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Application of two-dimensional high-performance liquid chromatography–mass spectrometry with particle beam interface

DeMing Song*, Kenneth Kohlhof

Clinical Research Associate, 50 Madison Ave, New York, NY 10010, USA

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Abstract

The use of a particle beam interface (PBI) in LC–MS has faded because of its poor resolution and low sensitivity for many compounds. It also does not perform well in reversed-phase systems with a high percentage of water. This manuscript describes two-dimensional LC to overcome the problems of PBI, both in the identification of unknown samples and in the quantitative determination of metabolites of vitamin D in human plasma. For the identification of unknown components, the reversed-phase solvent in the first dimension was transferred to an isocratic normal solvent system in second dimension by column switching. Moreover, only the peaks of interest (components) in the first dimension were transferred into the second LC column for mass analysis. For the quantitative determination of the metabolite of vitamin D, the peak width of analyte in the second dimension was greatly narrowed, and interference was excluded such that high sensitivity and resolution resulted. The limit of quantification for the test metabolite of vitamin D in human plasma can reach 0.050 ng/ml with injection volume of 50 μl.

Keywords: Particle beam interface; Vitamins

1. Introduction

The heart of LC–MS is the interface that makes two incompatible analytical techniques, liquid chromatography and mass spectrometry, compatible. Since the LC–MS instrument became available on the market, dozens of interfaces have been tried. The particle beam interface (PBI) was one of those popular interfaces and was used in our LC–MS laboratory.

The major problem of PBI encountered in our experiments involved peak resolution in the LC–MS chromatogram which was found to be very poor. The small peaks, which were clearly monitored by the UV detector, were lost or overlapped by the broadened large peaks in the LC chromatogram. Actually, the multiple peaks close in retention time in the LC chromatogram were all overlapped so that we could not identify any single component, let alone to do the quantitative analysis. It was also not efficient for PBI to separate the water mobile phase from the analyte. Accordingly, it is not advisable for PBI to use a reverse phase with a gradient program and high concentration of water. These disadvantages of PBI had also been cited in the articles by Brown and Draper [1], Apfell [2], and Tinkel et al. [3]. Consequently, PBI is not produced by major

*Corresponding author. Present address: Department of Treasure, US Customs Service, New York Laboratory 6 WCT, NY 10048, USA.

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manufacturers anymore[1], instead, Electron Spray Ionization (ESI), and Atmosphere Pressure Chemical Ionization (APCI) are advanced as the favorite interfaces today. However, PBI has been popular in the laboratory for years and has its specific advantages that are valuable now. For instance, the current market mass libraries are not applied to ESI and APCI but work well with PBI-MS. This is one of the reasons why PBI is still used in a number of laboratories. To overcome the shortcomings of PBI, we have developed a new method by employing two-dimensional HPLC. By two different experiments of identification and quantitative analysis, this manuscript demonstrates how to make the LC–PBI-MS system work well in most circumstances.

2. Experiments

2.1. I. Experiment of identification of unknown components in liquid chromatography

2.1.1. Method

This method is suitable for those samples that cannot be tested by GC–MS and whose molecular weight is less 2000. In this experiment, the unknown sample for identification was supplied by West Inc., USA in the acetonitrile solution. For the best separation, the solvent system of the first-dimensional LC must be a reversed-phase with a gradient program that is listed in Table 1.

Analysis was performed on an HP 1050 HPLC with a VWD UV detector, an HP 1050 auto sampler and HP HPLC[3d] DOS ChemStation. The instrument configuration is shown in Fig. 1.

Points of interest marked '*' in Fig. 2 were collected by a 2 ml sample loop and then transferred to an HP 59980B particle beam interface. The helium inlet pressure and the temperature of desolvation chamber of the PBI were set at 50 p.s.i. and 60°C, respectively. The molecules of analytes went into an HP 5989 MS Engine equipped with a Wiley138 mass spectrum library, a Model 7673 autosampler and a Model HP DOS G1034C MS ChemStation (Hewlett-Packard, Palo Alto, CA, USA) for identification. The mass spectrometer was set in electron impact ionization and scan acquisition mode monitoring m/z from 75 to 550. The temperatures of source and quadrupoles of the mass spec-

Table 1
Solvent system of two dimensional HPLC

First dimensional HPLC. Flow rate: 0.4 ml/min, Column: Beckman ultrasphere 5 μ 2.0 mm×15 cm			
Reversed-phase gradient solvent system	Acetonitrile (V%)	Water (V%)	Time (min.)
1	15.0	85.0	0.0
2	15.0	85.0	1.0
3	100	0	10.0
4	100	0	15.0
5	15.0	85.0	19.0
6	15.0	85.0	22.0
Second dimensional HPLC. Isocratic solvent acetonitrile flow-rate: 0.4 ml/min column Beckman ultrasphere 5 μ 2.0 mm×15 cm.			

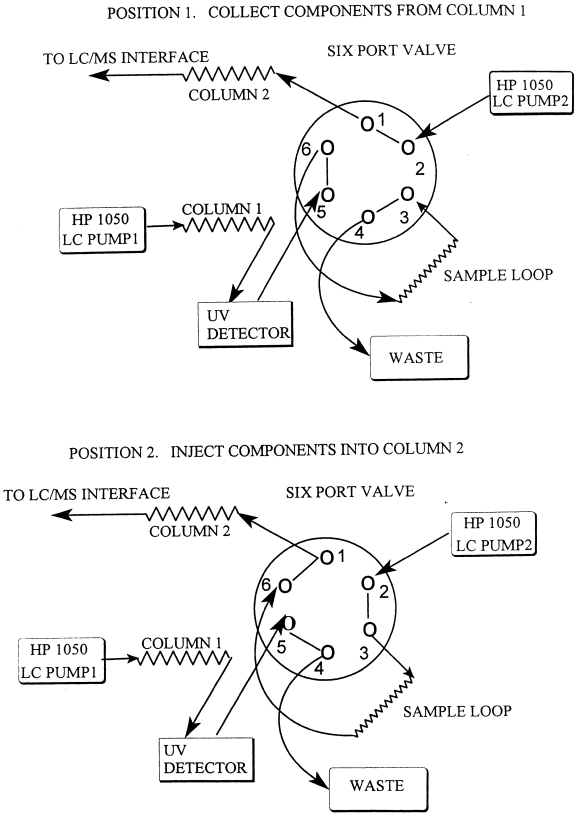

Fig. 1. The configuration of two-dimensional HPLC.

[1]Communication in the Eastern Analytical Symposium, 1997, NJ, USA and Pittsburgh Conference, 1998, New Orleans, LA, USA.

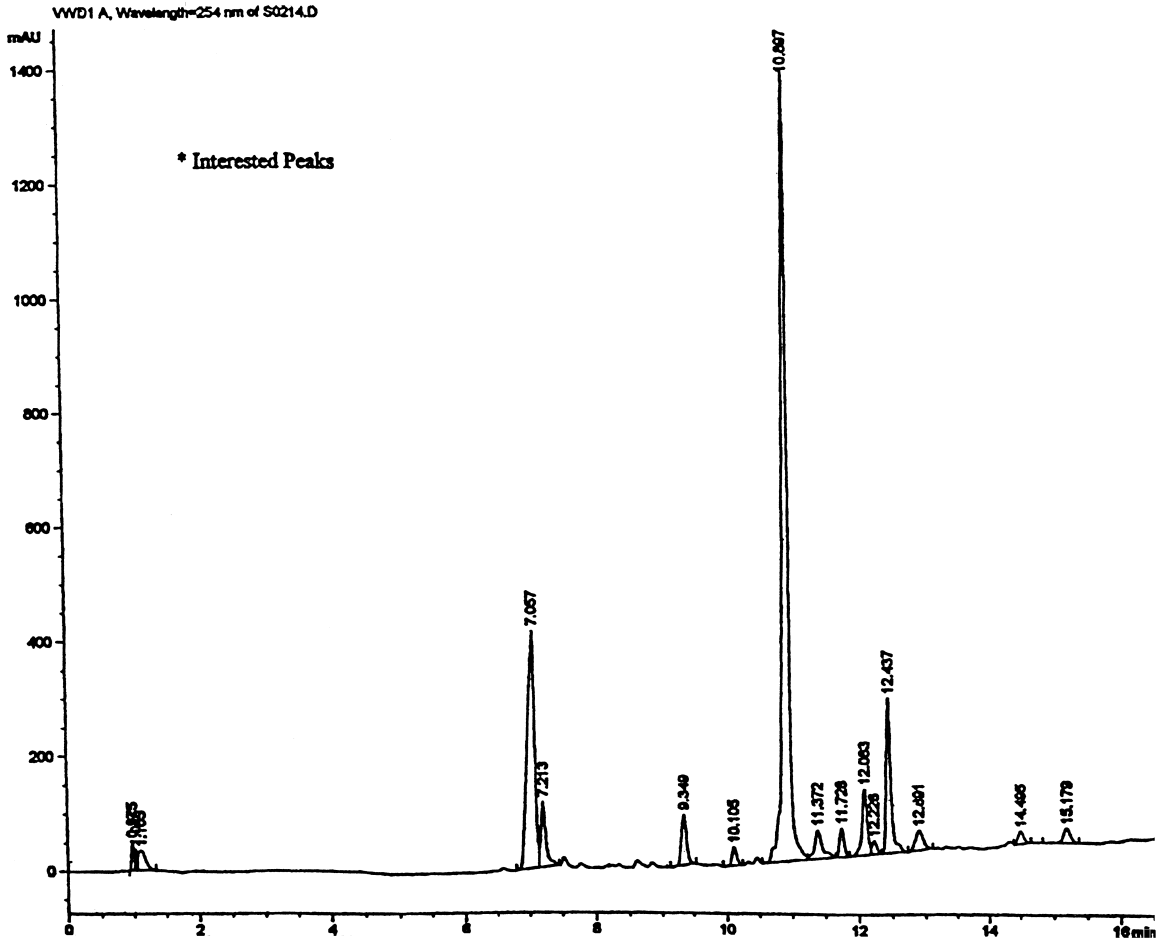

Fig. 2. A HPLC chromatogram of the sample for identification detected by a HP VWD UV detector at the wavelength 254 nm. The interested peaks were marked by '*'.

trometer were 150°C and 100°C, respectively. The energy of ionizing electrons and the voltage of electron multiplier were set at 70 eV and 2000 V, respectively.

2.2. II. Experiment of quantitative determination of the major metabolite of vitamin D in human plasma

2.2.1. Introduction

Vitamin D refers to a group of similar sterol compounds. There are 11 sterols having activity, but two of the most important are referred to as vitamin D_2 and D_3. D_3 is converted in the liver to 25-hydroxy-D_3 (m-vit-D_3), the major metabolite in circulation [4,5]. According to recent research, the concentration level of m-vit-D_3 in human plasma is an indicator for some diseases [5]. Since m-vit-D_3 is non-sensitive to UV and fluorescence detectors, and is neither well-separated by a GC column, we have chosen the LC–MS method for this job. The isotopically labeled compound m-vit-D_3-d_6 was used as the internal standard. The structures of D_3, m-vit-D_3 and m-vit-D_3-d_6 are shown in Fig. 3.

2.2.2. Chemicals

M-vit-D_3: Ro-24-2090, Internal standard m-vit-D_3-d_6, Ro-24-2090-d_6 and derivatization agent PFB-TADO (4-penta-fluorobenzyl-1,2,4-triazolidine-3,5

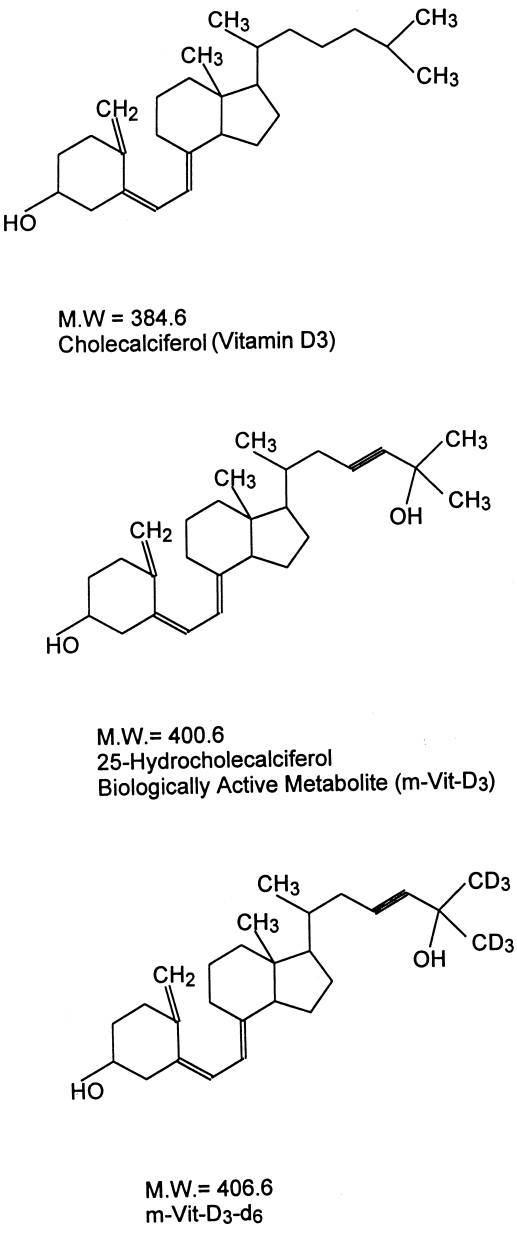

Fig. 3. The molecular structures of vitamin D_3, m-vit-D_3 and internal standard m-vit-D_3-d_6.

dione) were supplied by the Hoffman La Roche company (Nutley, NJ, USA). Dichloromethane, tetrahydrofunan, hexane, pentane, ethyl acetate and methanol were all HPLC grade and purchased from Baxter Healthcare (McGaw Park, IL, USA).

2.2.3. Sample preparation and derivatization[2]

To each 1 ml human plasma sample, add 5.0 ml of extraction solution (pentane–dichloromethane 85:15, v/v). Vortex mixing and centrifuge for 20 min each. The upper organic layer is pipetted into another test tube and then vapored to dryness under N_2 flow at 60°C. To each dry residue, add 150 μl acetonitrile and 50 μl PFB-TADO (4-penta-fluorobenzyl-1,2,4-triazolidine-3,5 dione) (dienophile). The reaction is instantaneous. Then add 100 μl methanol and vortex. After evaporating to dry again, add 65 μl reconstitution solvent (hexane–ethylacetate 1:1) and transfer to test vial for LC–MS analyses.

2.2.4. Method

The configuration of the instrument for quantitative determination is the same as for identification and shown in Fig. 1. Since m-vit-D_3 is not sensitive to UV, the UV detector can be bypassed. M-vit-D_3 and its internal standard m-vit-D_3-d_6 were eluted from column (1) in the time window from 7.8–8.4 min (Fig. 4). The sample loop was set to collect the elution of analytes from column (1) at this time-period by setting the contact of HPLC to control the six port valve. The fluid from the column (2) was transferred into a HP 59980B particle beam interface. The helium inlet pressure and the temperature of the desolvation chamber of the PBI were set at 60 p.s.i. and 45°C, respectively. The analyte went into a HP 5989 MS engine equipped with a Model 7673 autosampler and a Model HP DOS G1034C MS ChemStation (Hewlett-Packard, Palo Alto, CA, USA). The mass spectrometer mode was set as negative chemical ionization and SIM mode monitoring m/z 492.1 for m-vit-D_3 and 498.1 for m-vit-D_3-d_6. The temperatures of source and quadrupoles of the mass spectrometer were 250°C and 100°C, respectively. The energy of ionizing electrons and the voltage of the electron multiplier were set at 230 eV and 2300 V, respectively. The chemical ionization gas was pure ammonia and the best operating pressure was found to be 0.173–0.25 kPa. The solvent system of HPLC was as listed in Table 2.

[2]Procedures of sample preparation and derivatization were provided by Hoffman La Roche Inc.

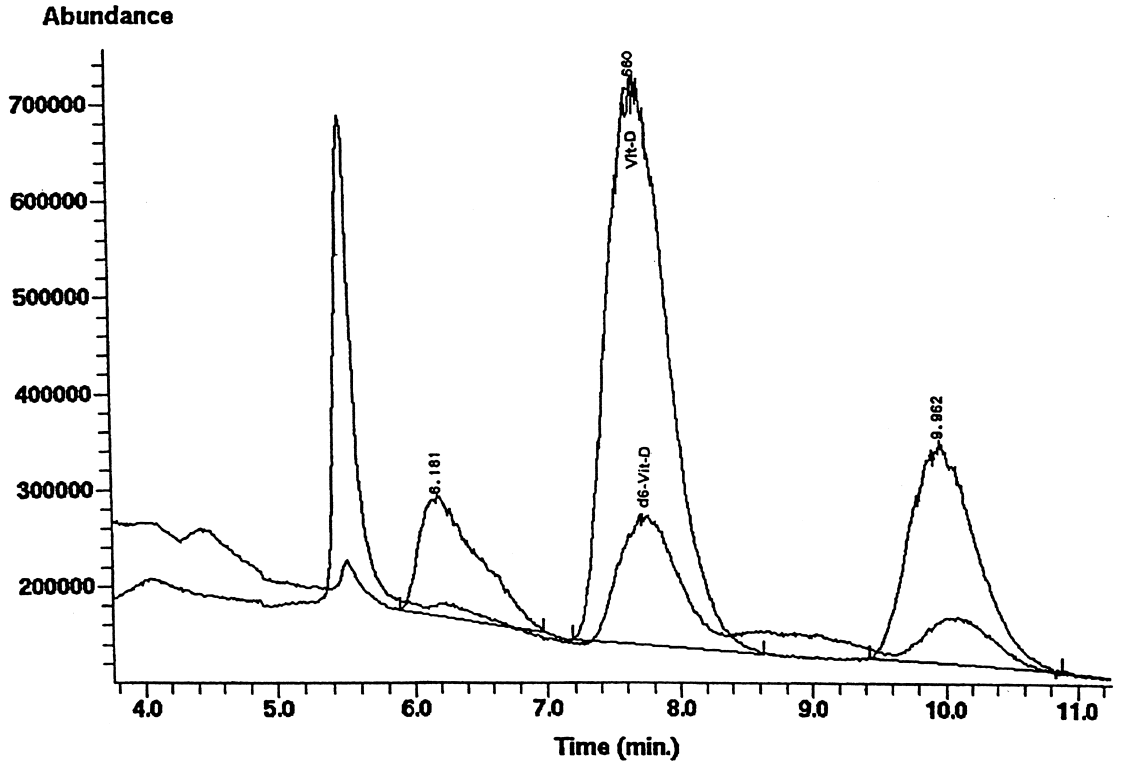

Fig. 4. The LC chromatogram of a m-vit-D_3 standard sample from column (1) detected by mass spectrometry. The concentration of m-vit-D_3 is 20 ng/ml.

3. Results and discussion

3.1. Results of the experiment of identification of unknown components in LC

A HPLC chromatogram of the sample detected by a HP VWD UV detector at the wavelength 254 nm is shown in Fig. 2. The retention times of the peaks of interest, which are marked '*', were t_R=9.349, 10.897 and 12.437 min, respectively. The LC chromatograms of column (2) and the mass spectra of these three peaks are shown in Fig. 5a, b and c, respectively. These three components, t_R=9.349; 10.897 and 12.437 min. were identified as, benz[b]thiophen-3(2H)-one, 2-(3-oxobenzo[b]thien-2(3H)-ylidene; 2,6-bis(1,1-dimethylethyl)-4-ethyl-

Table 2
The solvent systems of two-dimensional HPLC

	Column	Isocratic mobile phase	Flow rate
First dimension	Silica 3×100 mm, 3μ particle, 80 A pore	Hexane, ethyl acetate tetrahydrofunan 60:40:6, v/v/v	0.4 ml/min
Second dimension	Amino 3×150 mm, 3μ particle, 80 A pore	Tetrahydrofunan	0.4 ml/min

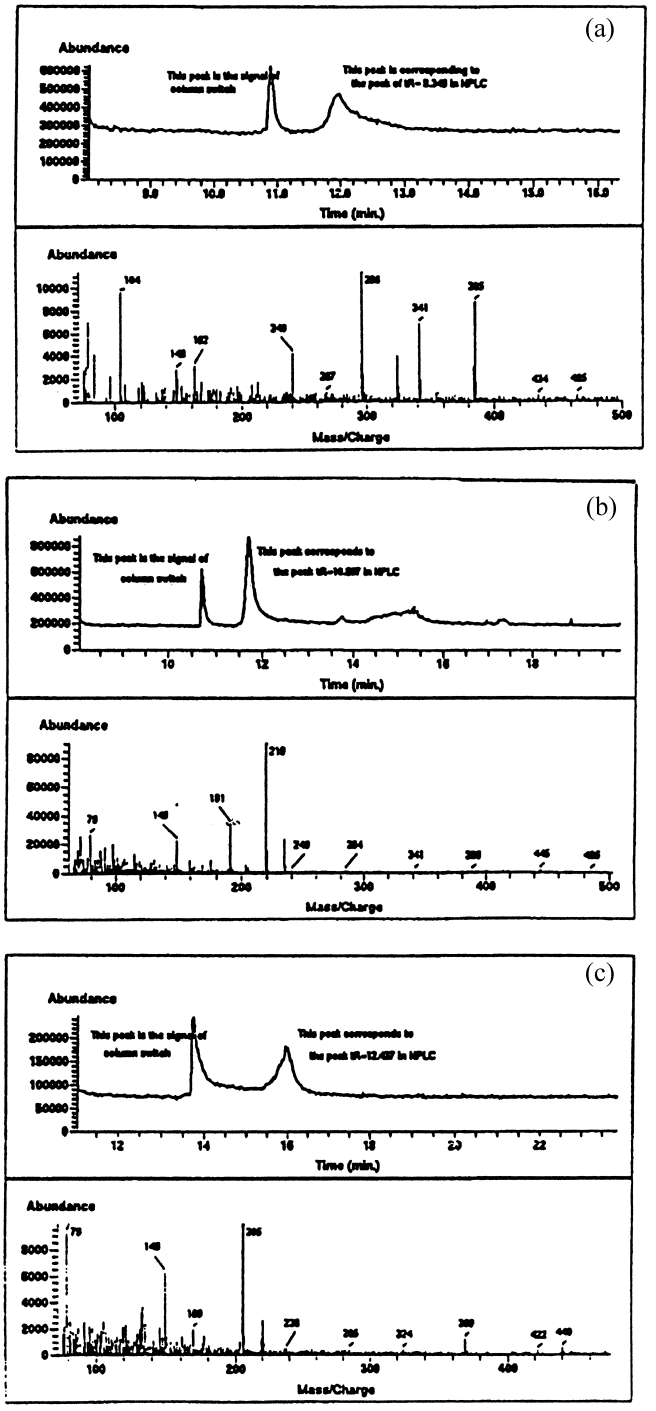

Fig. 5. The LC chromatogram of the identification sample from column (2) detected by mass spectrometer. The peaks in chromatograms, a, b, c, are related to the interested peaks '*', t_R=12.437 min, t_R=10.897 min, t_R=9.349 min, respectively in Fig. 2. The mass spectra in a, b, c are related to those three components for identification.

phenol and butylated hydroxytoluene respectively by the mass library Wiley138.

The first peak in Fig. 5a, b and c was due to the signal of column switch. When the six port valve switched, the composition of solvent into the PBI then changed and this change gave a signal to mass spectrometer and appeared as a peak.

3.2. Results of the experiment of quantitative determination of m-vit-d_3 in human plasma

Fig. 4 shows the LC–MS chromatogram of a standard sample with a concentration of 20 ng/ml from column (1). It was obtained by connecting the PBI directly to column (1) and bypassing the six port valve. It was important to get the time window for the elution of analyte from column (1). The time window for m-vit-D_3 and its internal standard in this case was 7.8–8.4 min. Fig. 6 is the LC–MS chromatogram of the same sample from column (2). It was obvious that, from the second dimension of LC, the peak width was much sharper and all other peaks in Fig. 4 were excluded. The normal concentration level of m-vit-D_3 of a healthy person is from 0 to 2 ng/ml. Fig. 7 shows the concentration of a m-vit-D_3 sample that was found to be 0.055 ng/ml. The limit of quantification is set to 0.050 ng/ml. The detection limit of the method can reach 0.025 ng/ml. The data for the method validation are shown in Table 3 that includes the standard curve calibration, the precision and accuracy of the method from the QC samples for intra-assay and inter-assays. All of them are better than 10%.

3.3. The limitations and disadvantages of the particle beam interface

PBI was developed by Willoughby and Browner as monodispersed aerosol generation interface [6,7]. In PBI the volatile and low-mass (low momentum), mobile phase vapor is drawn away by the vacuum pump but the more massive (high momentum) sample particles are not deflected and go to the mass spectrometer.

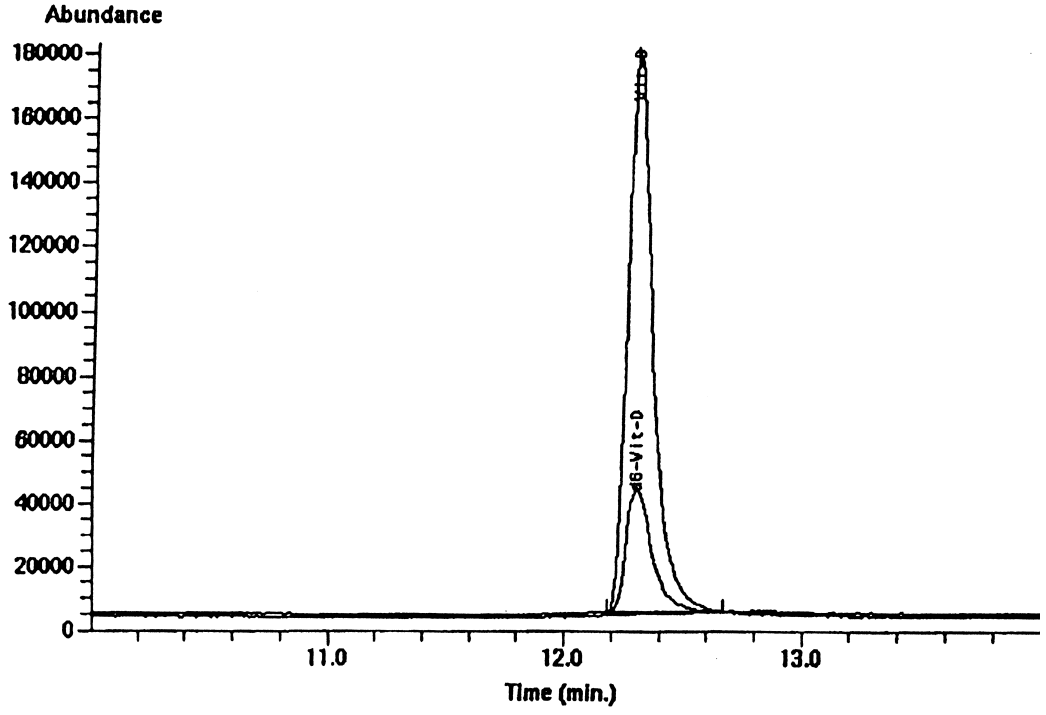

Fig. 6. The LC–MS chromatogram of the same m-vit-D_3 standard sample (shown in Fig. 3) from column (2) detected by mass spectrometer. The concentration of m-vit-D_3 is 20 ng/ml.

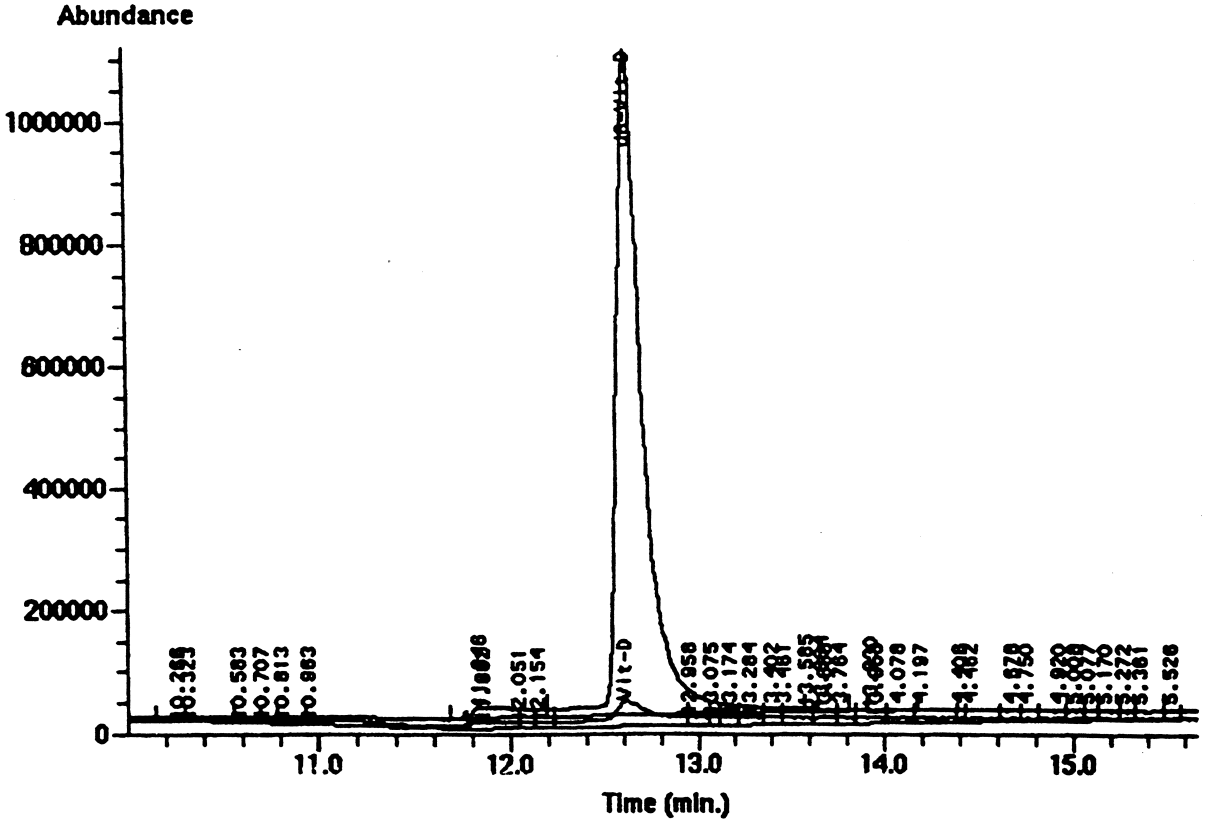

Fig. 7. The LC–MS chromatogram of a m-vit-D_3 sample from column (2). The concentration of m-vit-D_3 is 0.055 ng/ml.

In most cases in our experiments, the resolution was greater than 0.5 min in LC–PBI-MS. It was very poor compared to LC chromatograph by UV detector (less than 0.1 min). Some factors beside the interface, e.g. detector rise time and the geometry of ion source of the mass spectrometer may have contributions to the band broadening [8–10]. The main reason is the extra-column dispersion of the interface that reduces the efficiency of the column. Our experiment showed it took about 0.2 min for the analyte to spray from the nebulizer to the mass source in PBI. This dispersion of the analyte reduced the resolution of LC chromatography. The same study had been reported by Ahuja et al. [11]. The other studies were reported by Brown et al. [1] that the feature of peak broadening of PBI was related to the mode of ionization of the mass spectrometer. They found peak broadening was unacceptably large with NCI mode for testing phenols and their conjugates. Tinke et al. found that the peak broadening in PBI was dependent on the volatility of analyte [3].

PBI shows better sensitivity and works well when the mobile phase contains more organic modifier and less water [2]. It also reported that the S/N and peak height could decrease to 2/3 to 1/25 (depending on different analytes) when the solvent was changed from acetonitrile to acetonitrile buffer [1]. The reason is possibly that water is not highly volatile and difficult to pump away from the analyte in PBI. Our experiment also showed that there was no significant improvement from raising the temperature of the desolvation chamber (maximum temperature is 70°C). Moreover, it could take the risk of decomposing the analyte by raising the temperature of the

Table 3
Method validation data
A. The precision of calibration curve

Calibration standard samples for analyte: M-vit-D_3
Internal standard M-vit-D_3-d_6: 60 ng/ml

Sample ID replicate	Calibration concentration *A*	Concentration found (average) *B*	Error% $(B-A)/A$%
Standard-1	0.1	0.101	−0.5
Standard-2	0.2	0.212	6
Standard-3	0.5	0.469	−6.2
Standard-4	2.0	2.077	3.9
Standard-5	10.0	10.21	2.1
Standard-6	20.0	19.81	−1.0
$R^2=0.9968$			

B. Intra-assay precision[a] and accuracy[b] from quality control samples for M-vit-D_3 in human plasma:

Sample	Nominal concentration (ng/ml)	Concentration Founded (mean ±SD), (ng/ml)	C.V.%	% Theoretical value	*N*
QC_L	0.4	0.431±0.0439	10.21	107.7	5
QC_M	4.0	4.39±0.154	3.51	109.8	5
QC_H	16.0	15.06±0.734	4.88	94.1	5

C Inter-assay precision[a] and accuracy[b] from quality control samples for M-vit-D_3 in human plasma:

M-vit-D_3	QC_L founded (average) Nominal 2 ng/ml	QC_M founded (average) Nominal 8 ng/ml	QC_H founded (average) Nominal 20 ng/ml
Assay #1	0.441	4.436	14.7
Assay #2	0.457	4.252	15.54
Assay #3	0.448	4.299	15.65
Assay #4	0.392	4.477	14.75
Assay #5	0.421	4.422	14.84
Assay #6	0.438	4.491	14.63
Assay #7	0.410	4.307	15.44
Mean	0.429	4.38	15.1
SD	0.023	0.096	0.44
C.V.%	5.4	2.2	2.9
% Theoretical	107.3	109.6	94.3
n	7	7	7

[a] Precision is reflected by C.V.%.
[b] Accuracy and reproducibility is reflected by theoretical value %.

desolvation chamber too high. The insertion of water molecules into the mass source leads to their decomposition into hydrogen and oxygen as a result of the impact of high energy electrons. This not only reduces the life of the filament but also opens the possibility of chemical reactions with the analytes. The background noise was high when the reverse phase with high water concentration solvent system was employed.

Another major disadvantage for PBI is the low sensitivity for some kinds of samples. The recent research found that small particles are transported at a relatively low efficiency and result in low sensitivity for such compounds [12]. Derivatization of the

specific analyte may increase its volatility to reduce the peak broadening and raise the sensitivity in LC–PBI-MS [3]. This was proved in the test of m-vit-D_3 in this paper. We have tested m-vit-D_3 without derivatization by the same method and found the sensitivity was 6–7 times lower compared to that with derivatization. However, derivatization is not suitable for all compounds, especially to allow unknown components to be identified.

3.4. The resolution and sensitivity of two dimensional LC–PBI-MS

The operation of two-dimensional HPLC by column switch is shown in Fig. 1. The functions of the second-dimensional LC can solve the problems of PBI stated above.

Since only one component of interest is picked in the sample loop at a time and goes into PBI for analysis that excludes all other components including interferences. In such a way, it may solve the problem of the poor resolution of PBI.

In addition to the separation, the second column has also contribution for narrowing the peak width to raise the ratio of signal-to-noise (S/N) and the sensitivity. That is why we can reach a sensitivity of 2.75 pg on a column (50 pg/ml with an injection volume 55 μl) for testing m-vit-D_3 in human plasma by this method. This is the highest sensitivity so far presented for the LC-PBI-MS technique. For Atmospheric Pressure Ionization Electrical Spray Interface (API-ES), the detect limit was reported as 10 pg on a column for standard reserpine [13]. Although, there is no base to compare these two sensitivities for different analytes, the ultra high sensitivity for m-vit-D_3 by LC–PBI-MS shows PBI still has promise today.

Moreover, since column (2) is working under an isocratic normal-phase system, no matter what solvent components changes in column (1), the solvent into PBI would not change. As expected, the water solvent could also be isolated from PBI.

These advantages of second-dimensional HPLC may be seen by comparing Figs. 4 and 6. All other components or interference in Fig. 4 are excluded in Fig. 6 and the peak width in Fig. 6 is much narrower and sharper than that in Fig. 4.

4. Conclusion

Although PBI has its disadvantages and limitations, it still has life today in scientific fields. It has advantages. For instance, it is good for both EI and CI mass operation. It is useful for a number of compounds when using an isocratic normal-phase solvent. Moreover, the current mass libraries are applied to LC–PBI-MS but not to API–ES and APCI interfaces. The disadvantages of poor resolution and the problem with a gradient reversed-phase solvent containing high composition of water can be solved by two-dimensional HPLC. From the experiments described in this manuscript, two-dimensional HPLC–PBI-MS is a powerful instrument, not only for identification, but also for quantitative determination.

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