Journal of Chromatography, 308 (1984) 354—358
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2060

Note

Therapeutic drug monitoring using high-speed liquid chromatography and rapid sample preparation: an assay for serum theophylline

JAY L. BOCK*, STANLEY LAM and ARTHUR KARMEN

Department of Laboratory Medicine, Albert Einstein College of Medicine, Bronx, NY 10461 (U.S.A.)

(First received October 12th, 1983; revised manuscript received December 13th, 1983)

For routine serum drug measurements, chromatography often has the drawbacks of lengthy analysis and tedious sample preparation. Recently the speed of liquid chromatography (LC) has been increased, through use of smaller-sized silica particles for column packing and low-volume components to minimize band spreading [1]. However, sample requirements for such systems may be more stringent. Microscale protein precipitation with acetonitrile is a convenient preparation technique for conventional high-performance liquid chromatography (HPLC) [2], but Kabra and co-workers [3, 4] found that deproteinized serum causes clogging of a high-speed system.

Lam and co-workers [5, 6] have previously devised a method for serum deproteinization that uses zinc sulfate in combination with methanol or acetonitrile. It yields a visibly clearer supernatant than does either solvent alone. In the present communication we describe an application of high-speed LC for serum theophylline, using this precipitation method followed by chromatography on a short, 3-μm reversed-phase column.

MATERIALS AND METHODS

LC apparatus

We used a Series Two pump, an LC-85 UV detector having a 2.4-μl flow-cell and a 135-msec response time, and a 32 × 4.6 mm reversed-phase column packed with 3-μm C_{18}-bonded silica, all from Perkin-Elmer. Sample application was either manual, with a Rheodyne injector, or automatic with a Perkin-Elmer ISS-100 sampling system. In either case we used a 10-μl sample loop and a

0378-4347/84/$03.00 © 1984 Elsevier Science Publishers B.V

Perkin-Elmer bypass coil, which maintains column flow during valve switching to minimize pressure fluctuations during injection.

Sample preparation

To 50 μl of serum we added 10 μl of zinc sulfate solution (10%, w/v), followed by 75 μl of methanol containing 20 μg/ml of the internal standard, 8-chlorotheophylline. Each sample was mixed by vortexing for several seconds, then centrifuged for 2 min at 1000 *g*.

Sample analysis

A 10-μl volume of supernatant was injected onto the column. Elution was at 2.0 ml/min, ambient temperature, with mobile phase containing 50 ml acetonitrile, 30 ml tetrahydrofuran, and 0.5 ml glacial acetic acid per liter, pH adjusted to 4.9 with sodium hydroxide. Following detection at 273 nm, theo-

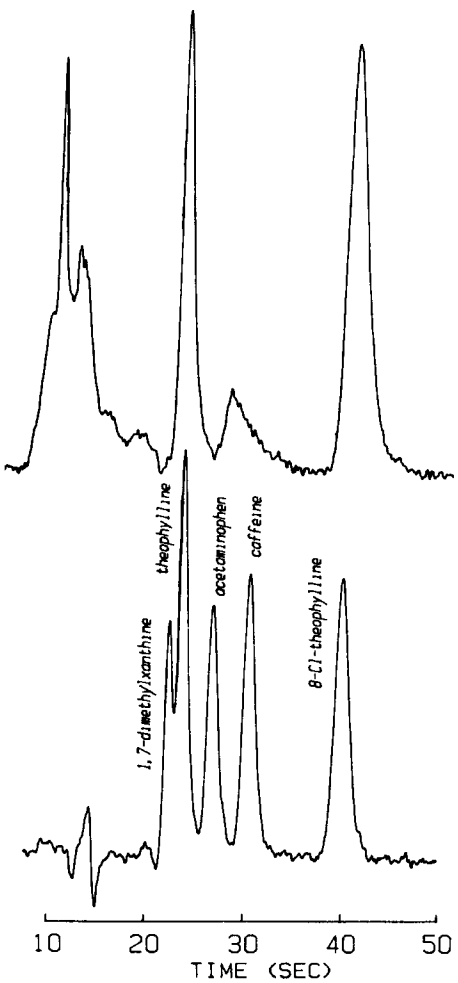

Fig. 1. High-speed LC of (bottom) an aqueous mixture of xanthines and acetaminophen, approximately 10 μg/ml each and (top) de-proteinized serum from a patient on theophylline, concentration measured at 12 μg/ml. Detection at 273 nm, 0.04 a.u.f.s.

phylline was determined from its relative peak height. Standards were prepared in theophylline-free serum.

Correlation with immunoassay

Ninety patient samples assayed by this LC procedure were also tested by an enzyme immunoassay (EMIT, Syva). The EMIT assays were performed using a Rotochem Model R2A centrifugal analyzer (American Instrument Company).

RESULTS

Fig. 1 shows chromatograms of an aqueous mixture of xanthines and acetaminophen, and of de-proteinized serum from a patient on theophylline. Although chromatography is complete in only 40 sec, the quality of separation, in terms of peak widths relative to retention times, is similar to that seen with conventional systems [2, 7]. Baseline resolution of theophylline from caffeine and acetaminophen is achieved, and the resolution from 1,7-dimethylxanthine (paraxanthine) is sufficient to avoid significant interference from this caffeine metabolite [8]. Serum specimens from twenty individuals not on theophylline showed no peaks in the region of theophylline or 8-chlorotheophylline. Substances tested for interference, which were found not to interfere, include theobromine, salicylate, phenobarbital, phenytoin, carbamazepine, procainamide, N-acetyl procainamide, quinidine, acetazolamide, trimethoprim, sulfamethoxazole and ampicillin.

Standard curves exhibited good linearity over a concentration range of 5—40 μg/ml, with correlation coefficients of 0.995—0.998. Precision was determined by repeat assays of a 15 μg/ml serum standard. Within-run coefficient of variation (C.V.) was 2.7% (n = 13), and between-day C.V. was 4.6% (n = 12). Good agreement was achieved between the LC assay and enzyme immunoassay by EMIT, as shown in Fig. 2.

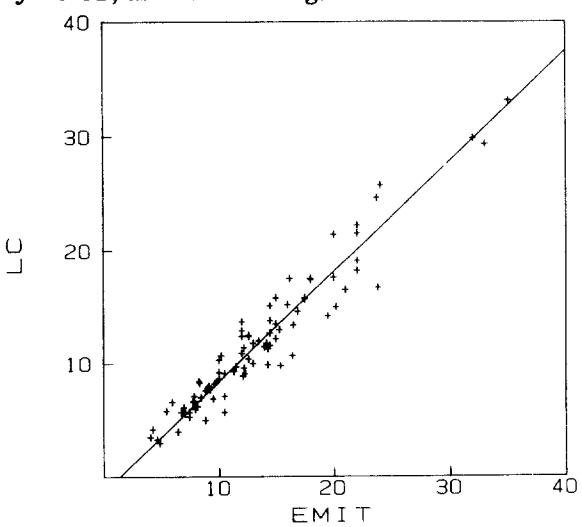

Fig. 2. Correlation between high-speed LC assay of theophylline and immunoassay by EMIT, in 90 patient samples submitted for analysis. Concentrations are in μg/ml. Regression line is LC = 0.970 × EMIT — 1.26, r = 0.959, standard error = 1.24.

During the useful life of each column, samples were injected at about 1-min intervals, and no significant fluctuations in the baseline were observed. However, after about 200 injections deterioration of resolution became noticeable. This deterioration was not related to the use of de-proteinized serum, as it also occurred with repeated injections of an aqueous standard (Fig. 3). It was also found that any sudden changes in flow had a deleterious effect on the performance of the column. Reversing the direction of flow through the column, or changing the packing material at the top, did not give any improvement. The pressure on the column, which was initially about 14 MPa, had generally increased to 20—30 MPa after 200 injections.

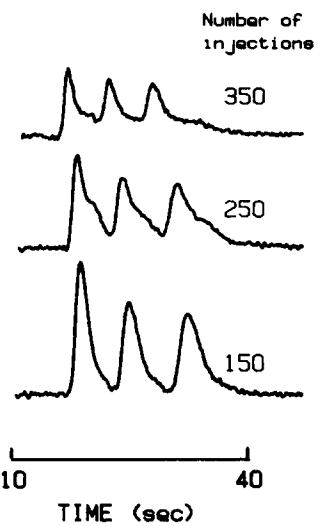

Fig. 3. Deterioration of chromatographic resolution after multiple injections, on a brand new column, of an aqueous standard. The standard contained (in order of elution) theophylline, caffeine, and 8-chlorotheophylline, each 10 μg/ml. The vertical scales for the three chromatograms are identical.

DISCUSSION

Serum theophylline is commonly measured using standard HPLC systems, which give analysis times of several minutes [9]. The procedure described here shortens the chromatographic analysis to 40 sec and uses a rapid method of sample preparation. The time saving not only allows large batches of samples to be processed quickly, but also makes it much easier to re-standardize frequently and perform quality control. For urgent specimens a technician can run the analysis in duplicate along with two standards or controls, and still have results within 10 min.

This procedure appears to be comparable to standard HPLC procedures for theophylline in precision, accuracy, and freedom from interferences. Although the sharpness of peaks enhances sensitivity, this is counterbalanced by a loss in signal-to-noise due to the small flow cell and the dilution that occurs in sample preparation. Reagent expense is minimal, but the economy of this method is currently limited by short lifetime of the column. We could not

pinpoint the cause of column deterioration, but believe it results from mechanical damage to the 3-μm silica. The absence of a more rigorous clean-up step in sample preparation did not appear to have any negative effect on the lifetime of the columns or performance of the system. In contrast, when serum protein precipitation is performed with acetonitrile alone, clogging of a high-speed LC system may result [3, 4].

In summary, the feasibility of high-speed LC for serum drug analysis, using microscale protein precipitation for sample preparation, has been demonstrated. Routine use of this approach would be facilitated by means for increasing the lifetime and decreasing the cost of 3-μm reversed-phase columns.

ACKNOWLEDGEMENTS

We wish to acknowledge the expert technical assistance of Ms. Alice Erling and Ms. June Pieratti.

REFERENCES

1 J.L. DiCesare, M.W. Dong and L.S. Ettre, Introduction to High-Speed Liquid Chromatography, Perkin-Elmer, Norwalk, CT, 1981
2 J.W. Nelson, A.L. Cordry, C.G. Aron and R.A. Bartell, Clin Chem., 23 (1977) 124.
3 P.M. Kabra and L.J. Marton, Clin. Chem., 28 (1982) 687.
4 P.M. Kabra, M.A. Nelson and L.J. Marton, Clin. Chem., 29 (1983) 473.
5 S. Lam, S. Rosenbaum and A. Karmen, Clin. Chem., 26 (1980) 963.
6 S. Lam and A. Karmen, Clin. Chem., 28 (1982) 1670.
7 N. Weidner, D.N. Dietzler, J.H. Ladenson, G. Kessler, L. Larson, C.H. Smith, T. James and J.M. McDonald, Amer. J. Clin. Pathol., 73 (1980) 79.
8 J.H.G. Jonkman, R.A. de Zeeuw and R. Schoenmaker, Clin. Chem., 28 (1982) 1987.
9 K.D. Thakker and L.T. Grady, in K. Florey (Editor), Analytical Profiles of Drug Substances, Vol. 11, Academic Press, New York, 1982, pp. 1—44.