Journal of Pharmaceutical & Biomedical Analysis
Vol. 4, No. 2, pp. 155–162, 1986
Printed in Great Britain

0731–7085/86 $3.00 + 0.00
Pergamon Journals Ltd.

Application of a new cholate liquid membrane electrode to the determination of the cholic acids pool in human bile*

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Abstract: A cholate-selective liquid membrane electrode has been applied to the determination of the pool of cholic acids in human bile. Pretreatment of the sample was necessary for reliable potentiometric determination; different procedures were tried for pretreatment of human bile.

Keywords: *Bile acids; cholic acids; ion-selective electrode; liquid membrane; pretreatments.*

Introduction

The potentiometric determination of cholic acids in matrixes such as aqueous solutions and commercial drugs, with a liquid membrane cholate electrode, was described in two previous papers [1, 2]. The same determination in human bile liquids, especially in cholecystic aspirates, should be of a marked practical interest [3, 4]; nevertheless a direct measurement cannot be performed because of chemical interference and practical difficulties. From theory it is possible to predict the interference from organic compounds such as proteins [5], that can seriously influence permeability of the Teflon membrane and the response of the electrode, from lipids (especially phospholipids and cholesterol [6]) and from inorganic anions [1] (especially chloride, hydrogen carbonate and phosphate, normally present in bile liquids). Other problems probably arise from the fact that cholic acids are conjugated either completely in fresh cholecystic bile or partially, due to hydrolysis, in aspirates and in stored bile samples.

From published information it is possible to perform a potentiometric determination with reference to a calibration experiment conducted with an aqueous solution containing a mixture of the free cholic acids in the same concentration ratios as those present (as sum of conjugated and unconjugated forms) in the bile. However, it is not possible to establish the concentration of each one of these acids in conjugated or unconjugated form because of the concurrent hydrolysis, unless analysis for each

*This work was presented at the Symposium on Electrochemical Sensors, Rome, 12–14 June 1984.
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component is performed. Finally it must be observed that most cholates present in human bile are solubilized in micelles [7] and that under these conditions permeability through the Teflon membrane is drastically changed. Experimentally, the direct measurement on bile samples by a cholate electrode is unsuccessful because of marked interference, poor precision and chemical attack on the membrane. Therefore, it was necessary to study some possible pretreatments. In the present paper, the results obtained on some human bile samples are reported, discussed, and compared with those obtained by the Talalay enzymatic–spectrophotometric method [8].

Experimental

Materials and apparatus

The experiments were performed on samples of human bile from ten patients who had cholecystectomies and on cholecystic aspirates of patients with presumed gallstones. The age of patients from whom bile liquids were extracted was 42–68; nine patients were female, one was male. All the reagents were of analytical grade. Sodium cholate, cholic acid, deoxycholic acid and chenodeoxycholic acid were supplied by Merck (Darmstadt, FRG). Enzymatic–spectrophotometric (UV) tests for bile salts were performed by Nyegaad and Co.A/S (Oslo, Norway). The assembly of the cholate liquid membrane electrode using benzyldimethylcetylammonium cholate in l-decanol as sensor, and its analytical and electrochemical characterization, are described in detail in a previous paper [1, 2]. The main data for standard sodium cholate solution relative to the electrode are: linearity range 4.00×10^{-5}–1.00×10^{-2} mol l^{-1}; slope -0.0577 (± 0.0006) V per decade of mean activity; response time <10 s; precision (as pooled standard deviation) 0.5%; error for concentrations of 1×10^{-4}–1×10^{-2} mol l^{-1} not higher than 3% by Gran's plot method. Electrochemical cell: Ag/AgCl/KCl 0.01 mol l^{-1}, sodium cholate 0.01 mol l^{-1}/benzyldimethylcetylammonium cholate 0.01 mol l^{-1} in 1-decanol/test solution/saturated calomel electrode.

An electrometer (Radiometer PHM 64) and a Varian G-14 A2 recorder were used for electromotive force measurements. An automatic burette (Radiometer ABU-11), coupled to the electrometer–recorder system was used for the titration curve. A saturated calomel electrode and a silver–silver chloride electrode were employed as reference electrodes. UV measurements were made with a Perkin–Elmer 320 spectrophotometer with 1-cm quartz cells.

Method

The possible pretreatment operations are: (i) deproteinization with ethanol; (ii) treatment with carbon black for removal of the bile pigments; (iii) strong alkaline hydrolysis to deconjugate bile acids; (iv) precipitation of cholic acids in acid medium, followed by their extraction with an organic solvent or alternatively by filtration of the precipitated acids on a Gooch filter, to free the acids from the micelles and to remove inorganic anions that can interfere; and (v) washing the cholate aqueous solution with ether to eliminate cholesterol and lipids still present. Two different procedures were applied in the experimental work.

Procedure "a"

To 1 ml of bile, 10 ml of ethanol is added [9]; after stirring, the protein precipitate is permitted to slowly sediment. After carbon black addition, the suspension is stirred; the

suspension is filtered, the filter is washed well with about 5 ml of ethanol, and the filtrate is evaporated to dryness in a rotary evaporator. If further precipitation is observed it is necessary to repeat the deproteinizing treatment. The residue is redissolved in 20 ml of 4 M NaOH and saponified in an autoclave at 120° for 1.5 h [10]. The hydrolysed sample is acidified to pH 1 with 6 M HCl and the precipitated bile acids are extracted from the reaction mixture with methanol–chloroform (1:2, v/v) so that a ratio chloroform–methanol–water (8:4:3, v/v/v) results [11]. The mixture is shaken and then centrifuged to separate the organic phase; otherwise the demixing of the emulsion is very slow; a further extraction on the aqueous phase is performed with methanol–chloroform (1:2, v/v). The two organic phases are combined, dried and evaporated in a rotary evaporator. The residue is redissolved at pH 11 first with about 30 ml of 10^{-3} M NaOH and then, as the pH shifts to an acid value, with a more concentrated solution of NaOH, so that the final volume is not greater than 50 ml. This solution is washed by shaking twice with 30 ml of ether to remove cholesterol and lipids. After centrifugation, the traces of ether are removed by a vacuum pump and the potentiometric measurements are performed on the resulting solution.

Procedure "b"

A 1 ml sample of bile is treated as in procedure (a) up to the step when after acidification at pH 1 the bile acids are precipitated; at this point the acids are not extracted but are centrifuged and filtered on a Gooch G4 filter. The precipitate is washed with 5 ml of water at pH 1 (with 0.1 M HCl) and redissolved as described previously. The solution is washed with ether as in procedure (a), and the potentiometric measurement is performed.

Calibration curve

To determine the cholate concentration in the unknown sample a potentiometric calibration curve must be recorded; for this purpose, a 10^{-2} mol l^{-1} aqueous solution of cholate at pH 11 is prepared. In this solution all the main cholic acids are present in the same concentration ratios as those in human bile, but the acids are all in unconjugated form. The mixture comprises 41% of cholic acid, 22% of deoxycholic acid and 37% of chenodeoxycholic acid [12]. Different volumes of this solution are added by an autoburette to 25 ml of water, at pH 11 with stirring, in which the cholate-indicating electrode and the reference electrode are dipped. From measurements of potential in the different solutions, a calibration curve can be obtained. In Fig. 1 a rectilinear regression

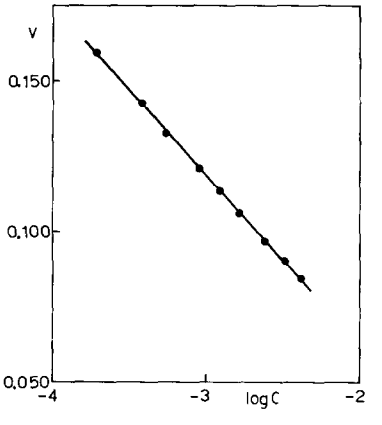

Figure 1
Regression line for the concentration range 2×10^{-4} to 4×10^{-3} mol l^{-1}. The regression equation is $E(V) = -0.0568\ (\pm 0.0024) \log C - 0.0514\ (\pm 0.0072)$; the precision of measurements, as pooled standard deviation = 2.8%.

is shown, the line representing the mean of four different calibrations; the useful concentration range and the precision of the measurements are also noted.

Enzymatic–spectrophotometric method

To characterize the new method and to evaluate the reliability of the results obtained by both procedures, the spectrophotometric–enzymatic technique of Talalay was employed for reference [8], since it was considered to be the most reliable method for the analysis of cholic acid solutions. The determination was performed as previously described [4] both on the bile samples, diluted if necessary so that suitable absorbance values are obtained, and on the samples after pretreatment. In addition, the correlation between the potentiometric and the enzymatic method was evaluated by measuring the total content of all the main cholic acids in some human bile samples.

Results and Discussion

Experiments were conducted to verify the possibility of employing the electrode directly on bile or on the sample after all or part of procedure (a) pretreatment. The results are compared in Table 1 with data obtained by the enzymatic method, both on original bile (sample 1) and on the solutions obtained from it by all or part of the pretreatment procedure.

Five observations can be made from the results. First, on untreated bile samples the potentiometric method cannot be applied as no meaningful potential value is obtained. Second, on the deproteinized sample and after hydrolysis potentiometric measurement is possible, but the value obtained is markedly affected by interference as shown by comparison with the enzymatic data. Third, after a complete series of pretreatments (deproteinization, hydrolysis, precipitation of cholic acids in acid medium, extraction with the above ternary mixture, washing of the final solution with ether) potentiometric measurement yields a result that agrees well with the enzymatic value. Fourth, as the pretreatment operations proceed, a progressive loss of cholic acid is observed by potentiometric and enzymatic methods. Fifth, potentiometric measurement is also possible if the washing operations with ether are performed on the hydrolysed solutions: however, in this case, the values obtained give higher negative errors than those obtained when washing operations are performed on the alkaline solution at the end of the

Table 1
Enzymatic and potentiometric results, as mean of three determinations, obtained for bile sample 1, untreated and after some pretreatment

Matrix	Enzymatic spectrophotometric method (mmol l^{-1})	(RSD %)	Potentiometric method (mmol l^{-1})	(RSD %)
Full bile sample	33.1	2.9	—*	
After deproteinization and hydrolysis	32.4	4.3	122.0	4.6
After complete pretreatment (shaking the final solution with ether)	24.8	2.0	25.0	6.4
After complete pretreatment (shaking the solution with ether after hydrolysis)	12.5	5.4	11.0	6.0

*On the untreated bile sample the potentiometric measurement cannot be performed.

pretreatment procedure. In addition separation of the ether and water phases is more difficult.

By examination of Table 1 it is concluded that, with the complete pretreatment procedure, an aqueous solution is obtained on which the potentiometric determination of cholate can be reliably performed. Nevertheless, the determined value is lower than that obtained by the enzymatic–spectrophotometric method performed on the untreated sample. To evaluate the loss during the pretreatment operations, the concentration in cholic acids of three different human bile samples, before and after all the pretreatment (procedure (a)), was determined by the enzymatic method; the values obtained are reported in Table 2. A mean loss of 24.1% was observed for the three samples. Therefore, the correlation between the potentiometric method (applied to treated samples) and the enzymatic method (directly applied to each sample) was investigated. Results for five different samples are reported in Table 3.

Table 2
Experimental evaluation of the mean loss of cholate by the full series of pretreatments

Pretreatment	Bile sample	Determined cholic acids concentration before pretreatments (mmol l^{-1})	Determined cholic acids concentrations after pretreatments (mmol l^{-1})	Loss (%)	Mean loss (%)	RSD (%)
Procedure (a)	1	33.1	24.8	25.1	24.1	± 1.0
	2	31.0	23.5	24.2		
	3	37.6	28.9	23.1		
Procedure (b)	6	123.2	82.2	33.3	27.3	± 5.5
	8	67.1	52.1	22.5		
	10	121.4	89.6	26.2		

From these results, the precision, as the relative standard deviation (RSD) of the measurements performed on the biological liquids, can also be evaluated. The analysis of data in Table 3 shows that the agreement between the results obtained by the two methods is not always satisfactory; in some cases the differences are as great as 25%. Among the causes of this disagreement, two can be readily identified: first, the difficulty of separation of the aqueous and the organic phases during the extraction operation since very stable emulsions are formed; second, the interference from chloride ions derived from HCl added to neutralize of 4N NaOH. However, H_2SO_4 cannot be employed in place of HCl as its use can result in sulphation of the $-OH$ group, especially in the 3α position, and consequently in an enhanced solubility so that acids could pass into the aqueous phase rather than into the organic one. To avoid these problems, the laborious extraction operation was eliminated by recovering the cholic acids precipitated in acidic medium by filtration on a Gooch filter. This was performed by procedure (b). The validity of this procedure (b) was confirmed by performing the analysis of five bile samples and correlating the experimental results of both methods (Table 4).

It can be observed that agreement is now much better ($\Delta\% < 7\%$); the experimental potentiometric values were corrected on the basis of the correction factor evaluated as described earlier (Table 2).

Table 3
Correlation between the values, as mean of three experiments, of cholate content, by enzymatic and potentiometric determination*

Bile sample	Type of sample	Enzymatic spectrophotometric method (mmol l^{-1})	(RSD %)	Potentiometric method (mmol l^{-1})	(RSD %)	Difference (%)
1	Cholecystic aspirate	33.1	2.9	31.0	6.4	−6.3
2	Cholecystic aspirate	31.0	4.2	32.9	5.7	+6.1
3	Gallbladder bile	37.6	0.5	27.7	7.1	−26.3
4	Gallbladder bile	29.1	2.0	25.8	6.0	−11.3
5	Gallbladder bile	23.8	4.6	29.4	5.1	+23.5

*Potentiometric determinations were performed after full pretreatment by procedure (a) and the results were corrected for loss during the pretreatment.

Table 4
Correlation between the values, as mean of three experiments, of cholate content, by enzymatic and potentiometric determination*

Bile sample	Type of sample	Enzymatic spectrophotometric method (mmol l^{-1})	(RSD %)	Potentiometric method (mmol l^{-1})	(RSD %)	Difference (%)
6	Gallbladder bile	123.2	1.5	125.9	5.0	+2.2
7	Gallbladder bile	206.2	1.5	196.0	1.7	−4.9
8	Gallbladder bile	67.1	0.5	69.1	3.0	+3.0
9	Cholecystic aspirate	8.5	4.1	8.6	1.6	+1.2
10	Gallbladder bile	121.4	1.0	129.8	3.2	+6.9

*Potentiometric determinations were performed after full pretreatment by procedure (b) and the results were corrected for loss during pretreatment.

Conclusions

It has been confirmed that it is not possible to perform the direct determination of the cholic acids on untreated bile samples; before the analysis it is necessary to isolate the cholates from all other interfering species present in bile. The series of the proposed pretreatments, particularly deproteinization and alkaline hydrolysis, has been established on the basis of the previous experience of other researchers [9, 10] who tried to determine cholic acids in bile by chromatography or spectrophotometry.

These pretreatments seem adequate for the removal of all sources of interference in the determination of the pool of the cholic acids by the potentiometric method. In particular, the alkaline hydrolysis of tauro- and glyco-conjugated cholates, performed under the drastic conditions proposed by Roseleur and Van Gent [10], ensures full deconjugation of the cholic acids.

It is also possible to employ a mixture of only free acids as a standard for the calibration curve. The behaviour of the cholate electrode in this solution is characterized by high precision and fast response time; the time was just twice that in solutions of only sodium cholate.

The two proposed procedures (a) and (b) differ in that extraction is involved in (a) whereas filtration and washing are employed in (b). The latter precedure results in faster and easier determinations that are more efficient in eliminating interference, but the cholate losses are a little higher than those for procedure (a) (Table 2).

Nevertheless these losses can be evaluated and thus easily corrected, by experimentally determining the mean loss or by a standard addition to the bile. It can also be concluded that, by adopting the described pretreatment procedures, it is possible to employ the proposed liquid membrane electrode to determine the pool of bile cholic acids. This method requires only a small amount of bile for the analysis and the experimental apparatus is commonly available; however, the time necessary to complete the analysis is not short and the procedure is laborious. In addition, automation would be difficult. For all these reasons this method cannot be considered as a routine alternative to the spectrophotometric–enzymatic method, although Talalay's method [8] is more expensive. The method proposed in the present work can be useful where there is a lack of reagents for the enzymatic–spectrophotometric method. In such a case, it is to be preferred for its higher accuracy and precision to other methods such as spectrophotometric [13, 14] techniques which use toxic reagents that are poorly specific.

Acknowledgement: This work was financially supported by the Italian C.N.R.

References

[1] L. Campanella, L. Sorrentino and M. Tomassetti, *Analyst* **108**, 1490–1494 (1983).
[2] L. Campanella, L. Sorrentino and M. Tomassetti, *Ann. Chim.* **74**, 483–497 (1984).
[3] M. C. Carey and D. M. Small, *J. Clin. Invest.* **61**, 998–1026 (1978).
[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] R. T. Holzbach, A. Kibe, E. Thiel, J. H. Howell, M. Marsh and R. E. Herman, *J. Clin. Invest.* **73**, 35–45 (1984).
[6] T. J. Gilligan III, E. L. Cussler and D. F. Evans, *Biochim. Biophys. Acta* **497**, 627–630 (1977).
[7] D. M. Small, S. A. Penkett and D. Chapman, *Biochim. Biophys. Acta* **176**, 178–198 (1969).
[8] P. Talalay, *Methods Biochem. Anal.* **8**, 119–143 (1960).
[9] J. Reinhold and W. Wilson, *J. Biol. Chem.* **96**, 637–646 (1932).
[10] O. J. Roseleur and C. M. Van Gent, *Clin. Chim. Acta* **66**, 269–272 (1976).

[11] J. Folch, M. Lees and F. H. Sloane Stanley, *J. Biol. Chem.* **226**, 497–509 (1957).
[12] K. W. Heaton, *Bile Salts in Health and Disease*, Churchill-Livingstone, Edinburgh (1972).
[13] J. L. Irvin, C. G. Johnston and J. Kopala, *J. Biol. Chem.* **153**, 439–457 (1944).
[14] U. Biader Ceipidor, R. Curini, G. D'Ascenzo and M. Tomassetti, *Thermochim. Acta* **46**, 269–278 (1981).

[Received for review 23 July 1984; revised manuscript received 20 November 1984]