Journal of Chromatography, 221 (1980) 271—277
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 675

ANALYSIS OF SERUM IRON BY GEL PERMEATION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received April 1st, 1980; revised manuscript received July 17th, 1980)

SUMMARY

A high-performance liquid chromatographic procedure for the determination of serum iron is reported. Serum iron extracted with methyl isobutyl ketone was converted to dibenzoylmethane chelate (molecular weight 725), and it was separated from excess dibenzoylmethane (molecular weight 224) by gel permeation chromatography. The chelate was determined by measuring ultraviolet absorption at 280 nm. Good reproducibility, recovery, and correlation with the conventional colorimetric method were observed.

INTRODUCTION

Analysis of serum iron is one of the important clinical tests, and has mostly been performed by a colorimetric method using bathophenanthroline sulfonic acid; however, this method requires a larger quantity of serum compared to other tests. Recently, high-performance liquid chromatography (HPLC) has been introduced into the field of analytical chemistry. Previously, analysis of iron(III) by gel permeation HPLC was reported by Yuki et al. [1]. In this method, iron(III) was extracted into methyl isobutyl ketone (MIBK) from 4—6 *N* hydrochloric acid solution, and was reacted with dibenzoylmethane (DBM), molecular weight 224, to form a chelate compound, iron(III)—$(DBM)_3$, of molecular weight 725. The difference in molecular weights between the chelate and DBM was large enough for the separation of these compounds by gel permeation HPLC. This method, which was simple and sensitive, was successfully applied to the determination of serum iron. Details of these experiments are presented in this article.

EXPERIMENTAL

Reagents and materials

Hydrochloric acid: concentrated hydrochloric acid (reagent grade) was

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mixed with the same volume of water and was distilled twice. To the constant boiling fraction collected was added one-tenth volume of MIBK, and the mixture was shaken well in a separating funnel to remove trace iron. After standing, the hydrochloric acid layer was taken and used. The concentration of the hydrochloric acid after MIBK extraction was 95% of the initial concentration. Hereafter, this MIBK-treated hydrochloric acid will be designated simply as MIBK—HCl. MIBK, a commercial product of reagent grade (Wako, Osaka, Japan) was purified by distillation.

Tetrahydrofuran (THF) was a commercial product prepared for HPLC (Ishidzu, Osaka, Japan). DBM, a reagent grade material (Nakarai, Kyoto, Japan) was purified by distillation at reduced pressure.

Octa-*p*-nitrobenzoyl sucrose was synthesized as reported in the previous paper [1], and was used as an internal standard (I.S.) (molecular weight = 1535.14).

DBM solution: 60 mg of DBM and 50 μg of I.S. were dissolved in 10 ml of THF.

n-Butylamine was a reagent grade material (Wako) and was purified by distillation and used as a 5% solution in THF. Ammonium ferric sulfate was reagent grade (Nakarai).

Control serum was Nescol X from Nihon Shoji (Osaka, Japan). Pooled serum and patients' sera were kindly supplied by Y. Aoki, Toho University Hospital.

Deionized distilled water was used throughout these experiments.

Apparatus

A Shimadzu LC-2 liquid chromatograph was used equipped with a sample injection valve of 0.2 ml capacity, a prepacked HSG-15 column (500 × 7.9 mm; particle size 10 μm; polystyrene type), and a spectrophotometric detector SPD-1 (path length 6 mm, volume 6.4 μl).

Standard procedure for the analysis of serum iron

A mixture of 0.2 ml of serum, 0.8 ml of MIBK—HCl, and 1 ml of MIBK was shaken longitudinally in a glass-stoppered test tube (10 ml) placed horizontally on a reciprocating shaker at 100 strokes/min for 15 min. One-half millilitre of the upper layer was mixed with 1 ml of DBM and 0.1 ml of butylamine solution. After standing for 10 min, 0.2 ml of the mixture was injected into the liquid chromatograph. Elution was carried out with THF at a flow-rate of 0.8 ml/min at room temperature. The I.S. and the chelate were eluted at retention times of 18 and 20 min, respectively. Peaks were detected at 280 nm, and iron was determined by measuring its peak height in comparison with that of the I.S. Sensitivity = 0.08 a.u.f.s.

Calibration curve

To 0.2 ml of standard solutions of iron prepared by dissolving ammonium ferric sulfate in water were added 0.8 ml of MIBK—HCl and 1 ml of MIBK; the mixtures were treated in the same way as given under Standard procedure. A straight line was observed for iron concentrations up to 53.7 μmol/l (300 μg per 100 ml).

RESULTS AND DISCUSSION

Since serum iron is usually bound to protein in the form of transferrin, it is necessary to liberate it from the protein prior to analysis. In the colorimetric method, protein-bound iron is liberated by treatment of serum with 1 *N* hydrochloric acid at 100°C for 1—2 min. As reported in the previous paper [1], 4—6 *N* hydrochloric acid was found to be optimum for MIBK extraction. It was found that the protein-bound iron was dissociated by treatment with 4—6 *N* hydrochloric acid without heating, and the liberated iron was conveniently extracted into MIBK.

Fig. 1 shows the effect of the volume of MIBK—HCl on the MIBK extraction of iron from serum. Addition of 0.8 ml of MIBK—HCl to 0.2 ml of serum gave complete extraction of iron into MIBK. Vigorous mixing of the mixture using a thermomixer resulted in the formation of an emulsion from which it was difficult to separate the organic layer. Thus, conditions for extraction were set as follows: the mixtures were shaken longitudinally in glass-stoppered test-tubes, laid horizontally on a reciprocating shaker, at 100 strokes/min for 15 min. The effect of the shaking time on extraction is illustrated in Fig. 2.

After MIBK extraction, DBM was added to form a chelate. However, as the MIBK extract contained a small amount of hydrochloric acid, DBM did not form a chelate with iron due to the strongly acidic conditions. Although, in

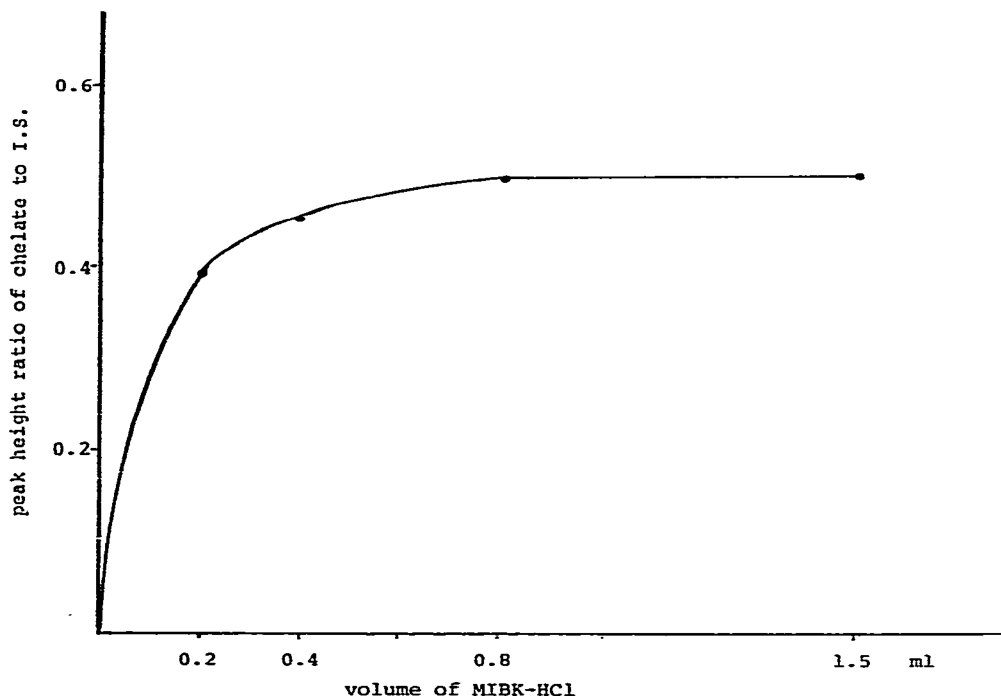

Fig. 1. Effect of volume of MIBK—HCl on extraction of iron(III) into MIBK. To 0.2 ml of serum were added the specified volume of MIBK—HCl and 1 ml of MIBK, and the mixtures were shaken as described in the text. After standing, 0.5 ml of supernatant, 1.0 ml of MIBK, and 0.1 ml of 5% butylamine were mixed, and 0.2 ml of the mixture was injected into the HPLC apparatus. Chromatographic conditions are referred to the text.

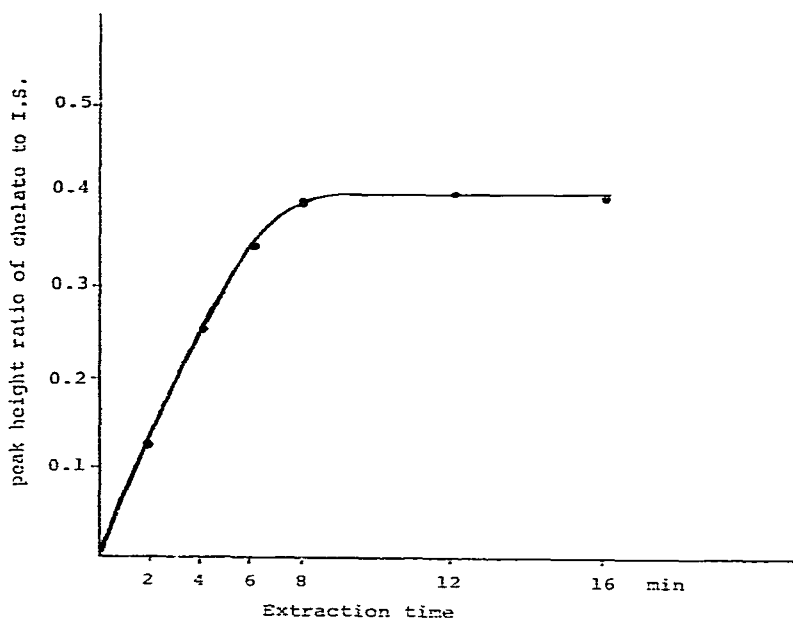

Fig. 2. Relationship between extraction time and extraction efficiency. To 0.2 ml of control serum were added 0.8 ml of MIBK—HCl and 1 ml of MIBK. The mixtures were shaken as described in the text for 2, 4, 6, 8, 12, or 16 min, then treated according to the standard procedure.

the previous study [1], propylamine was added to neutralize the hydrochloric acid extracted, this amine gave rise to a white precipitate, probably propylamine hydrochloride. The formation of a precipitate is undesirable for the subsequent procedures. It was found that addition of butylamine did not give any precipitate, while triethylamine, diisopropylamine, laurylamine, piperidine, and morpholine did. Aromatic amines were not tested because of their ultraviolet absorption.

Absorption at 254 nm was used for the detection of the chelate in the previous experiments [1]. However, with serum samples there was not a good separation between the peaks of the chelate and other substances. As measurements at different wavelengths give different chromatograms, the selection of wavelength is an important factor for quantitative analysis. After a number of experiments, a wavelength of 280 nm was chosen, as it showed the best resolution of the chelate although the sensitivity was slightly lower. Fig. 3 shows chromatograms obtained at 254 and 280 nm for comparison. A straight calibration line was observed up to 53.7 μmol/l using a peak height method measured at 280 nm. A blank test gave a small peak at the same retention time as that of the chelate. Treatment of constant-boiling hydrochloric acid with MIBK made the blank peak smaller. However, in spite of purification of reagents and cleaning of the glassware, complete elimination of the blank peak has so far been unsuccessful. Thus, the peak height of the blank test had to be subtracted from that of the samples run.

Within-day reproducibility was examined with pooled serum. Results obtained with ten runs gave 15.4 ± 0.446 μmol/l (mean ± S.D.) and the coefficient

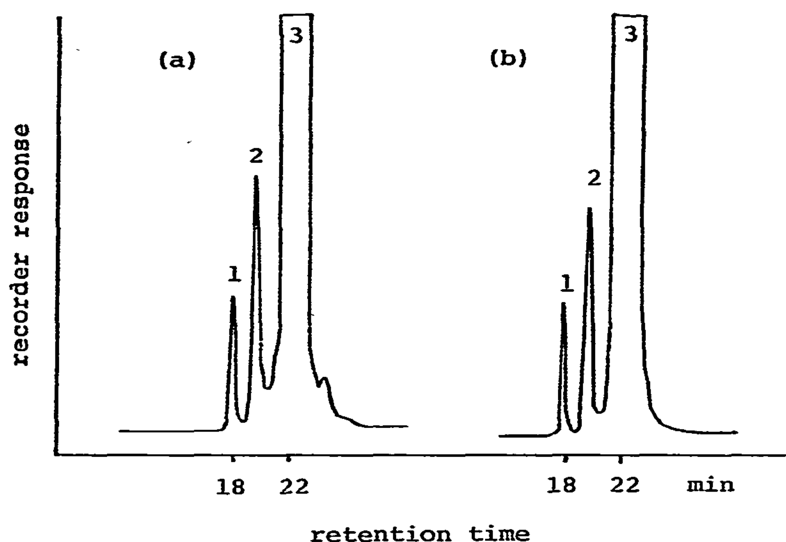

Fig. 3. Difference between chromatograms measured at 254 nm (a) and at 280 nm (b). Control serum (0.2 ml) was treated as described in the text, and chromatograms were prepared at 254 and 280 nm. Peaks: 1 = I.S.; 2 = iron—DBM chelate; 3 = DBM and others.

of variation (C.V.) was 2.83%. Day-to-day reproducibility was examined for ten days with the same serum. Mean ± S.D. was 15.2 ± 0.456 μmol/l, and the C.V. was 2.96%. For the recovery test, dried control serum (17.9 μmol iron per l when dissolved in a specified volume of water) was dissolved by the addition of different concentrations of ammonium ferric sulfate solution, and these sera were analysed by HPLC. Satisfactory recovery was obtained, as shown in Table I.

TABLE I

RECOVERY TEST

Exp. No.	Iron added to control serum (μmol/l)	Iron found (μmol/l)	Recovery (%)
1	0	17.7	
2	0	17.9	
3	0	18.1	
Mean		17.9	
4	9.0	26.3	93.3
5	9.0	26.6	96.7
6	9.0	26.0	90.0
7	9.0	26.8	98.9
Mean			94.7
8	17.9	35.6	98.9
9	17.9	34.3	91.6
10	17.9	35.3	97.2
11	17.9	36.0	101.1
Mean			97.2

Sixty-nine samples were analysed by the proposed HPLC method and a conventional colorimetric method [2], and the two methods were compared. The results are shown in Fig. 4. The regression line obtained was $y = 0.953x + 0.50$ (μmol/l), where y represents the results obtained by the HPLC method and x those obtained by the colorimetric method ($n = 69$). The correlation coefficient (r) was 0.961.

Serum contains some other metals such as calcium, magnesium, copper, zinc, etc. However, MIBK extraction is extremely selective for iron as reported by Goto and Sudo [3] and Tajima and Kurobe [4,5]. This was also stated in the previous paper [1] in which it was demonstrated that a ten-fold concentration by weight of the above ions did not interfere with the determination of iron by gel permeation HPLC. Thus, the values obtained by this method should unambiguously be those of serum iron. This was further evidenced by the correlation coefficient and regression line obtained with patients' sera compared with that obtained with the colorimetric method.

Thus, it was demonstrated that the analysis of serum iron by gel permeation HPLC was selective and sensitive, and the whole procedure could be performed

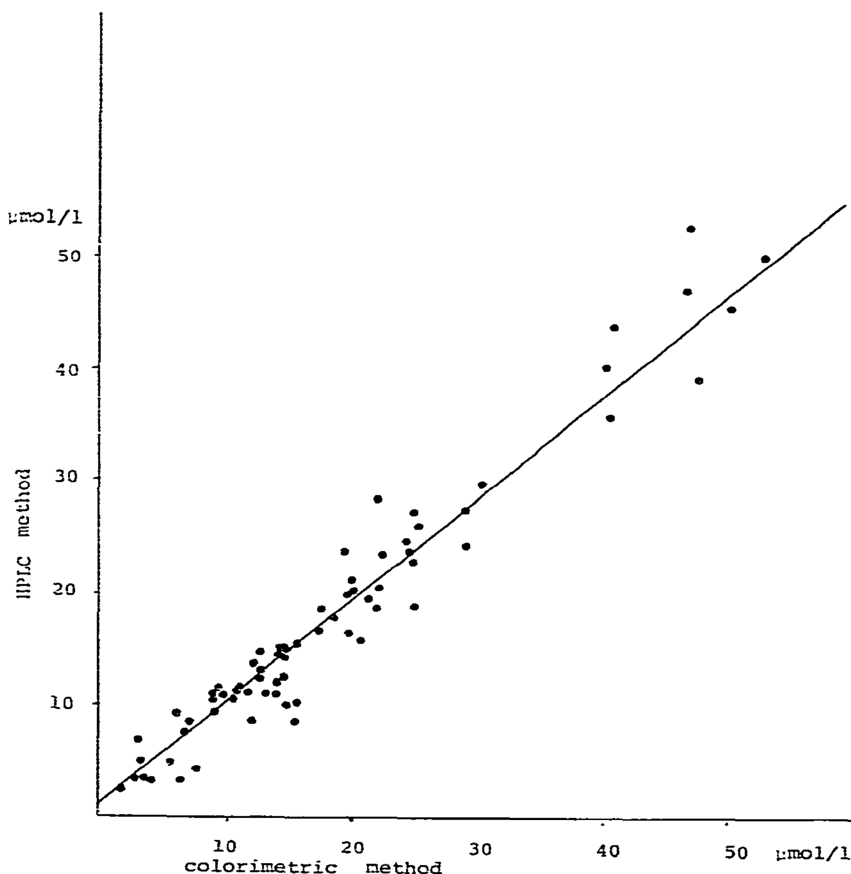

Fig. 4. Sixty-nine patients' sera were analysed by the HPLC method, and the results were compared with those from the colorimetric method. $n = 69$; $y = 0.953x + 0.50$; $r = 0.961$.

within 1 h per sample. Since the first peak appears at 18 min after injection and all UV-absorbing substances are eluted within 30 min, sample injection could be repeated every 15 min. The chelate formed after addition of butylamine is so stable for at least 6 h [1] or more, that a number of samples can be prepared at a time. In the experiments reported here, 0.2 ml of serum was used, but half of this volume was found to be sufficient for routine analysis. Elimination of the blank peak would make the analysis possible with much less serum. It was also expected that elution with a higher flow-rate would reduce the chromatographic time and the interval between sample injections. These possibilities are currently under investigation.

In conclusion, this method is simple since it does not involve a deproteinization step, and can be used in clinical laboratories in its present form. Further improvements suggested above would increase the practical value of the method.

REFERENCES

1 H. Yuki, M. Okuno, H. Kawasaki and A. Yamaji, Bunseki Kagaku (Jap. Anal.), 27 (1978) 27.
2 International Committee for Standardization in Hematology, Blood, 37 (1971) 598.
3 H. Goto and E. Sudo, Bunseki Kagaku (Jap. Anal.), 9 (1960) 213.
4 N. Tajima and S. Kurobe, Bunseki Kagaku (Jap. Anal.), 9 (1960) 399.
5 N. Tajima and S. Kurobe, Bunseki Kagaku (Jap. Anal.), 9 (1960) 798.