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Short Communication

Multidimensional high-performance liquid chromatography on Pinkerton ISRP and RP18 columns: direct serum injection to quantify creatinine

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ABSTRACT

A two-dimensional high-performance liquid chromatographic method for the determination of creatinine with direct serum injection without sample pretreatment has been developed. The column-switching technique allowed a switch from columns packed with internal surface reversed-phase (ISRP) material to columns of almost any other material, even if the eluents necessary in a particular case do not appear to be directly compatible. A Pinkerton ISRP column, which stands out because of its very good stability when loaded with undiluted serum samples, was used as precolumn. The creatinine-containing fraction was switched to a reversed-phase Shandon RP18 column and was focused there by alteration of the eluent from pH 6.5 to phosphoric acid–ion-pair reagent. The separation occurs via a pH gradient, with ultraviolet detection at 234 nm. This method stands out particularly for its good long-term stability, simple sample handling without pretreatment, high selectivity, a broad linearity (0.3–30 mg/dl creatinine), good reproducibility (inter-assay coefficient of variation less than 3%) and high recovery (97–100%) relative to values obtained with gas chromatography–mass spectrometry.

INTRODUCTION

Different high-performance liquid chromatographic (HPLC) procedures for the determination of creatinine in serum have been described in recent years [1–9]. The majority of these methods are associated with laborious sample preparation to separate proteins (protein precipitation, ultrafiltration or solid-phase extraction), which require external standards [8] or time-consuming correcting calculations [9]. Furthermore, calibration inaccuracies caused by matrix effects may occur in some cases. The direct injection of serum samples is possible with only a few procedures [1–3], the precolumns used allowing only short periods of system standstill as they have not been designed for a serum protein load.

The use of the Pinkerton ISRP (internal surface reversed-phase) technique allows direct injection of serum samples with a high protein content without impairing the separation capacity, even with numerous samples. This technique was described by Hagestam and Pinkerton [10] in 1985, and has since been used for the quantification of a great number of serum constituents [11–13]. Owing to the poor chemical stability of the

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phase, the range of available eluents is rather limited; strongly polar substances, such as creatinine, cannot be sufficiently retarded and separated (Fig. 1). The separation of creatinine from the remaining low-molecular-mass components, however, is easily achieved by ion-pair chromatography on an RP18 column in combination with a pH gradient.

By combining the two separation techniques (ISRP and reversed-phase ion-pair), a selective and stable HPLC system can be constructed that combines the high separating capacity of the RP18 material and the stability of the Pinkerton ISRP material with direct serum injection.

The technique described here allows column switching from ISRP material to almost any other material, even if the eluent systems required do not seem to be directly compatible.

EXPERIMENTAL

Chemicals and solutions

The following chemicals and standards were used: creatinine reference material SRM 914 and lyophilized human serum reference material SRM 909 (National Institute of Standards and Technology, Gaithersburg, MD, USA); phosphoric acid (A.R.), sodium hydroxide (A.R.) and sodium dihydrogenphosphate (A.R.) (Merck, Darmstadt, Germany); sodium dodecylsulphate (Serva, Heidelberg, Germany); acetonitrile, HPLC-grade (Baker, Gross-Gerau, Germany); Milli-Q-purified water (Millipore, Eschborn, Germany). The solutions were prepared as follows: (A) 27.6 g of sodium dihydrogenphosphate were dissolved in 1950 ml of deionized water, and the pH was adjusted to 6.5 with sodium hydroxide; (B) deionized water was added to 5.7 g of phosphoric acid and 0.4 g of sodium dodecylsulphate to give 1000 ml; (C) 13.8 g of sodium dihydrogenphosphate and 0.4 g of sodium dodecylsulphate were dissolved in 1950 ml of deionized water, the pH was adjusted to 3.0 with phosphoric acid, and deionized water was added to give 2000 ml; (D) 13.8 g of sodium dihydrogenphosphate and 0.4 g of sodium dodecylsulphate were dissolved in 1950 ml of deionized water, the pH was adjusted to 6.0 with sodium hydroxide, and deionized water was added to give 2000 ml.

Standard solutions in the concentration range 0.3–30 mg/dl were prepared by diluting a creatinine starting solution of 30 mg of creatinine SRM 914 dissolved in 100 ml of deionized water.

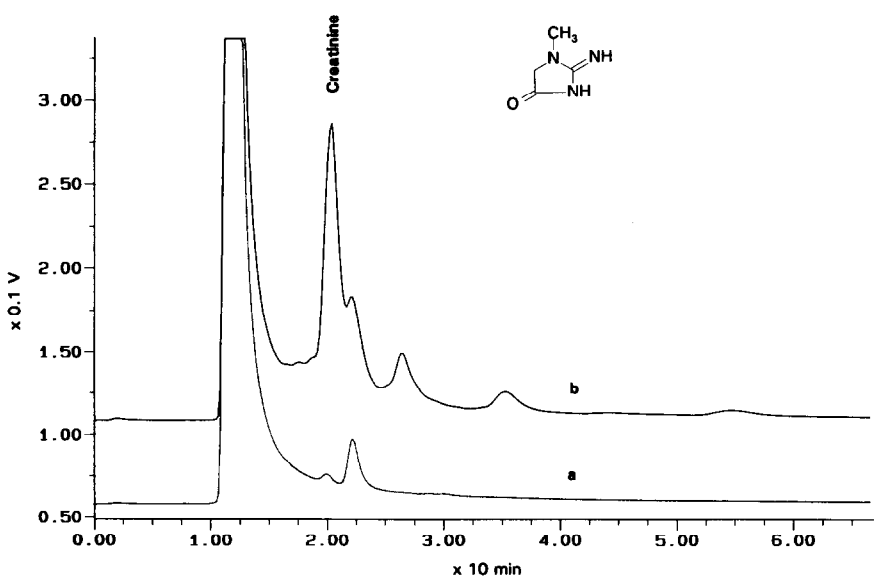

Fig. 1. Chromatograms of serum samples, with separation on Pinkerton ISRP only: (a) normal serum; (b) serum of a dialysis patient.

Samples

Lyophilized human serum SRM 909 was reconstituted according to the manufacturer's instructions. Native human sera were obtained from in-patients. In the sampler of the HPLC system the samples were cooled to 10°C.

Columns

The first column was a Pinkerton ISRP GFF-S5-80, particle size 5 μm, 250 mm × 4.6 mm I.D., from ICT (Frankfurt, Germany). The second column was a Shandon RP18, particle size 5 μm, 125 mm × 4.6 mm I.D., from Bischoff (Leonberg, Germany).

Apparatus

The HPLC system consisted of an HP 1090 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) with a DR 5 solvent-delivery system, a thermostat-equipped autosampler and an autoinjector. The complete system, including the column-switching valves, was controlled via an HP 85 data system. Pumps A and B were Gynkotek-HPLC pumps 300B. Data were collected and evaluated with a Maxima 820 chromatography data system on an IBM AT computer (Waters, Eschborn, Germany). A column-switching valve Rheodyne No. 7010P (ERC, Regensburg, Germany), a T-piece 0.159 cm Swagelok No. SS-100-3 (B.E.S.T., Munich, Germany), a relay box for control of pneumatic valves (laboratory-made), and pneumatic valves (Festo, Murnau, Germany) were also used.

Conditions for separation

The flow diagram in Fig. 2 shows the connection of columns, column-switching valves, HPLC pumps and the HP 1090 gradient system. The position for injection, separation on the ISRP and separation on the RP18 was position A (t = 0.0–3.0 and 3.75–20.0 min), the metering of the sample into the autosampler injection system occurred in position B, and the transfer of the creatinine-containing fraction from the ISRP to the RP18 column occurred in position C (t = 3.0–3.75 min).

Eluent A was used for injection and separation on the ISRP. It was fed via pump A at a flow-rate of 1.0 ml/min. Pump A was directly connected to the eluent intake of the original switching valve B of the HP 1090 autoinjector. The HP 1090 solvent-delivery system was connected to the switching valve A, and delivered the following gradient for separation on the RP18: 0.0–4.49 min, eluent

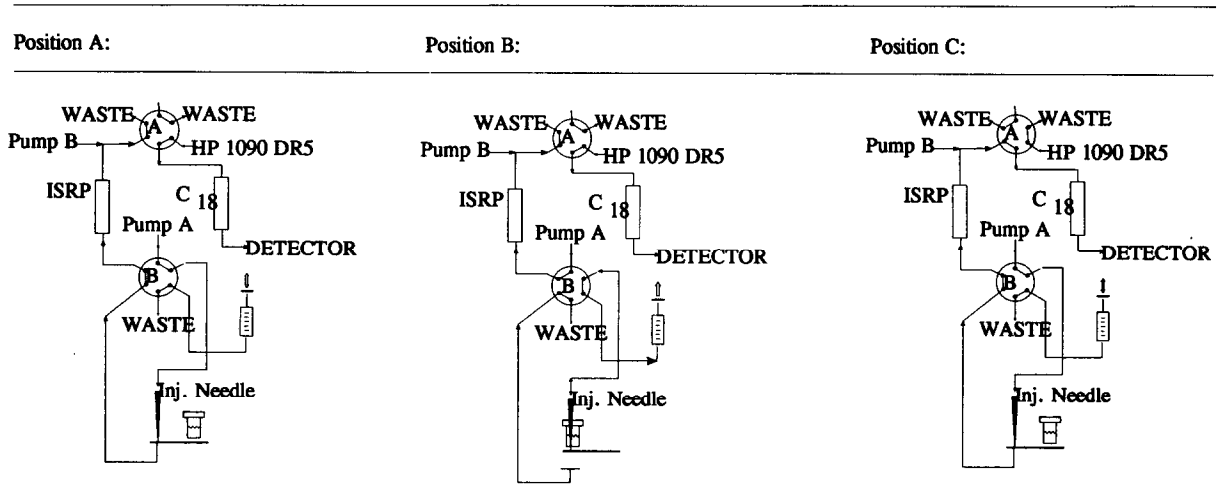

Fig. 2. Flow diagrams for the column-switching system: position A, injection and separation; position B, metering of sample; position C, transfer of the creatinine fraction to RP18.

C; 4.49–7.0 min, linear gradient with eluent D; 7.0–14.0 min, eluent D; 14.0–16.0 min, resetting to eluent C; and 16.0–20.0 min equilibrating with eluent C. The total time is 20.0 min. Pump B fed eluent B at a flow-rate of 1.0 ml/min. It was used to adjust the pH value and the sodium dodecylsulphate concentration to the starting conditions of the RP18 separation.

RESULTS AND DISCUSSION

Multidimensional HPLC using Pinkerton ISRP material and RP18 is made difficult by the limited number of eluents suitable for use with the ISRP material. The limitations are the pH range (pH 6.5–7.5), ionic strength (0.1–0.2 *M*), and the fact that the use of organic eluents is restricted (tetrahydrofuran 10%, acetonitrile 20%, 2-propanol 20%, and methanol 20%, maximum total content of organic constituents 20%). These conditions for separation usually do not allow adequate retardation and thus focusing of polar substances when switching to other stationary phases. Ion exchangers, for example, require extreme pH conditions or ionic strength differences, whereas polar phases (silica, diol, etc.) only work with a high percentage of organic eluents, and RP material generally requires ion-pair formers and extreme pH conditions to retard polar substances.

In the analysis of creatinine in human serum described in this example, a very low pH (phosphoric acid) combined with an ion-pair reagent (sodium dodecylsulphate) is required for focusing on the RP18 column, which is in principle not possible on ISRP material. The necessary conditions can, however, be adhered to, if adequate amounts of phosphoric acid and sodium dodecylsulphate are added to the eluent flow at the outlet of the ISRP column. When the sample is transferred to the RP18 column, the RP18 column is exposed to a rather strong pressure due to increased flow-rates; the results, however, show that the performance of the column is nevertheless sufficiently good and the stability is maintained for a long period of time (more than 400 injections). The ISRP column, which is sensitive to pressure, is used continuously under constant pressure and flow conditions.

Fig. 1a shows that creatinine is separated from

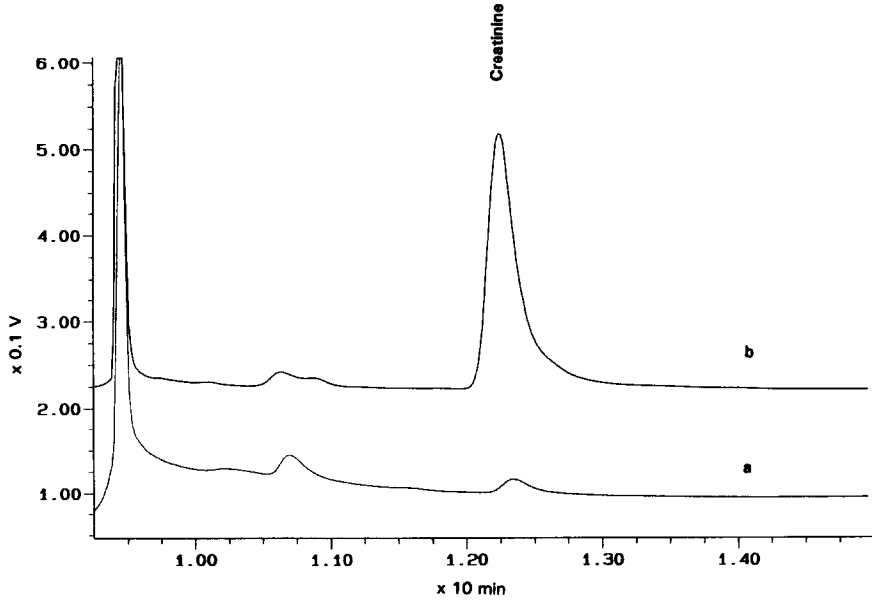

Fig. 3. Chromatograms of serum samples, with separation on the column-switching system: (a) normal serum; (b) serum of a dialysis patient.

a great deal of the other serum constituents (protein and low-molecular-mass components) on the ISRP column. The separation is, however, not sufficient for quantification, because considerable peak overlapping is observed, particularly with pathological serum samples (Fig. 1b). Fig. 3a and b show chromatograms of the same samples when using the column-switching technique, and there is no overlapping of peaks. The linearity range of the method is between 0.3 and 30 mg/dl ($y = -16\,755.9 + 49\,516.5x$, $r^2 = 0.9997$) of creatinine in serum, and thus all the usual clinical samples can be used without prior dilution.

The performance of the procedure was checked with lyophilized human serum SRM 909, and a relative inter-assay standard deviation of 2.9% ($n = 16$ over three independent measuring series) was obtained. The recovery relative to the values assigned by gas chromatography–mass spectrometry was 97%.

The results show that, with an adequate column-switching technique, the chemically rather unstable Pinkerton ISRP material can easily be combined with other HPLC separating materials. The typical disadvantages of this material, such as poor selectivity for strongly polar substances, poor separating capacity and strong tailing of the protein peak, can thus be compensated for. Through combination, HPLC systems are obtained that are stable with respect to direct serum injection and also show high separating capacity and selectivity.

REFERENCES

1 E. Wiedemann, E. Hägele, J. Siedel and J. Ziegenhorn, *Z. Anal. Chem.*, 324 (1986) 333.
2 A. Zhiri, O. Houot, M. Wellmann-Bednawska and G. Siest, *Clin. Chem.*, 31 (1985) 109.
3 G. Werner, V. Schneider and J. Emmert, *J. Chromatogr.*, 525 (1990) 265.
4 A. Harmoinen, P. Sillanaukee and H. Jokela, *Clin. Chem.*, 37 (1991) 563.
5 S. Ekelund and P. Paby, *Scand. J. Clin. Lab. Invest.*, 51 (1991) 67.
6 T. G. Rosano, R. T. Ambrose, A. H. Wu, T. A. Swift and P. Yadegri, *Clin. Chem.*, 36 (1990) 1951.
7 R. Paroni, C. Arcelloni, I. Fermo and P. A. Bonini, *Clin. Chem.*, 36 (1990) 830.
8 J. M. Guy and E. F. Legg, *Ann. Clin. Biochem.*, 27 (1990) 223.
9 G. Schumann, *Fresenius' Z. Anal. Chem.*, 324 (1986) 209.
10 I. H. Hagestam and T. C. Pinkerton, *Anal. Chem.*, 57 (1985) 1757.
11 J. A. Perry, *J. Liq. Chromatogr.*, 13 (1990) 1047.
12 L. E. Mathes, G. Mushik, L. Demby, P. Polas, D. W. Mellini, H. J. Issaq and R. Sams, *J. Chromatogr.*, 432 (1988) 346.
13 Ya-Qin Chu and I. W. Wainer, *Pharm. Res.*, 5 (1988) 680.