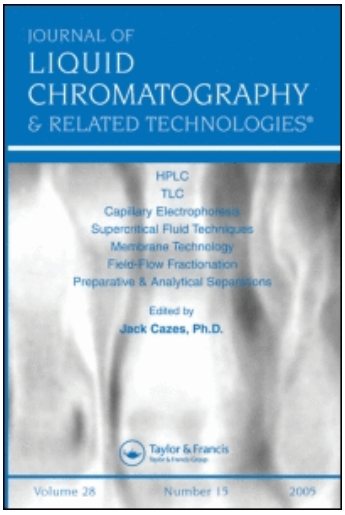

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High Performance Liquid Chromatographic Assessment of the Quality of Materials Employed as Calibration Standards in Creatinine, Urea, Uric Acid and Bilirubin Determinations

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSESSMENT OF THE QUALITY OF MATERIALS EMPLOYED AS CALIBRATION STANDARDS IN CREATININE, UREA, URIC ACID AND BILIRUBIN DETERMINATIONS

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ABSTRACT

High performance liquid chromatography has been used to analyse various commercial urea, creatinine, bilirubin and uric acid standards. This technique has demonstrated variation in the purity of standards from different manufacturers and also the instability of urea, bilirubin and uric acid standards following storage.

INTRODUCTION

Accurate analytical results are essential in the clinical laboratory. Inaccuracy in an analytical result may be due to a

number of factors such as the specificity of the analytical procedure, its susceptibility to interference, and the material used as a calibration standard. The stated value of a calibration standard must be as accurate as possible since error or uncertainty in this value will be reflected in the results of patients specimens (1, 2).

Calibration standards in clinical chemistry can be of two types:-

(a) an aqueous or primary standard solution in which the concentration is determined solely by dissolving a weighed amount of standard material in an appropriate solvent and making it up to a stated volume or weight, and

(b) a secondary standard solution in which the concentration is determined by repeated chemical analysis. An example of the latter type is the serum based standard (1). These are based on serum from which all dialysable solutes have been removed and known amounts of constituents then added. Thus whilst there are advantages and disadvantages to both types of standard (3, 4) both rely on the addition of analytes to a matrix whether aqueous or serum and therefore the quality of both is dependent on the quality of the added constituents. The two important factors determining the quality of these standards, are their purity and stability. Purity may be assessed by a range of chemical or physical techniques either singly or in combination. For example physical methods used to assess the purity of standard reference materials (SRM) include neutron activation analysis, polarography, atomic absorption

spectroscopy, paper and thin-layer chromatography, differential scanning calorimetry, phase solubility analysis and emission spectrometric analysis. Gas chromatography has also been used to identify and measure impurities, e.g., those present in the buffer, 2-amino-2-methyl-1-propanol which is used in alkaline phosphatase assays (5). Finally, Clark and Kricka (6) have demonstrated the use of reversed phase high performance liquid chromatography (HPLC) for monitoring the purity of substances used to prepare calibration standards.

In most cases, stability of a standard is assessed by chemical analysis after storage in a controlled environment. Thus enzyme activity may be measured in a standard stored for a period of time (hours-years) at a specified temperature. Accelerated stability studies (e.g., exposure to higher temperatures) may be useful in investigating the long term stability of a standard (7). However, chemical analysis in stability studies may not provide information on the relative importance and analytical effect of breakdown products.

The object of this study was to investigate the purity and stability of materials commonly used as standards for urea, uric acid, creatinine and bilirubin determinations by means of high performance liquid chromatography.

MATERIALS

Standards

Samples of analytical grade urea, uric acid, creatinine and bilirubin were obtained from various manufacturers (Table 1) and

TABLE 1 Details of Standards Used

Standard	Manufacturer	Product Number	Lot Number
Urea	1	66612	500687
	2	U-1250	57C-0026
	3	45204	6371390
	4	-	912
Uric acid	1	6671	600285
	2	U-2625	67C-0001
	4	-	913
	5	893000	61274
Creatinine	1	2390	602143
	2	C-6257	45C-0436
	3	37049	6560110
	5	350200	58804
Bilirubin	1	2011	602527
	3	44009	2524990
	5	219240	14097

Key

1. Calbiochem Ltd., 79-81 South St., Bishops Stortford, CM23 2AC England.
2. Sigma London Chemical Co. Ltd., Fancy Road., Poole, BH17, England
3. B.D.H. Chemicals Ltd., Poole, BH12 4NN, England.
4. National Bureau of Standards, Standard Reference Material.
5. Hopkin and Williams, Romford, RM1 1HA, England

samples of Standard Reference Materials (SRM) of urea and uric acid were obtained from the Office of Standard Reference Materials, National Bureau of Standards, Washington DC., 20234, U.S.A.

Standard solutions for HPLC analysis

Urea (100 mmol/l in distilled water); creatinine (10 mmol/l in 0.1 mol/l acetic acid); uric acid (10 mmol/l) containing lithium carbonate 8 mmol/l, formaldehyde (2 ml/l) and glacial acetic acid (1.5 ml/l); a second uric acid standard (500 µmol/l) was made up in 8 mmol/l aqueous lithium carbonate; bilirubin (25 µmol/l in HPLC grade methanol).

METHODS

HPLC Analysis

This was carried out using a Pye Unicam LC3 with a variable wavelength detector and under the following conditions: Column 25 cm x 5 mm I.D. Hypersil ODS (5µ) (Shandon Southern Ltd., Runcorn, Cheshire); ambient temperature, mobile phase (degassed) of 0.01 mol/l KH_2PO_4 : methanol (3 : 1 v/v) (HPLC grade, Rathburn Chemicals, Walkerburn, Scotland); flow rate 1 ml/min; wavelength of 210 nm, 292 and 453 nm, sample size 10 µl. Samples were introduced onto the column using either valve (Pye Unicam) or septum injection (Shandon Southern). All analyses were performed in duplicate and on two Hypersil ODS (5µ) columns. Analysis of samples on two columns and using two different modes of injection was undertaken in order to eliminate any artefacts due to possible "memory" effects by the injector or column. Bacteriologically contaminated standards, as judged below under Bacteriological Studies, were filtered through a

0.45 μm filter (Millipore Filter Corp., Mass. USA) to remove bacteria.

Stability Studies

HPLC analysis was used to assess the effect of storage of the various standard solutions under the following conditions: (a) 48 h at 37°C; and 6 weeks at (b) 4°C in diffuse light (c) ambient temperature in the dark (d) ambient temperature in daylight. Conditions, b, c and d were chosen to simulate a typical range of laboratory storage conditions.

Bacteriological Studies

No bacteriostatic agents were added to any of the standard solutions studied and solutions were therefore tested for bacterial contamination by plating out on yeast extract agar and incubation under the conditions used for storage of that particular solution. Further identification tests were carried out as appropriate.

RESULTS

HPLC analyses of solutions of the various standards demonstrated the presence of multiple components. The finding of multiple components on analysis can be an HPLC artefact possibly due to impure solvents, poor injection, high dead volume couplings, poorly packed columns or residual silanol groups on the column packing. Analytical runs in which each of the above factors were tested in turn indicated that the multiple components were genuine and not due to instrumental or chromatographic factors.

Urea

HPLC analysis demonstrated a variation in the number of UV absorbing components in standard solutions prepared from urea obtained from different manufacturers (Figures 1a-d). Standard solutions were found to be stable and, for each manufacturer, differences in the number of peaks were shown between the freshly prepared solutions and those that had been stored, particularly those stored at either 37°C for 48 hours or at 4°C in diffuse light for 6 weeks (Figures 1e-h).

Bacteriological screening revealed gross bacterial contamination of all the urea solutions except those stored at 4°C. Six types of bacteria were isolated, 5 were gram negative rods and one gram negative coccibacilli. None of the bacteria could be shown to utilise urea as a sole carbon source.

Creatinine

One UV absorbing peaks was obtained for each freshly prepared creatinine standard from each manufacturer. Long term storage (6 weeks) of the creatinine standard in the dark and light at room temperature resulted in splitting of the main creatinine peak. Storage at 37°C for 48 hours did not result in any changes (Figure 2). No bacterial contaminants were detected in any of the creatinine solutions either fresh or stored.

Uric Acid

Fresh solutions of uric acid from various manufacturers and that from NBS (made up in Li_2CO_3) showed a single peak on HPLC analysis (Figure 3a), with no variation between manufacturers either at

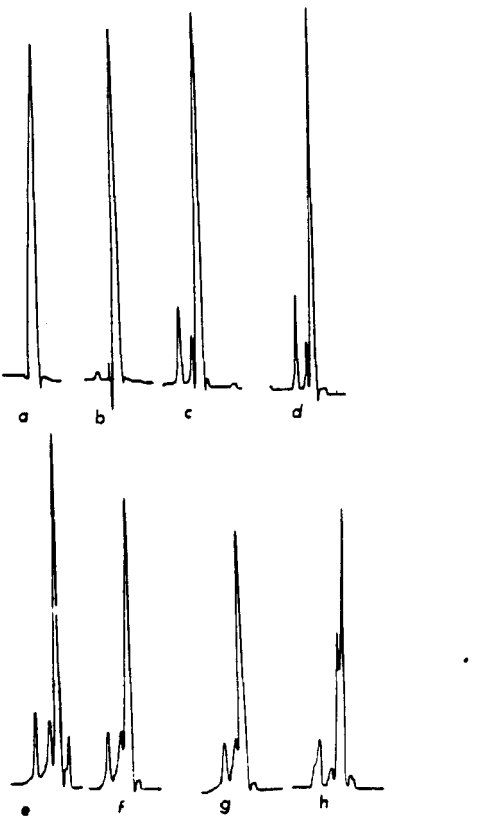

Figure 1 HPLC Chromatograms of Fresh and Aged Urea Standards

Key: Fresh standard solutions (a) NBS SRM (b) B.D.H. (c) Sigma (d) Calibiochem. Storage of the Calibiochem standard solution at (e) 4°C in diffuse daylight for 6 weeks (f) room temperature in daylight and (g) in the dark and (h) 37°C for 48 h. Detector wavelength 210 nm.

210 nm or 292 nm (the latter a wavelength at which uric acid shows maximum absorption). Fresh solutions of uric acid from various manufacturers (made up in Li_2CO_3/HCHO/CH_3COOH) showed three main peaks and the relative proportions of these varied from manufacturer to manufacturer, particularly at 292 nm (Figures 3c-f).

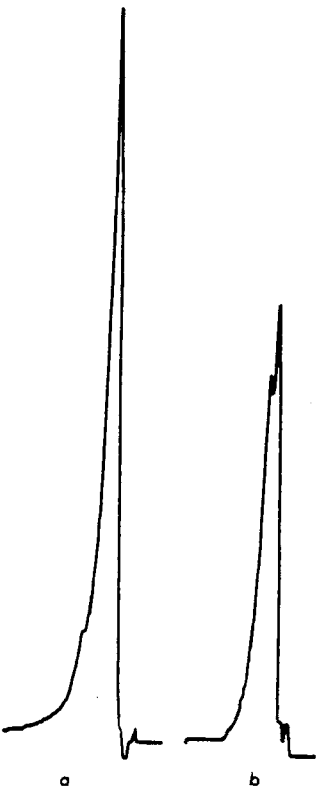

Figure 2 HPLC Chromatograms of Fresh and Aged Creatinine Standards

Key: (a) Fresh standard solution (b) after storage for 6 weeks at room temperature in the dark. Manufacturer: Hopkin and Williams. Detector wavelength 210 nm.

Stability studies of uric acid solutions made up in Li_2CO_3 showed an increase in the number of UV absorbing constituents (210 nm) and a decrease in peak height on storage (Figure 3b). This was not the case at 292 nm when only one peak was detected.

Solutions made up in Li_2CO_3/HCHO/CH_3COOH for each manufacturer showed a loss in one peak at 210 nm (and a similar loss at 292 nm)

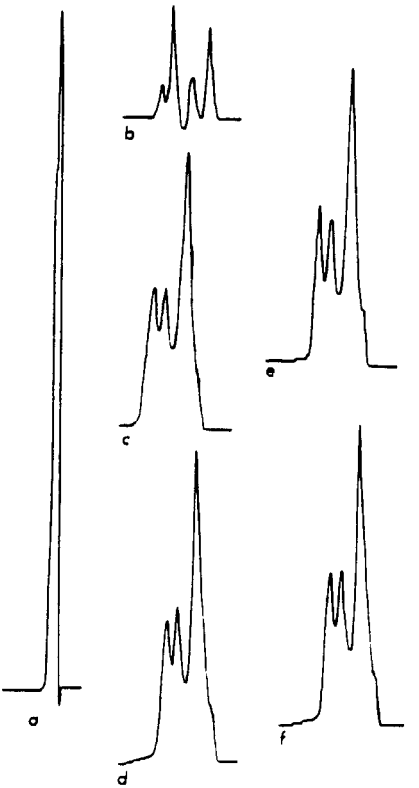

Figure 3 HPLC Chromatograms of Uric Acid Standards

Key: (a) Fresh standard (Calbiochem) (made up in lithium carbonate solution) (b) Effect of storage at 37°C for 48 h on (a) Fresh standards of uric acid made up in acetic acid/formaldehyde/ lithium carbonate solution from the following manufacturers: (c) Calbiochem (d) Hopkin and Williams (e) Sigma and (f) NBS. Detector wavelength 210 nm, (a), (b); 292 nm, (c)-(f).

after storage of the solutions at room temperature in light and dark for 6 weeks (Figure 4). Analysis of the preservatives themselves showed no significant peaks. No bacterial contamination was detected in any of the uric acid standards.

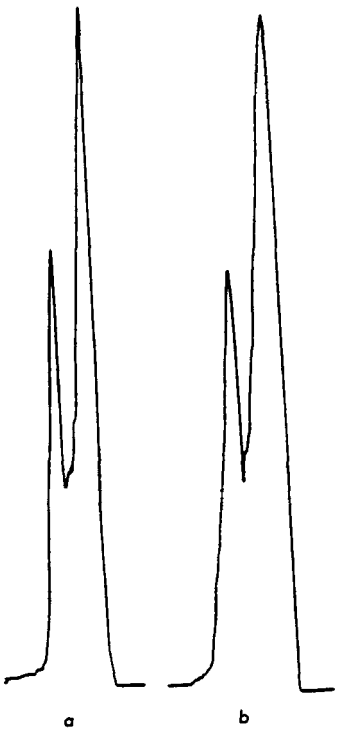

Figure 4 HPLC Chromatograms of Aged Uric Acid Standard Solutions

Key: (a) Effect of storage standard solution for 6 weeks at room temperature in the light and (b) in the dark. Detector wavelength 210 nm. Manufacturer: Calbiochem.

Bilirubin

At a detector wavelength of 210 nm two peaks were obtained on HPLC analysis of bilirubin standards with little variation between manufacturers (e.g. Figure 5a). On storage, under all the conditions investigated, all the manufacturers bilirubin standards showed an increase in the number of UV absorbing peaks (Figure 5b, c).

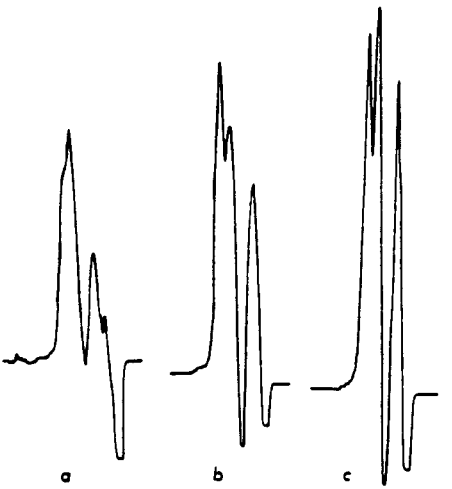

Figure 5 HPLC Chromatograms of Fresh and Aged Bilirubin Standards

Key: (a) Fresh standard solution and after storage for 6 weeks (b) at 4°C in diffuse daylight (c) room temperature in the light.

No bacterial contaminants were detected in any of the bilirubin solutions either fresh or stored.

DISCUSSION

Most of the interest in the quality of standards has understandably centered on those substances which are unstable or difficult to obtain in a pure state, e.g. enzymes and other proteins. The quality of the more readily available standards commonly employed in the clinical laboratory seems not to have been questioned.

Studies presented here have shown that standards, especially urea, uric acid and bilirubin currently employed as calibration

standards, contain more than one UV absorbing constituent and the proportion of these varies between manufacturers.

Urea showed two or three major UV absorbing components and the pattern and relative intensities of the components varied between manufacturers. The identity of these components is unknown. Industrially urea may be prepared either by the thermal degradation of ammonium carbonate formed from liquid carbon dioxide and liquid ammonia or by the partial hydrolysis of cyanamide in acidic solution (8). Reaction intermediates from these processes, isomers (ammonium isocyanate) or thermal degradation products (biuret) may persist in urea preparations and give rise to the additional UV absorbing components found on HPLC analysis of urea standards.

Interpretation of the chromatograms of aged urea solutions is complicated by the presence of bacteria. However, the similarity between the results of the accelerated stability study and the longer term study indicate that there are no arterfacts attributable to the presence of bacterial metabolites in the specimens.

Creatinine standards showed only one peak on HPLC analysis and little variation from manufacturer to manufacturer. Long term storage of the creatinine resulted in the appearance of a double peak on HPLC analysis and this may reflect the presence of creatinine in the solution which was not found in the accelerated stability study.

Similarly, bilirubin showed numerous peaks on HPLC analysis and variation between manufacturers was found. Commercial bilirubins are usually prepared from gallstones or bile and are thus liable to

contamination from other tetrapyrroles. Bilirubin is known to be extremely light sensitive and is readily converted to various photo-products. The multiplicity of UV absorbing peaks obtained both in fresh and aged standards reflects this conversion and also the occurrence of various isomeric forms of bilirubin (9, 10, 11).

In the literature there are several opinions regarding the stability of uric acid solutions (12, 13) different authors recommending the inclusion of various preservatives and differing storage conditions. HPLC analysis of uric acid standards has proved complex. When prepared in lithium carbonate alone only one peak was found and when prepared in Li_2CO_3/HCHO/CH_3COOH three peaks were found both at 292 nm and 210 nm. It is unlikely that the two extra peaks were due to the added preservatives for several reasons: (i) At the concentrations used, none of the preservatives gave significant peaks at 210 nm and no response at all at 292 nm. (ii) The retention times of the small peaks due to the preservatives do not correspond to any of these multiple peaks. (iii) The multiple peaks were also found using the wavelength 292 nm. It would therefore seem that the multiple peaks are various forms of uric acid which have arisen as a result of the presence of the preservatives acetic acid and formaldehyde but which are not the preservatives themselves. Other authors have noted the appearance of a second UV absorbing peak when uric acid standards are made up in formaldehyde solution (14).

Stability studies of uric acid solutions made up in Li_2CO_3 showed a dramatic increase in the number of peaks and reduction in

peak height (at 210 nm) on storage. However, although a decrease in peak height on storage was demonstrated at 292 nm no new peaks were found. This would seem to indicate the breakdown of uric acid, which absorbs maximally at 292 nm, to UV absorbing components.

Solutions of uric acid made up in the presence of all three preservatives, Li_2CO_3/HCHO/CH_3COOH showed a loss of one peak on storage. This peak could not be identified as one of the preservatives, but this loss may represent a re-equilibration of the uric acid forms with the preservatives.

This study has demonstrated the use of HPLC analysis to monitor the stability of standards. This is particularly important as few published methods recommend conditions of storage of standards. This study has also shown that great variation in the purity and stability of calibration standards of urea, bilirubin and uric acid from various manufacturers exists, as demonstrated by HPLC. Whilst it has not been possible to identify the UV absorbing impurities and hence determine their relative concentrations in all cases the NBS SRM gave the fewest peaks on HPLC analysis. The significance of these impurities to the routine methods for the estimation of urea, uric acid, creatinine and bilirubin is under investigation, and it is expected that their relevance may depend on the analytical method used.

CONCLUSION

HPLC analysis offers a sensitive and convenient technique for monitoring the quality of substances used to prepare calibration

standards and would appear suited to the routine assessment of standards employed in chemical analyses.

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REFERENCES

1. J. Buttner, R. Borth, J.H. Boutwell and P.M.G. Broughton Clin. Chim. Acta 69 (1977) F11-F20.

2. J. Buttner, R. Borth, J.H. Boutwell and P.M.G. Broughton Clin. Chim. Acta 75 (1977) F11-F20.

3. P.M.G. Broughton Lab-Lore 6 (1975) 369-372.

4. D. Stamm Z. Klin. Chem. Klin. Biochem. 12 (1974) 137-145.

5. J.C. Thompson Ann. Biol. Clin. 36 (1978) 329-330 (Abstr.).

6. P.M.S. Clark and L.J. Kricka Ann. Biol. Clin. 36 (1978) (Abstr.).

7. M. Grassel in Industrial Aspects of Biochemistry (B. Spencer, ed) Federation of European Biochemical Societies, (1974) p 891-911.

8. I.L. Finar Organic Chemistry, 6th edn., Vol 1, Longmans, London (1973).

9. H Fog Scand. J. Clin. Lab. Invest. 16 (1974) 49-54.

10. A.F. MacDonagh and F. Assisi FEBS Letters 18 (1971) 315.

11. I.A.D. Bouchier and B.H. Billing Bilirubin Metabolism, Blackwell, Oxford (1967).

12. R.J. Henry Clinical Chemistry, Principles and Techniques. 2nd edn., Hoeber Medical Division, Harper and Row, New York (1974).

13. T. Feichtmeir and H. Wrenn Am. J. Clin. Path. 25 (1955) 833-845.

14. E.J. Kiser, G.F. Johnson and D.L. Witte Clin. Chem. 24 (1978) 536-540.