CHROM. 6168

METHODS FOR THE QUANTITATIVE ANALYSIS OF SULPHUR-CONTAINING COMPOUNDS IN PHYSIOLOGICAL FLUIDS

B. FOWLER AND A. J. ROBINS*

Willink Biochemical Genetics Laboratory, Royal Manchester Children's Hospital, Pendlebury, Manchester, and Department of Medical Biochemistry, University of Manchester, Manchester (Great Britain)

(Received May 23rd, 1972)

SUMMARY

A continuous flow system utilizing iodoplatinate (H_2PtCl_6–KI) for the detection and quantitation of sulphur compounds in column eluates after ion-exchange separation is described. The conditions of reaction of several sulphur-containing compounds were established. An analytical procedure for the separation of cysteic and homocysteic acids by anion-exchange chromatography is also described. These systems are suitable for biochemical investigations of physiological fluids from subjects with abnormalities of sulphur amino acid metabolism and can be readily incorporated into automated analyzers in current usage.

INTRODUCTION

The necessity for a specific, quantitative method for the analysis of small amounts of homocystine, cysteine-homocysteine, cystine and other sulphur-containing compounds in a study of metabolism of methionine in heterozygotes for homocystinuria has led to the development of an automated system using the iodoplatinate reagent (chloroplatinic acid–potassium iodide). The iodoplatinate reagent is decolourised by organic sulphides[1] and has been used as a spray reagent for paper chromatograms[2]. This facilitates the parallel monitoring of the column effluent of an amino acid analyzer with iodoplatinate in addition to ninhydrin, thus allowing reliable identification and quantitation of relatively small amounts of sulphur-containing compounds in physiological fluids. Most compounds containing sulphur in the II or IV oxidation state react with the reagent, which therefore detects a wider range of sulphur-containing compounds than, for example, the sodium cyanide–sodium nitroprusside reagent. The use of ninhydrin alone in the amino acid analysis of urine is unsatisfactory for the unequivocal identification of small amounts of sulphur-containing amino acids, particularly homocystine, owing to the abundance of ninhydrin-positive compounds in this physiological fluid.

The conditions of reaction of several sulphur-containing compounds with the iodoplatinate reagent have been determined.

* Present address: Department of Biochemistry, Manchester Royal Infirmary, Manchester 13, Great Britain.

An ion-exchange chromatographic method for the determination of cysteic acid and homocysteic acid in oxidized urine is described. This method gives a better separation than that obtained with the method of SCHRAM *et al.*[3].

METHODS

Chemicals

The sulphur compounds used in this study are commercially available (B.D.H. Ltd., Koch-Light Laboratories Ltd., Sigma London Chemical Co. Ltd.) except for S-pyridylcysteine, which was synthesised[4]. Chloroplatinic acid was obtained from Johnson Matthey Chemicals Ltd. All other chemicals were A.R. grade.

Iodoplatinate analytical system

The iodoplatinate reagent used was modified from that of AWWAD AND ADELSTEIN[5] (Table I). The arrangements of and flow-rates in the pump tubes are shown in Fig. 1. The time delay coil is constructed with PTFE tubing, I.D. 1.5 mm, supported

TABLE I

COMPOSITION OF IODOPLATINATE REAGENT

Component	*Amount*
Chloroplatinic acid (10 mg/ml)	15 ml
Potassium iodide (AnalaR)	5.52 g
Glacial acetic acid (AnalaR)	2000 ml
Distilled water	2800 ml

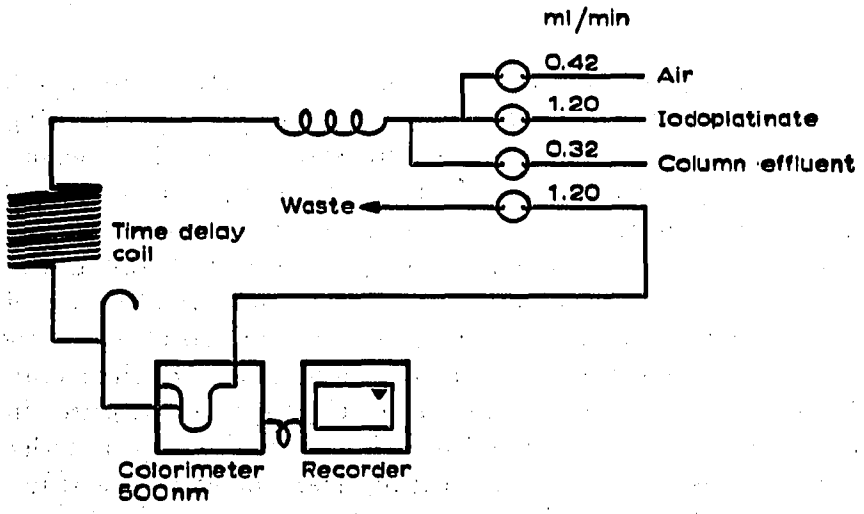


Fig. 1. The iodoplatinate analytical system showing the flow-rates of pump tubes.

in a Perspex frame. The length of the coil is sufficient to give a time delay of 15 min with the pump tubes used. The extinction is then measured at 500 nm using a Technicon colorimeter–recorder system or a Vitatron colorimeter coupled to a Leeds & Northrup Speedomax W multipoint recorder. The output from the colorimeters is adjusted to give an extinction of 0.70 when the reagent is passing through the colorimeter flow cell. The area of peaks obtained with sulphur-containing compounds is

calculated by trapezia summation, the area under the peak being subtracted from the area under the baseline. Suitable internal standards are cysteinesulphinic acid, which is frontally eluted and is loaded on to the ion-exchange column separate from and prior to loading the sample, and S-pyridylcysteine[4], which is eluted close to histidine. As thiodiglycol reacts with iodoplatinate, it is necessary to omit this anti-oxidant from the eluting buffer solutions.

Determination of reaction conditions

The relationship between the decrease in absorbance of the reagent and the concentration of the sulphur-containing compound was demonstrated by automatic sampling of standard solutions. The time course of the reaction was determined by adding 1 ml of sulphur-containing compound (1.0 m*M*) to 5 ml of reagent and monitoring the decrease in extinction at 500 nm in a Unicam SP-800 recording spectrophotometer. The effect of pH on the decolourisation of iodoplatinate by methionine was determined over the pH range 2.0–7.0. The effect of varying the potassium iodide concentration of the reagent on the decolourisation of iodoplatinate by methionine was determined as shown in Table II.

Separation of cysteic acid and homocysteic acid

The chromatographic separation of cysteic acid and homocysteic acid is performed on a 60 × 0.6 cm column of Amberlite CG-4B anion-exchange resin (B.D.H. Ltd.) in the chloride form operated at room temperature and 200 p.s.i. pressure. The positive displacement pump is adjusted to give a column flow-rate of 1.0 ml/min. The column is prepared for analysis by pumping 1.0 *M* HCl for 10 min followed by 0.1 *M* HCl for 40 min. The sample is loaded and amino acids are eluted with 0.1 *M* HCl. The detection system used is ninhydrin–hydrazine.

RESULTS

The relationship between the concentration of sulphur-containing compounds and the decrease in extinction at 500 nm is shown in Fig. 2. The relationship was linear in the range 0–0.15 m*M* for cysteine, lanthionine and methiopropionic acid,

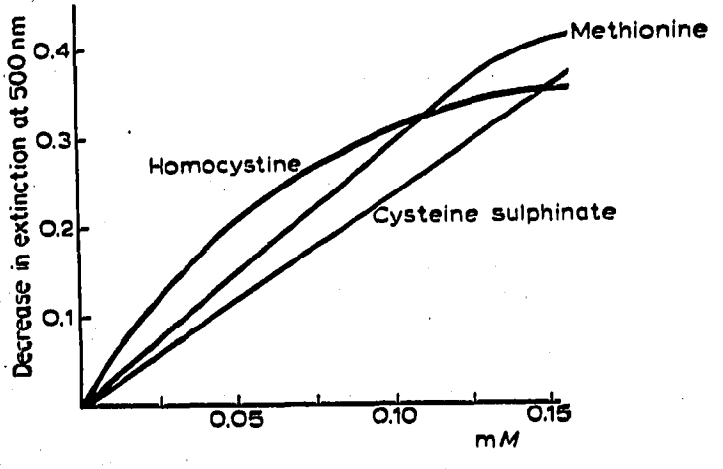
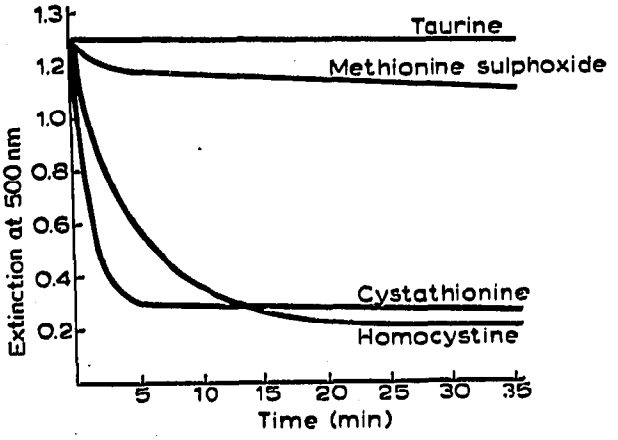


Fig. 2. The relationship between decrease in extinction of iodoplatinate and concentration of sulphur compounds.

while the relationship for cystine and djenkolic acid was similar to that for homocystine.

The time course of the reaction (Fig. 3) for cystine and lanthionine followed homocystine, while that of methionine, cysteine, S-methylcysteine, glutathione, cysteinesulphinic acid and djenkolic acid followed cystathionine. Taurine, which does not react, contains sulphur in the VI oxidation state. An anomalous finding is that methionine sulphoxide reacts to a slight extent whereas cysteinesulphinic acid gives a good reaction with iodoplatinate. Both compounds contain sulphur in the IV oxidation state and the same degree of reaction would be expected for these compounds.



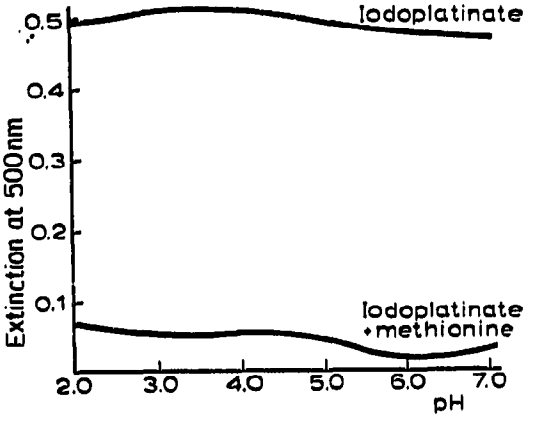


Fig. 3. Time course of the reaction of sulphur compounds with iodoplatinate.

Fig. 4. Effect of varying pH on the decolourisation of iodoplatinate by methionine.

Examination of molecular models suggests that there might be a degree of steric hindrance that reduces the ability of the iodoplatinate ion to react with the sulphur atom of methionine sulphoxide but not with cysteinesulphinic acid. It would appear that disulphides react more slowly than thioethers and thiols. Fig. 3 shows that the reaction of homocystine with iodoplatinate reaches completion in 20–25 min whereas that of cystathionine is completed in 5 min. It is evident that a time delay of 15 min is satisfactory for this reaction, as a high degree of reproducibility is achieved between standards and samples in such an automated system. The effect of pH on the reaction is minimal below pH 7.0 (Fig. 4); the iodoplatinate ion does not exist in alkaline solution. The acetic acid content of the reagent is sufficient to ensure adequate

TABLE II

EFFECT OF VARYING THE CONCENTRATION OF POTASSIUM IODIDE IN THE IODOPLATINATE REAGENT ON THE DECOLOURISATION OF METHIONINE

Chloroplatinic acid (mmole)	*Potassium iodide (mmole)*	*Decrease in extinction at 500 nm with 1 μmole of L-methionine*	*Black material*
0.01	0.3	0.535	Absent
0.01	0.2	0.465	Absent
0.01	0.1	0.285	Absent
0.01	0.06	0.275	Present

buffering of the column effluent, even at the buffer pH of 12.0 used during the later stages of the 5-h amino acid analysis that is used in this laboratory for physiological fluids.

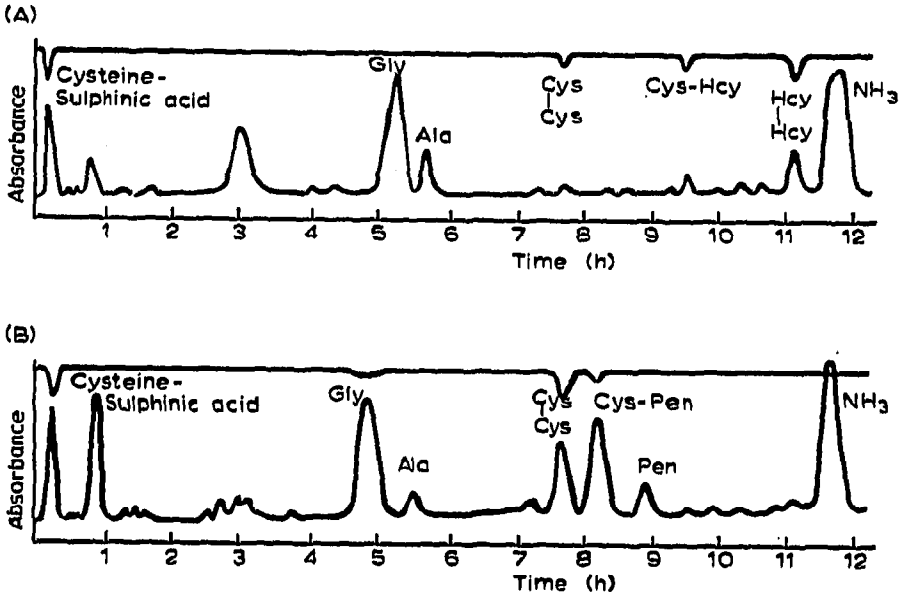


Fig. 5. Tracing of ion-exchange chromatograms obtained with (A) urine from a homocystinuric patient; (B) urine from a cystinuric patient treated with penicillamine. Upper trace, iodoplatinate reaction at 500 nm; lower trace, ninhydrin reaction at 570 nm. 1.0 ml of urine and 0.2 μmole of cysteinesulphinic acid (internal standard) analysed. CysCys = cystine, Cys-Hcy = cysteinehomocysteine disulphide, HcyHcy = homocystine, CysPen = cysteinepenicillamine disulphide, PenPen = penicillamine disulphide.

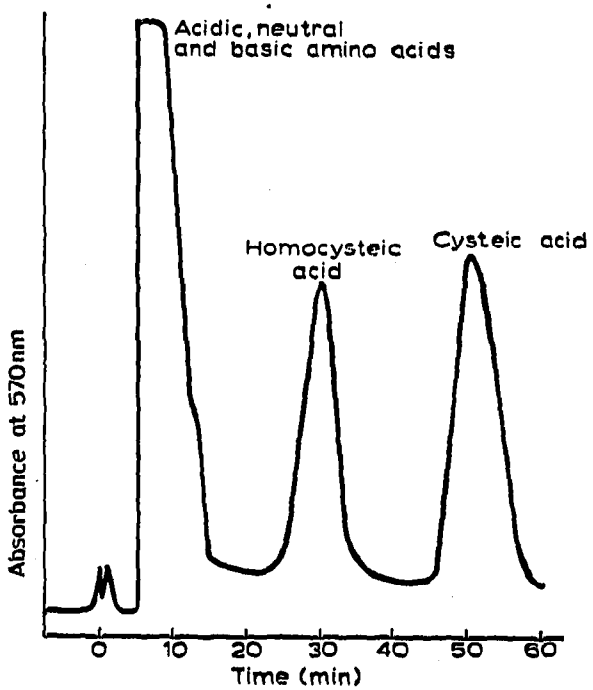


Fig. 6. Separation of cysteic acid and homocysteic acid on an Amberlite CG-4B anion-exchange resin column. 0.2 μmole of each amino acid analysed. Trace corresponds to ninhydrin reaction at 570 nm.

The effect of varying the potassium iodide concentration of the reagent is illustrated in Table II. An excess of potassium iodide relative to chloroplatinic acid is imperative in order to obtain maximum sensitivity and to prevent the deposition of black material in the transmission lines and reaction coils.

Ion-exchange chromatograms obtained with urine from a homocystinuric patient receiving a restricted methionine intake and urine from a cystinuric patient treated with D-penicillamine are illustrated in Fig. 5. The sensitivity of the method is such that homocystine in concentrations as low as 0.002 μmole/ml can be detected. It is evident that with disulphides of penicillamine (β,β-dimethylcysteine), steric factors prevent reaction of the sulphur atom with iodoplatinate.

The separation of cysteic acid and homocysteic acid obtained by ion-exchange chromatography on Amberlite CG-4B resin is shown in Fig. 6. The column of this resin produced a maximum back-pressure of 200 p.s.i. when freshly packed and approximately 100 h of operation is possible before an increase in pressure due to settling down of the non-spherical resin necessitates repacking of the column.

DISCUSSION

The iodoplatinate system has been used successfully in this laboratory for over 2 years. The system is extremely useful for any investigation of sulphur-containing amino acids in physiological fluids, particularly when they are present in small amounts. The system has been used to investigate sulphur metabolism in heterozygotes for homocystinuria and normal individuals following administration of L-methionine[6]; to monitor dietary and drug therapy in homocystinuric and cystinuric patients; to investigate sulphur-containing peptides in homocystinuric urine[7]; and to study the metabolism of L-cysteine in homocystinuric patients[8].

Noise-free baselines have been obtained with the reagent described, although a reagent described by BARBER[9] containing equal amounts (by weight) of chloroplatinic acid and potassium iodide is totally unsatisfactory in a flow system. It has been shown (Table II) that unless the reagent contains a great excess of potassium iodide, a loss of sensitivity and a deposition of black material in the reaction tubing occurs, which leads to an unacceptable baseline. The method could also be readily used with amino acid analysers in which the gas segmentation technique is not used.

The determination of cystine in proteins as cysteic acid by using a column of Dowex 2 X10 in the chloroacetate form and elution with 0.1 M chloroacetic acid has been described[3]. The elution of homocysteic acid and cysteic acid in a similar manner using a flow-rate of 1 ml/min at 25° but with automated monitoring of the eluate with ninhydrin in a continuous flow system resulted in broad, flat peaks, thereby reducing the sensitivity of the method. The use of an Amberlite CG-4B column in the method described results in a marked improvement in resolution and peak shape, resulting in greater sensitivity. This ion-exchange chromatographic system has been operated by a fully automated gradient elution device[10] and would seem to be easily adapted for use in other automated amino acid analyzers.

ACKNOWLEDGEMENTS

The financial support of the Children's Research Fund, Liverpool, and the National Fund for Research into Crippling Diseases (Action for the Crippled Child)

is gratefully acknowledged. The authors wish to thank Dr. G. M. KOMROWER (Director) and Dr. I. B. SARDHARWALLA for encouragement and facilities at the Willink Biochemical Genetics Laboratory, and Dr. D. B. GORDON for help and advice.

REFERENCES

1 T. L. SEASE, T. LEE, G. HOLMAN, E. H. SWIFT AND C. NEINMANN, *Anal. Chem.*, 20 (1948) 431.
2 G. TOENNIES AND J. J. KOLB, *Anal. Chem.*, 23 (1951) 823.
3 E. SCHRAM, S. MOORE AND E. J. BIGWOOD, *Biochem. J.*, 57 (1954) 33.
4 J. F. CAVINS AND M. FRIEDMAN, *Anal. Biochem.*, 35 (1970) 489.
5 H. K. AWWAD AND S. J. ADELSTEIN, *Anal. Biochem.*, 16 (1966) 433.
6 B. FOWLER, I. B. SARDHARWALLA AND A. J. ROBINS, *Biochem. J.*, 122 (1971) 23P.
7 P. C. SHERLOCK, *M.Sc. Thesis*, University of Manchester, 1971.
8 I. B. SARDHARWALLA AND B. FOWLER, *Arch. Dis. Childhood*, 47 (1972) 150.
9 G. W. BARBER, *Technicon Symposium, New York, 1966*, p. 401.
10 A. J. THOMAS, in A. BAILLIE AND R. J. GILBERT (Editors), *Automation, Mechanisation and Data Handling in Microbiology*, Academic Press, London, 1970, p. 107.