Basic features offered by HPPLC

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Because of the complexity of many real-life samples which need HPLC for chromatographic analysis, using a single mode of analysis may fail. Peak overlapping is the common source of systematic analytical errors. Thus, a second liquid chromatography concept might be helpful.

High pressure planar liquid chromatography (HPPLC) (Fig. 1) is a rapid, digital separation mode of liquid chromatography under high mechanical pressure. In contrast to the one-dimensional HPLC elution technique with conventionally packed columns online, a flow-through detector HPPLC uses a flat bed of 100 to 250 μm thickness and about 100 mm circular diameter. This circular bed is packed with any of the commercially available stationary HPLC phases. Ready-made HPTLC plates can be used directly on glass.

The HPPLC "column" is a flat circular system covered completely by glass. The "peaks" of a single sample develop chromatography as concentric circles of substances. The substances remain separated from each other by their differing radius. The chromatogram is stored substantially and appears as if "frozen." After removal of the mobile phase, it can be detected/quantitated using image-processing in a proper light region or by optical "in situ scanning," using a computerized HPTLC scanner. The mobile phase flow is constant and digitized into limited volume portions. Digitization here means "mobile phase digitization," which is a new dimension in chromatography. Therefore, HPPLC is incompatible with TLC, HPTLC and OPLC [over pressured layer chro-

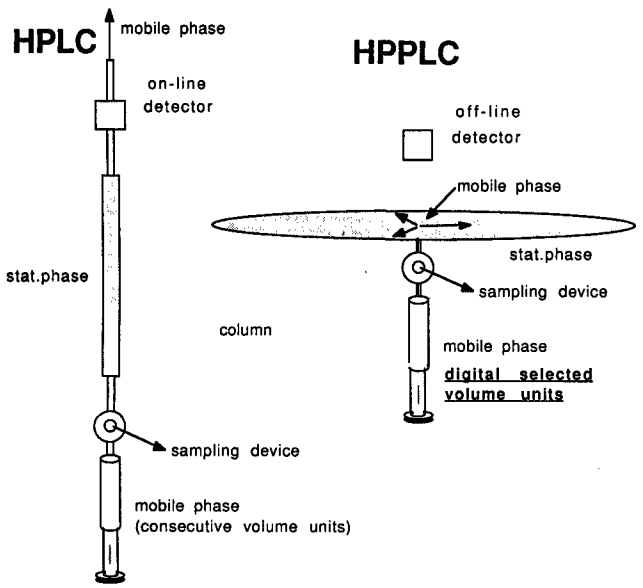

FIG. 1. HPPLC principle in comparison to HPLC.

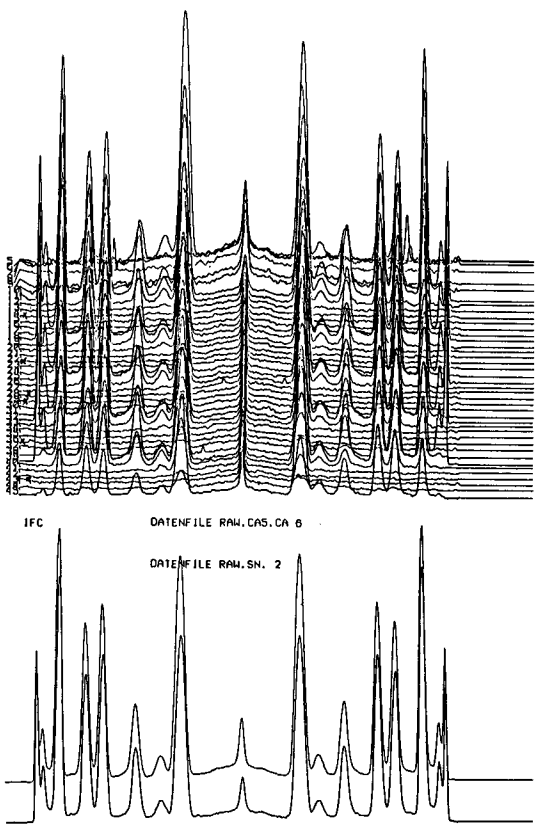

FIG. 2. Computer averaging of the chromatogram signal by addition of the complete raw data sets scanned at changed angles of the circular chromatogram and calculation of the raw data mean = CAT. The mean raw data set is then integrated. This concept reduces structure signal without falsifying circle positions or other data polishment by noise rejection or other factors.

matography, as introduced by E. Tyihak and coworkers in the 1970s (1)].

Most substance mixtures show a stochastic chromatogram after separation; there is no regularity for the peak position except in synthetic samples made up of homologue series. In 1986, C. Giddings of the U.S. noted that a 1000-component mixture would need 10^{12} theoretical plates for a complete separation. These components theoretically coexist in a mixture of 0.1% each, but in a real-life sample, probably coexist at the parts per million (ppm) level. There are no separation systems available that offer more than one millionth of the number of plates theoretically necessary for such a complete separation. A chromatogram of 500-meter length must show peak width in half height of 1 mm in order to approximately represent 10^{12} theoretical plates; or, we would need a 50-m long thin layer plate to accept only 0.1-mm wide spots or substance lines all over the plate length.

Peak overlapping, therefore, is common in real-life chromatography. In high resolution capillary gas chromatography (GC), we must consider the possible analytical danger of having 3-4 substances overlapped under each GC peak separated. In HPLC, we must consider the danger of up to 350 substances coeluting under one peak in the worst case of a ppm-level analysis. In cases in which detection becomes dependent on mixtures and the equipment has been calibrated with clean chemical compounds, we may have to consider the possibility of correction factor falsification. We already have found this effect as disastrous systematic error in quantitative high resolution capillary GC. Even by "round-robin" tests, systematic analytical errors remain undetected (2). In this critical situation, the use of more than one analytical method is the only way to handle these problems.

HPPLC is applicable one dimensionally as a further quantitative LC analysis method but also is useful as a two-dimensional separation technique. Two-dimensional separation modes, especially when coupled with one-dimensional pre-separation modes, offer the largest separation power known. HPTLC, the precursor of HPPLC, turned out to be more "quantitative" than classical TLC. However, even TLC can be quite qualified with respect to quantitative analysis. International round-robin tests ended up with excellent data for the accuracy of HPTLC and TLC results, often better than those found with GC or HPLC (3).

The following arguments explain why a new level of relative standard deviation can be reached at the repeatability, comparability and accuracy level of modern quantitative HPLC using circular HPPLC in the single-sample mode of circular separation:

- Samples are injected exactly as in HPLC, but without the stress of high back pressure.
- Separation basically is done in the same environment of mobile phase, stationary phase and particulate materials as in HPLC.
- Channeling and wall effects noted in HPLC do not exist in HPPLC. There is no wall effect because

the layer (200 μm) is narrow enough. Here the same laws exist as with GC in micro-packed columns (4) and packed capillary column LC (5).

• The separation in HPPLC is comparable to mobile phase programming in HPLC but, in fact, runs under constant conditions of flow and polarity; it is isocratic.

• In contrast to HPLC, the HPPLC chromatogram may be started after complete removal of the sample solvent. The sample starting volume may be too large when using the full sample working range of 100 nanoliters to 100 μl in HPPLC, so the starting volume may be a broad band circle. Refocusing the sample circle to a 0.2 mm narrow band is possible. If solubility remains, the final chromatogram can begin with such a narrow (sharp) band or circle of sample. It then can be refocused using further differing mobile phases consecutively with or without intermediate mobile phase removal.

• The separated substances remain fixed onto the stationary phase, and there is no dilution by eluting into a voluminous detector cell as often occurs with HPLC.

• Whether image-processing or normal in situ optical scanning of the chromatogram is done (after a final mobile phase removal), sharp bands or circles may remain on the layer as material for quantitation. However, the layer consists of particulate matter and produces a chromatogram consisting of a stochastic but completely stable fixed base plane. We call its scanner signal the "structure signal." This overlays the substance signal and systematically falsifies it. This systematic error in planar chromatography quantification is highly constant and easily measured, and thus can be removed. Structure can be measured prior to analysis, stored in a laboratory computer and subtracted after the final overlaid chromatogram signal is stored. Or, we can get rid of it by "computer averaging" multiple circular scans at slightly differing angle positions (6).

• As the HPPLC chromatogram stays constant, repeating and comparing quantitations are a question of a proper laboratory computer and intelligent software to handle these steps rapidly and accurately.

HPPLC is comparably qualified with HPLC for quantitative analysis. Of course, there are sensitivity limits, but further improvements depend on possible post-run sample derivatizations. The planar field is a quantitative detector per se offering thousands of modes including post-run chemical treatment—which is quite limited in HPLC.

Details

One-sample analysis is possible with HPPLC, and post-run chemical treatment is simple. One sample may be injected as in normal HPLC, or up to 24 samples may be applied before the separation or before pre-chromatographic sample treatment.

When one sample is used, calibration is available, as the substance of interest may be injected "a few μl mobile phase" before and after the analytical sample

has been injected. Thus, the substance circle of interest is "enclosed" by one circle ahead and one behind the analytical substance. HPPLC, with its two-dimensional chromatographic fields, makes it possible to overlay a sample circle with a calibration substance ellipse before separation. Both can be applied by spraying onto the dry plate before separation.

The sprayed-on circles also may be acid or basic compounds, thus splitting an analytical sample mixture into specific substance classes. To free one chemical-bonded sample part, one can neutralize the sprayed-on "blocker" circle. The separation then is continued without further sampling. Of course, there are limitations. Only simple mixtures with a limited number of compounds are applicable for such manipulations, and only chemically stable substances can be used for class separation steps. In the latter case, we did not find complexity limitations; most complex mixtures allowed separations into substance classes.

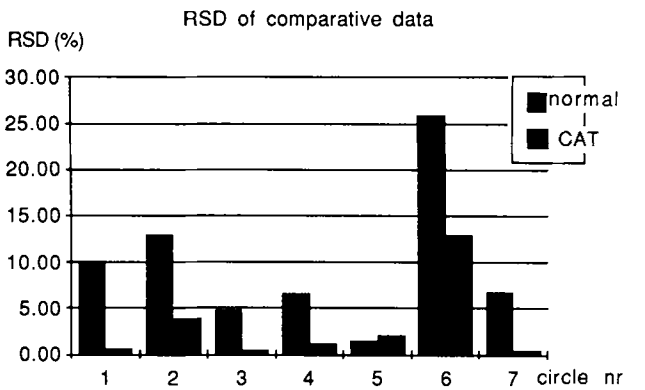

FIG. 3. Results of integrated computer averaging of the chromatogram signal (CAT).

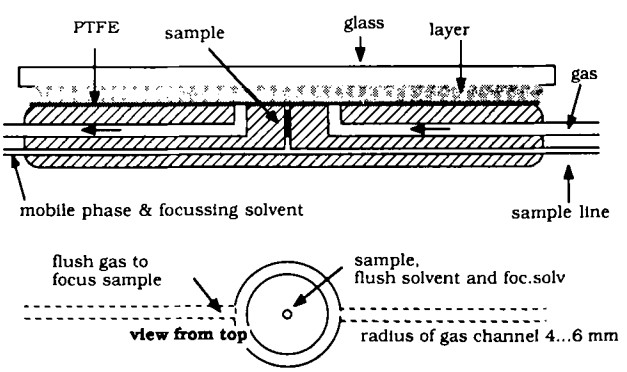

FIG. 4. Sampling combined with "in-plate" sample preparation, extraction and focusing into a sharp starting circle. This tool was used to directly inject blood or serum of 100 µ volume but still kept the sample area small and the starting substance circle sharp. If vacuum is used instead of flush gas, the sample pumped into the plate can be washed. If gas is used for flushing, the sample field can be extracted and the extract focused as sharp circle in the dimension shown from top. Actual circle diameter can be 20 mm or less. (Diploma thesis Anja Bolz and Gerhard Hartmann, Prof. W. Funk, Fachhochschule Gießen, Fachbereich Technisches Gesundheltswesen, D-6300 Gießen, F.R.G.). The authors thank Anja Hartmann, Gerhart Hartmann and W. Funk for their contributions with HPPLC.

The separation runs either under complete computer control or is done by hand. In the latter, the mobile phase is pressed by hand through a syringe into the glass-covered plate. A Hamilton "gas-tight" 1000 µl syringe, for instance, survives 20-70 bar pressure, although certified only for 7 bar pressure. Post-run chemical sample reactions may dramatically enhance the selectivity or sensitivity of the detection. For 1-24 samples separated in 1-2 minutes, the analyst needs 500 to 1000 µl mobile phase. Any LC mobile phase can be used. The quantitation is done in the absence of the mobile phase. More mobile phases are available for HPPLC than for the HPLC elution technique.

The "column" is completely inert, covered by glass on both flat sides. It is opened before quantitation and thus allows direct access to the separated substances. The planar stationary phase bed is a substance-storing (data-) bank. The critical limit of HPPLC is that it cannot be applied either for separating volatile compounds or for substances destroyed by air or light if decomposed into volatiles.

Comparisons

Basically, HPPLC is an isocratic LC process which results in a programmed separation. It is finished within 60-120 seconds regardless of the number of samples applied. The separation of up to 24 samples runs simultaneously. HPPLC uses the circular separation mode by development or is used in a flow-through mode. The use of pressure in planar chromatography was introduced in the 1970s by E. Tyihak, who is considered the pioneer of HPPLC. HPPLC differs from OPLC, HPLC, TLC and HPTLC in the following:

(a) The HPPLC plate is cleaned chemically under high pressure in minutes.

(b) One sample is injected, or many samples are stamped onto a circle line; the latter mode uses David Fenimore's circular transpotter (7).

(c) The mobile phase is injected quantitatively by volume under digital control.

(d) Quantification is done after removal of the mobile phase at changed angles, ending up under conditions of comparability with only one sample circle, the data summarized using the NMR computer-averaging technique (CAT) (Figs. 2-3).

(e) Sample capacity is 1-24 at 100 nl to 100 µl (48 samples anti-circular).

(f) On-plate enrichment and focusing are possible and are necessary at the 100 µl per sample solution level.

(g) Substance capacity ranges from 10 mg to several femtograms.

(h) Separation mode is circular at one minute separation time, 600 µl phase.

(i) The qualitative chromatographic range is within k>0.1 to k at 1000.

(j) K-data transfer quality HPPLC to HPLC is within 1-2% accuracy.

(k) Separation system is completely inert using glass as the total environment.

(l) HPPLC is digital and fully instrumentalized, but can easily be run by hand as the mechanical steps necessary are rapidly finished.

HPPLC differs from HPLC because (c), (d), (f), (h), (i) and (l) are not available with HPLC. HPPLC differs from TLC and partially from HPTLC because (a), (b), (c), (f), (g), (j) and (l) are not available with TLC/HPTLC. HPPLC differs from OPLC because (b), (c), (d), (f), (h), (i), (k) and (l) are not available with OPLC.

Practical steps

In the simplest operation, one sample is injected in HPPLC, just as with HPLC tools into HPLC-packed columns. Single-sample analysis allows absolute quantitation as well as external standard analysis. The standard and main substances of interest appear at different radius positions although the "external standard" substance may be chemically the same substance of analytical interest. This is possible by the so-called deferred mode of calibration used in elution chromatography applicable in HPPLC as development chromatography due to the digital character of HPPLC. Figure 4 shows how samples solved in volatile solvents may be applied and enriched in the stationary-phase field using flowing gas as the concentrator. The sample, or its extracts from any complex matrix, can be enriched on a sharp starting circle.

Figure 5 shows multiple scans of the sample and its calibration standard, applied before sampling. Figure 6 shows the sharpness of circular signals possible when using consecutive sampling steps of the same substance with intermediate digital mobile phase injections. It gives an idea about the possible separation efficiency of circular planar chromatography under high pressure.

Due to high pressure (3000 kg on 7090 mm^2 glass/stationary phase/glass sandwich), the mobile phase flow into the circular planar field is 10 times faster than normal. This high pressure mode allows the combination of any useful pair of mobile phase with stationary phase and provides chromatographic freedom to optimize a specific separation. In addition, HPPLC allows multiple development, taking approximately 120 seconds per mobile phase chosen.

HPPLC under the one-sample separation condition is rapid; sampling 24 to 48 spots, although time-consuming, is possible. The latter, however, is limited to substances stable in air and light. Material consumption per sample is small. Total material consumption reaches 1 ml clean-up solvent (to bring the stationary phase of ready-made HPTLC glass plates into a repeatable chemically clean status), plus 1-2 ml mobile phase for the final separation step, plus 1 plate per run. This can be expensive if the one-sample mode is used with chemically bonded phases of costly plates, but clever clean-up may allow reuse of the same plate.

HPPLC is especially useful for "dirty" samples,

or samples which simply cannot be injected into an HPLC column more than several times. Enriching or pretreatment of samples is possible (Fig. 4). Partial or total overlapping, mixing during sampling without starting chromatography and many more steps to solve specific analytical problems show the flexibility of planar chromatography. HPPLC offers the widest range of sample volume application of any chromatography technique. It can be used for classical HPLC and TLC applications, as well as direct blood, serum, sputum and urine analysis without any external sample preparation.

The separation may end up with 10 to 30 concentric circles. The limit of the peak capacity (number of base-line separated circles) reaches 50 under optimum conditions.

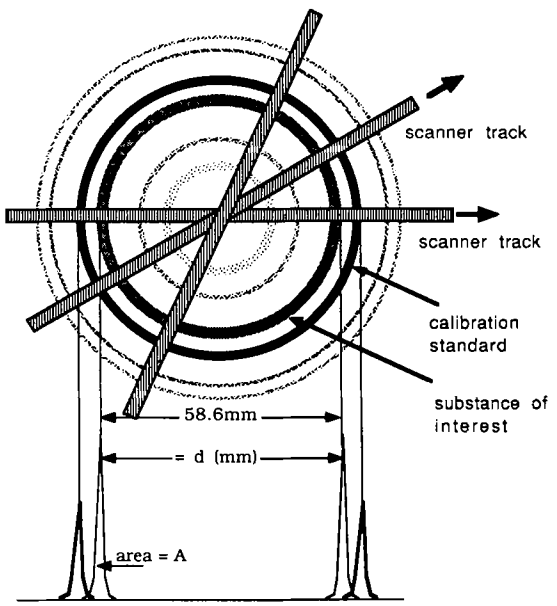

FIG. 5. Quantitation concept in single sample analysis. Area A, mean of the part of the total circular substance line A. This part represents (2 × π × dA × A)/w of the total substance amount present in the layer; w, width of the scanner light beam window; dA, diameter of analyte circle. It can easily be calculated into the total substance signal. The latter is to be compared with the total standard signal (2 × π × dSt × St)/w. dSt = diameter of standard substance circle.

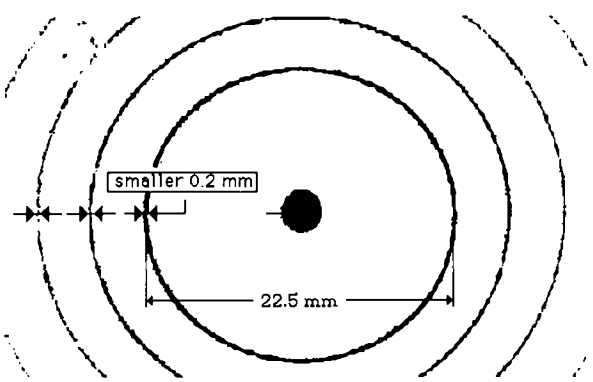

FIG. 6. Direct enlarged computer image of a part of the HPPLC layer. The circles represent sharpness and positions of consecutive injections of the same substance but using a mobile phase of utmost elution strength. The substance runs at the front of the consecutive injected mobile phase portions. The circles are so sharp that conventional scanners cannot quantitate without data falsification. Direct image processing, however, can quantitate such sharp HPPL chromatograms and will be used when layers based upon sharper cuts of smaller particulate matter are available commercially.

Material data

Any mobile phase can be used, although the more volatile ones are preferred. Normally, detection is performed after removal of the mobile phase. Therefore, we need not consider cut-off values for optical characteristics in the 200-800 nm light wave length range.

Mobile phases with melting points below 150°C are applicable. Multi-mobile phase application is possible with or without removal between phase changes.

For stationary phases, silica gel chemically bonded phases with the functional groups $-CH_2-OH$, -phenyl, -phenol-OH, $-NH_2$, -CN, $-(CH_2)_7-CH_3$, $-(CH_2)_{17}-CH_3$, chiral phases on ready-made glass plates are applicable. All must be cleaned under flow-through conditions under pressure. Removal of the 0.2 mm-thick porous layer during the clean-up phase is a rapid process when gas and heat are used.

HPLC is a perfect prechromatography step, not only for HPPLC sampling. This is also true for any planar chromatography mode, in which reconcentration of the transferred substances is possible by removing the mobile phase used in HPLC and refocusing the application spot, band or circle. Circular planar chromatographic techniques, however, are preferable, or else refocusing may be too time-and material-consuming.

Due to simple access to the opened planar chromatography field, dipping and molecular spray-on techniques allow post-run reactions. These can be done after mobile phase removal in a gas phase reaction chamber under very high electrical forces, using any level of temperature that the substances in the layer can accept, and even for long periods.

Biochemical reactions, with their specificity, are as useful as reactions done under the closest contact of the planar chromatography field with a planar-emitting field (a "sending" field) for substances. The latter may touch the HPPLC plate, applying heat on the sending side and cooling on the receiving side. Or, both layers may be kept several μm apart, isolated by the gas phase. Concentrated H_2SO_4 or other aggressive agents may be "molecular-sprayed" without damage in this manner.

Detection and measurement

Detection is done with or without post-run chemical derivatizations of the separated substances on the planar field by direct image-processing (8-10) or by in situ optical scanning. There currently is one commercially available scanner that fulfills the require-

ments of quantitative HPPLC: the CAMAG HPTLC scanner model II with digitizer and RS 232C interface (CAMAG, Muttenz, Switzerland).

The HPPLC plate, in its circular holder, is mounted onto the circular scanning device within the CAMAG scanner and quantitated at the optimum wavelength for the substance(s) to be detected. The scanning dimension is adjusted to a light-sensing window of about 2 to 5 mm × 0.1 to 0.3 mm. This window is moved linearly above the HPPLC plate at a speed of 10 to 20 mm per second, amounting to about 2.5 to 5 seconds of scanning time per track. One or a few of those tracks are scanned at changed plate positions. The scan mode can be linear, circular or tangential and at one or many differing wavelengths. Approximately 500 to 1000 raw data of 12-bit resolution are stored per track onto a hard disk or data disks using the scanner-controlling laboratory computer.

Specificity of detection is selected by the chosen wavelength of remitted UV light within a band width of 10-30 nm, or by digitization of the intensity of emitted fluorescence light induced by sharply filtered UV light. In case the substances are directly (or after derivatization) active within a visible range of light, cheap optical RAM with 64,000 light-sensing pixels can be used for a rapid total-area quantitation. An Apple IIe or Apple IIGS computer is capable of controlling the scanner, or handling the optical RAM signals. Fast IBM PC (clones) with 80386 microprocessors at 25 MHz offer a data treatment speed of 6.13 MIPS and make rapid post-run integration of the many HPPLC data tracks. However, 80286/12 MHz computers or a Macintosh II with 2.5 MIP offer acceptably fast data reduction as well. Even an Apple IIe or Macintosh plus or a normal IBM PC AT allow acceptably rapid data reduction. Macintoshes support the detection and quantitation elegantly by their graphics capability, important when working with huge amounts of data. In fact, HPPLC is the most data-intensive version of all chromatographic techniques.

Because properly selected laboratory computers are cheaper than classical laboratory recorders and intelligent planar chromatography software is available (11), HPPLC quantitation is a rapid and elegant process. Without laboratory computer application, however, the time for quantitation easily exceeds a factor of 100 for the separation time. In case of simultaneous multiple-sample separation HPPLC, detection, quantitation and calibration are done as in normal HPTLC. In case of single sample HPPLC, calibration is done using a standard—sample—standard injection of equal volumes with equal mobile phase injections between the three injections. A "distance" of 50 µl mobile phase, for example, is used between the beginning of the analysis and consecutive sample—standard injections. The three circles of the analytical sample and the standards consist of equal chemicals; therefore, we do not need substance-specific correction factors. However, a position-dependent correction factor is necessary; the quantitative signal of

a given amount of substance depends on the local position of the substance in planar chromatography (7).

Application

Some practical applications for HPPLC include the following:

- Hard drugs in blood, serum and urine at the normal concentration level (1-100 ppm).
- Specific compounds such as cholesterol or caffeine in blood, urine and saliva (at the 3-200 mg/100 ml level).
- Round-robin tests for cholesterol in a synthetic sample compared with quantitative GC (HPPLC cholesterol data—11.4 ± 0.26 mg/g; GC cholesterol data—11.3 ± 0.5 mg/g).
- Vitamin B_{12} in a complex organic environment not applicable to HPLC. The data quality of a nonlinear calibration curve in the 1 to 5 × 10^{-9} g/spot level had an overall statistical quality of 1.3% ± 0.7%. The 1.3% means the mean difference between found and calculated amount; the 0.7% is the mean standard deviation from data point to data point of the third degree nonlinear calibration curve.

HPPLC is directly compatible with micro-packed HPLC fused silica capillaries. The flow through such an HPLC capillary is quantitatively accepted by a well selected HPPLC plate at a local transfer speed of 1 mm/s in direct mechanical contact. The outlet of the HPLC capillary "writes" onto the circular moving plate surface sorbing quantitatively the 1 to 20 µl/min flow. A further and then second dimension separation run under HPPLC conditions may follow after proper refocusing of the transfer line from the HPLC system. The first dimension might have used an amino-bonded stationary phase. The second dimension on the HPPLC plate might use a chiral phase, separating the first dimension subtances into D, L species.

This new technique is capable of offering rapid ion chromatographic separations, as the entire instrumental environment around the stationary phase is glass. The HPPL-chromatograph working at 3000 kg pressure is commercially available (11).

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