapping, 512 and 64 scans were acquired, respectively. Spectrum (C) was acquired under the same NMR acquisition conditions when 1-OH-tacrine peak was back flushed into the NMR flow cell after column trapping. After 64 scans, spectrum (C) showed clear NMR signals, while it was noisy at the same total acquisition time without column

trapping, spectrum (B). A spectrum with similar S/N ratio required 512 scans without column trapping, therefore, increased NMR spectrometer time by 8-fold. Fig. 6 D shows the region near water signal of the same spectrum. The presence of a methine multiplet at 5.05 ppm identified 1-OH-tacrine as the major tacrine metabolite in the dog microsomal incubate, consistent with previous dog urine results [16,21]. These results clearly demonstrate the value of column trapping-NMR–MS system as a drug metabolite structure determination tool.

The present column trapping system can be easily switched to a peak collection mode [1], where fractions eluted from trapping column can be stored in capillary loops for later analysis. The loop volume of analyte collector is 130 μl, which is comparable with the volume of trapped peaks

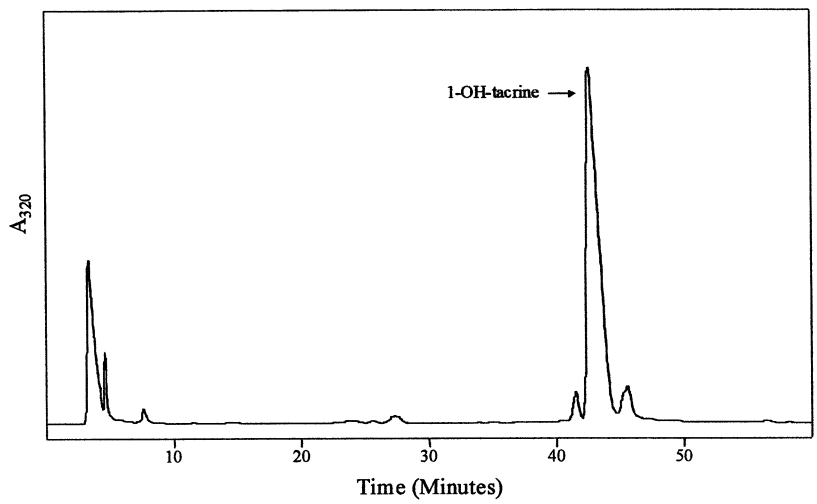

Fig. 4. UV chromatogram of dog microsomal incubate with tacrine under the conditions described in Section 2.4.

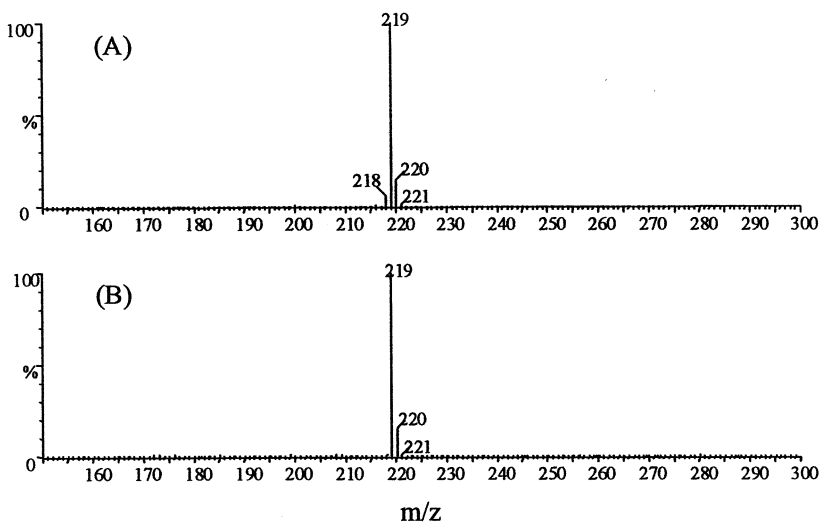

Fig. 5. Mass spectra of (A) 1-OH-tacrine standard in D_2O and (B) the major metabolite peak at 42.5 min in Fig. 4 after column trapping.

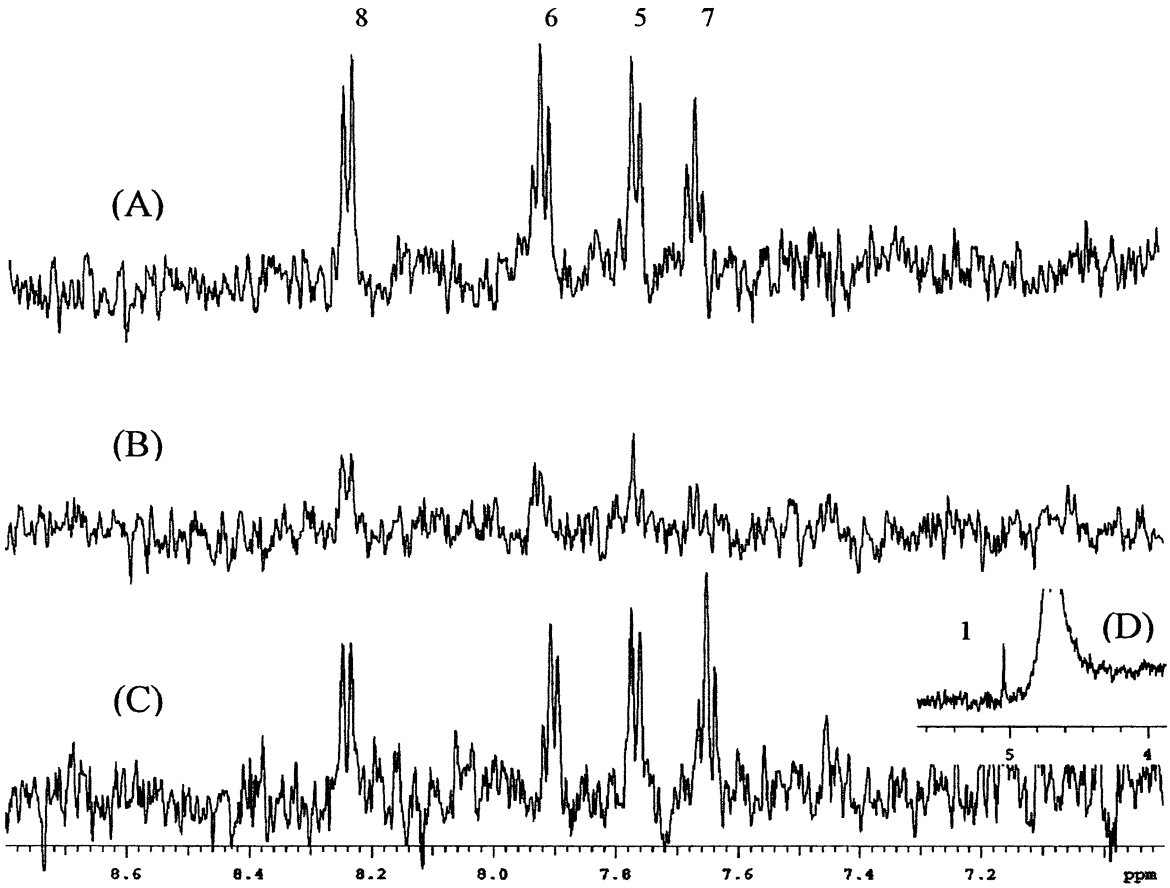

Fig. 6. Aromatic region of the ^{1}H NMR spectra (600 MHz) of 1-OH-tacrine from dog microsomal incubate. (A) LC–NMR in stop-flow mode, 512 scans acquired, (B) 64 acquired, (C) with column trapping, 64 scans acquired. All other NMR acquisition parameters were same as described in Section 2.5, with same amount of injection on analytical column. (D) Expansion of the region near water of the bottom spectrum, showing the methine resonance of 1-OH-tacrine.

in the NMR flow cell, thus minimize sample lost between transferring to and from loop collector.

The column trapping-NMR system is most useful where the amount of analyte is limited and chromatography peaks are well separated. In case of overlapping, a mixture of analytes will complicate NMR spectrum interpretation. Also, during the back flush of trapping column, the analytical column is still running. If a second peak is following too close (less than 1 min), it will be passed to waste. In addition, the analyte of interest must have lower mobility on the trapping column as compared with the analytical column when the same mobile phase is used for both. Further development in software and hardware is necessary to improve the column trapping system.

4. Conclusions

The present column trapping method involves a rapid, on-line concentration of target chromatography peak on a trapping column, followed by a back flush of the target analyte into the flow cell of the NMR spectrometer. The UV–VIS analysis of trapped peaks showed over three-fold increase in peak intensity on tested compounds, which would result in dramatic saving in NMR acquisi-

tion time as demonstrated by the analysis of the 1-OH-tacrine metabolite in dog microsomal post reaction incubate.

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