Journal of Pharmaceutical & Biomedical Analysis
Vol. 2, Nos 3/4, pp. 417–424, 1984
Printed in Great Britain

0731–7085/84 $3.00 + 0.00

Total phosphorus determination in human bile. Comparison between two spectrometric methods

MAURO TOMASSETTI*[1], LUIGI CAMPANELLA[1], ANNA MARIA SALVI[1], GIUSEPPE D'ASCENZO[2] and ROBERTA CURINI[2]

[1] *Dipartimento di Chimice, Università di Roma, Piazza A. Moro 5, 00185 Rome, Italy*
[2] *Dip. di Scienze Chimiche, Università di Camerino, Italy*

Abstract: The two most commonly used spectrometric methods for the determination of the phosphorus content of human bile are compared. The optimum experimental conditions are studied, and the analytical characteristics of the two methods, using both standard samples and human bile, are evaluated. The methods are compared on the basis of their sensitivity, precision and accuracy, and the correlation between the two techniques demonstrated using fifteen samples of human bile. Data obtained by both methods have been used to calculate lithogenic index values.

Keywords: *Bile phosphorus levels; lithogenic index; colorimetric analysis of phosphorus.*

Introduction

The determination of total phosphorus in human bile is of increasing interest [1, 2], as the lithogenic index can be calculated from the results if cholesterol and biliary acid concentrations are also known [3, 4]. The accuracy and precision of the phosphorus determination obviously affect the errors in the lithogenic index values [4], so particular attention must be paid to the choice of the experimental method. In view of the complicated composition of bile the possible interferences for each analytical method must also be studied. With the aim of identifying the best method, the Bartlett–Carey ([5], M. C. Carey, personal communication) and Murison *et al.* [6] methods were investigated and the results obtained in the analysis of different bile samples were compared.

Experimental

Materials and apparatus

Fifteen human bile samples from different patients with gallstones (six men and nine women, aged between 25 and 60), nine from duodenal aspirations, the others from surgery, were analysed. Standards were prepared using potassium dihydrogen phosphate (E. Merck). Ammonium molybdate and nitric and perchloric acids were Hoechst

* To whom correspondence should be addressed.

products; ammonium vanadate, hydrogen peroxide and sulphuric acid were E. Merck products. Fiske and Subbarow (F.S.) reagent [7] containing 1-amino-2-naphthol-4-sulphonic acid as reducer, was supplied by the Sigma Chemical Company (Saint Louis, USA). An enzymatic UV test for bile salts was obtained from Nyegaard (Oslo): an enzymatic colorimetric test for total cholesterol was obtained from Boehringer Mannheim. Spectrometric measurements were performed with Beckman DK-2A and Perkin–Elmer 320 instruments with 1.0 cm cuvettes and a thermostatic system providing a temperature control of ±0.1°C.

Methods

The Bartlett–Carey method was performed as follows. A 100 μl sample, appropriately diluted in a frosted glass-stoppered test tube, was heated at 120°C for about 1 h in order to eliminate water, and cooled to room temperature.

After addition of 0.50 ml 70% w/v perchloric acid the open test tube was heated at 170°C for 1.5 h and again cooled to room temperature. Then 4.5 ml aqueous ammonium molybdate (2.20 g l^{-1}) and 0.20 ml Fiske–Subbarow (158.7 g l^{-1}) reagent were added, and the solution was stirred in the stoppered tube on a boiling water bath for 8 min. After cooling to room temperature, the absorbance was measured at 820 nm against distilled water. A blank was studied by the same procedure, but with 100 μl 0.9% sodium chloride solution as sample, and gave an absorbance of 0.017. A calibration graph was established using potassium dihydrogen phosphate solutions.

Murison's method was performed as follows: 500 μl of diluted sample in a frosted glass-stoppered test tube, 0.50 ml of 70% w/v perchloric acid and 0.20 ml of 30% hydrogen peroxide were added. The open tube was heated at 180°C for 3 h (shorter heating times might be sufficient in some cases but the heating time must be the same for bile and standard solutions, so a fixed time of 3 h was used) and cooled to room temperature. The volume was adjusted to 0.5 ml with distilled water and 1.0 ml ammonium molybdate solution (49.4 g l^{-1}) containing 11.4% sulphuric acid, 1.0 ml vanadate solution (2.46 g l^{-1}) containing 1.8% nitric acid, and 2.0 ml of distilled water were added. The solution was shaken and allowed to stand for 10 min at 25°C. The absorbance was measured at 405 nm against distilled water in 1.0 cm cuvettes: in blank measurements the sample was replaced by 0.50 ml 0.9% sodium chloride solution, giving an absorbance of 0.070. A calibration curve was also obtained as above.

Results and Discussion

For Bartlett's method the influence of the experimental conditions on the spectra was investigated, with particular reference to the heating time and to the added volumes of the Fiske–Subarrow reagent and the 70% perchloric acid. Each condition was varied in turn, while the others remained constant (M. C. Carey, personal communication). The data obtained (Fig. 1) largely confirm the choice of conditions for the method, though the wavelength can profitably be changed from 830 to 820 nm (Fig. 2). These results led to the procedure described in the experimental section. Using this procedure a straight regression line over the concentration range 0.20–2.00 mM was obtained; its equation was $y = 0.524\,x + 0.015$ (x = concentration of the standard solutions in mmol l^{-1}, y = absorbance at 820 nm) with a correlation coefficient, r, of 0.996. The reproducibility and accuracy of the method were determined using standards and bile samples (Tables 1, 2 and 3).

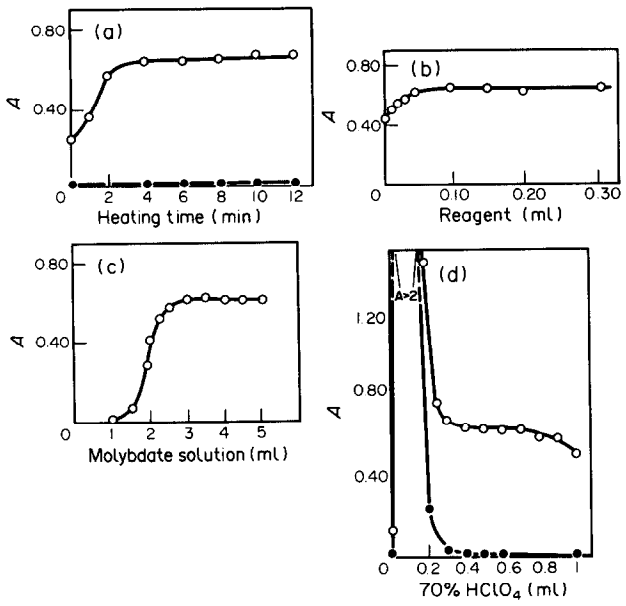

Figure 1
Bartlett's method: Effects on absorbance at 820 nm of (a) heating time (min) on boiling water bath. ○ = standard potassium dihydrogen phosphate solution (1.20 mM); ● = reagents only. (b) Added volume (ml) of Fiske–Subbarow reagent (158.7 g l^{-1}). (c) Added volume (ml) of molybdate (2.20 g l^{-1}). (d) Added volume (ml) of 70% perchloric acid, ○ = standard potassium dihydrogen phosphate solutions (1.20 mM); ● = reagents only.

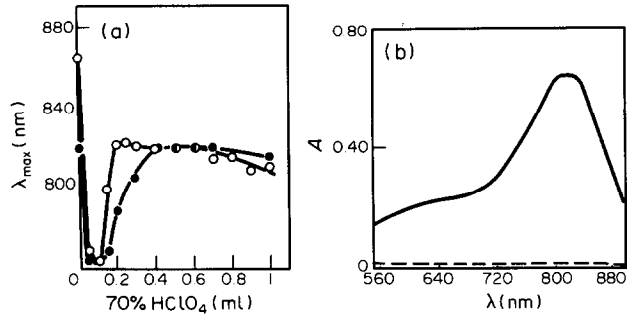

Figure 2
Bartlett's method (a) λ_{max} (nm) vs volume (ml) of perchloric acid, ○ = potassium dihydrogen phosphate solution (1.20 mM); ● = reagents only. (b) Spectra against water. Continuous curve = potassium dihydrogen phosphate (1.20 mM); broken line = blank with reagents only.

The experimental conditions in Murison's method, particularly the volumes of the reagents used (Fig. 3), were also checked. The data obtained support Murison's recommendations [6] and show that the use of 0.50 ml perchloric acid is a compromise between the acidity needed for mineralization and the requirement for a sufficiently high absorbance value. The experimental wavelength was chosen to ensure good sensitivity and reproducibility, but does not correspond to a spectral maximum. These results produced the procedure for bile analysis described in the experimental section. The calibration curve was studied over the concentration range 0.50–3.00 mM, and found to be $y = 0.236x + 0.071$, where x is the concentration of the standard solutions (mM)

Table 1
Reproducibility and accuracy of phosphate determination using Bartlett's method

Standard concentration (mM)	Experimental mean concentration* (mM)	R.S.D. (%)
0.200	0.201	2.7
0.600	0.588	1.7
1.000	1.003	1.1
1.400	1.408	0.8
1.800	1.797	2.5

* Each value is the mean of five determinations.

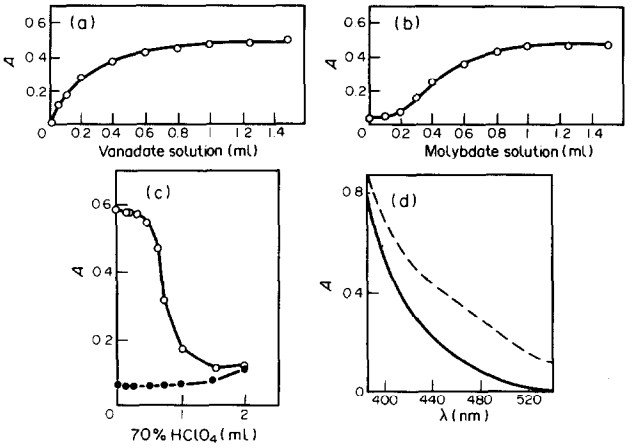

Figure 3
Murison's method: Effects on absorbance at 405 nm of (a) added volume (ml) of vanadate (2.46 g l^{-1}, in 1.8% nitric acid). (b) Added volume (ml) of molybdate (49.4 g l^{-1} in 11.4% sulphuric acid). (c) Added volume (ml) of 70% perchloric acid; ○ = standard potassium dihydrogen phosphate solutions; ● = reagents only. (a) and (b) 1.8 mM potassium dihydrogen phosphate; (c) 2.0 mM potassium dihydrogen phosphate; (d) spectra against water. Continuous curve, potassium dihydrogen phosphate (1.80 mM); broken line, same solution containing traces of hydrogen peroxide.

Table 2
Reproducibility of total phosphorus determination in human bile using Bartlett's method

Sample*	P concentration† (mM)	R.S.D. (%)
1 (s)	39.90	1.5
2 (d)	14.36	3.3
3 (s)	16.12	3.1
4 (d)	8.84	0.9
5 (s)	27.95	1.1

* d = duodenal aspirate, s = bile from surgery.
† Each value is the mean of six determinations.

Table 3
Recovery of phosphate in human bile using Bartlett's methods

Sample*	Total phosphorus found (mM)	KH_2PO_4 addition (mM)	Found after KH_2PO_4 addition†	Recovery (%)
2	14.30	8.00	22.32	100.1
		24.00	38.20	99.7
		40.00	54.80	100.9
		56.00	72.13	102.6
3	16.13	8.00	23.87	98.9
		24.00	40.65	101.3
		40.00	55.66	99.2
		56.00	73.13	101.4
4	8.84	8.00	17.00	101.0
		24.00	32.13	97.8
		40.00	48.67	99.7
		56.00	65.60	101.2

* For details, see Table 2.
† Each value is the mean of three determinations.

and y the absorbance at 405 nm. The correlation coefficient, r, was 0.9985. The precision and accuracy of the method [4] are summarized in Table 4. As suggested by Fig. 3c, neutralization of the perchloric acid with sodium hydroxide after heating at 180°C gives a small increase of the absorbance values. It is important that the hydrogen peroxide added during the mineralization procedure is completely destroyed, since it interferes with the colour formation (Fig. 3d).

The comparison between Bartlett's and Murison's methods considered their precision, accuracy, concentration range and sensitivity (Table 5) and the correlation of the values found by both methods for the bile samples from fifteen patients (Table 6). The bile salt pool and total cholesterol values were also determined for the samples, using the Talalay [4, 8] and Roeschlau [9, 10] enzymatic methods respectively. Lithogenic index (IL) values were calculated by using Small polynomial coefficients [3], and IL standard

Table 4
Precision and accuracy of phosphorus determinations in human bile using Murison's method

Reproducibility in standard phosphate solutions			Reproducibility and recoveries in human bile				
Standard concentrations (mM)	Found (mM)	R.S.D. (%)	Type of sample	Found (mM)	R.S.D. (%)	KH_2PO_4 addition (mM)	Recovery (%)
0.500	0.497	1.2	d	7.18	1.8		
1.000	1.043	2.8	d	5.48	2.6	10.0, 25.0	103.0, 96.3
1.500	1.475	1.5	s	32.31	2.6		
2.000	2.000	2.3	s	40.00	4.6	30.0, 75.0	101.9, 98.6
2.500	2.483	1.9	s	13.85	2.2	25.0, 52.5	102.7, 100.1
3.000	3.070	2.7					

Each value is the mean of at least three determinations.

Table 5
Comparison of the Bartlett and Murison Methods

Method	Range (mM)	Sensitivity as slope of calibration curve (mM)	P.S.D. (%)* of bile analysis	Mean error or bile recoveries (%)
Bartlett	0.2–1.8	0.52	2.3	0.28
Murison	0.5–3.0	0.24	4.1	0.34

* P.S.D. = pooled standard deviation.

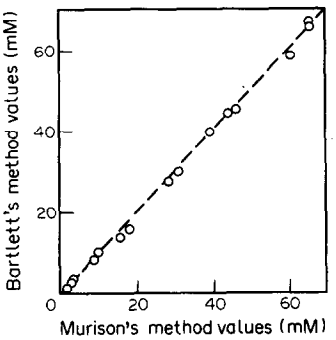

Figure 4
Correlation between the total phosphorus (mM) values determined by Bartlett's and Murison's methods on fifteen human bile samples. The regression line is $y = 1.00\,x - 0.407$; $r = 0.9995$.

relative errors were calculated as previously described [4]. The values of percentage differences $|\Delta\ IL/\overline{IL}\ \%|$ between the results from the two analysis methods are also given.

Conclusions

Bartlett's method is more precise and more sensitive than Murison's method, both for standard phosphate and for bile samples. In Murison's method absorbance is not measured at a spectral maximum. Murison's method is also affected by the problem of incomplete elimination of hydrogen peroxide. Addition of sodium hydroxide to neutralize the perchloric acid results in a 7% higher sensitivity, but the precision is the same and the addition complicates the operating procedure. The apparatus required is almost the same for the two methods, but reagent cost is higher for Bartlett's method. The accuracy of both methods is high and their results correlate well (Fig. 4). No significant differences are observed for the IL values obtained by these two methods of total phosphorus determination.

Acknowledgements: The authors thank Prof. A. Alessandrini of the 2nd Medical Clinic of Rome University for human bile samples and Prof. M. C. Carey for his helpful suggestions on Bartlett's procedure. This work was supported by the National Research Council (CNR) of Italy.

References

[1] J. Wiegard and G. M. Murphy, *Clin. Chim. Acta* **89**, 169–171 (1978).
[2] L. Campanella, M. Tomassetti, G. D'Ascenzo, G. De Angelis, R. Morabito and L. Sorrentino, *J. Pharm. Biomed. Analysis* **1**, 163–167 (1983).
[3] M. C. Carey and D. M. Small, *Clin. Invest.* **61**, 998–1026 (1978).

Table 6
Analysis of fifteen human bile samples for total phosphorus by Bartlett's and Murison's methods

Sample No.	Sample type	Total phosphorus, Bartlett's method (mM)	Total phosphorus, Murison's method (mM)	% differences	Total cholesterol (mM)	Bile salt pool (mM)	IL, Bartlett's method	IL, Murison's method	$\frac{S_{IL}}{IL}$ %	$\lvert\Delta IL\rvert$	$\left\lvert\frac{\Delta IL}{IL}\right\rvert$ %
1	s	39.90	39.75	0.4	23.4	18.4	sat.*	sat.	—	—	—
2	d	14.36	15.60	−7.9	23.7	4.8	sat.	sat.	—	—	—
3	s	16.12	17.88	−9.8	10.4	100.0	1.73	1.61	3.5	0.12	7.2
4	d	8.84	9.25	−4.4	7.9	41.5	2.92	2.81	3.6	0.11	3.8
5	s	27.95	28.40	−1.6	20.5	69.2	2.31	2.29	1.9	0.02	0.9
6	d	46.00	46.50	−1.1	23.0	13.3	sat.	sat.	—	—	—
7	s	59.00	61.00	−3.3	21.9	15.3	sat.	sat.	—	—	—
8	d	67.00	66.00	1.5	26.8	12.5	sat.	sat.	—	—	—
9	d	1.72	1.70	1.2	1.2	13.5	3.36	3.36	2.8	0	0
10	d	10.50	10.35	1.4	5.1	28.8	1.92	1.93	2.9	0.01	0.5
11	d	3.70	3.65	1.4	0.4	11.5	0.57	0.57	3.9	0	0
12	d	30.00	31.00	−3.2	10.6	87.6	1.16	1.14	2.6	0.02	1.7
13	s	66.00	66.00	0.0	23.3	52.0	sat.	sat.	—	—	—
14	d	3.26	3.13	4.2	0.9	7.1	1.93	1.89	0.8	0.04	2.1
15	s	44.50	44.50	0.0	12.7	54.4	1.49	1.49	1.0	0	0

sat = saturated, see Ref [4].

[4] U. Biader Ceipidor, R. Curini, G. D'Ascenzo, M. Tomassetti, A. Alessandrini and C. Montesani, *Giorn It. Chim. Clin.* **5**, 127–151 (1980).
[5] G. R. Bartlett, *J. Biol. Chem.* **234**, 466–468 (1959).
[6] J. Murison, D. Festi, P. E. Ross and I. A. D. Bouchier, *Clin. Chim. Acta* **68**, 159–166 (1976).
[7] C. H. Fiske and Y. Subbarow, *J. Biol. Chem.* **66**, 375–400 (1925).
[8] P. Talalay, *Methods Biochem. Anal.* **8**, 119–143 (1960).
[9] P. Roeschlau, E. Bernt and W. Gruber, *Z. Klin. Chem. Klin. Biochem.* **12**, 403–407 (1974).
[10] U. Biader Ceipidor, R. Curini, G. D'Ascenzo, M.Tomassetti and M. Alessandrini, *Giorn. It. Chim. Clin.* **4**, 355–369 (1979).

[*Received 20 February 1983*]