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Note

New optimized method for the determination of esterolytic activity in serum by gas—solid chromatography

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Serum levels of esterolytic activity have been a useful tool in the diagnosis of pathological disorders, especially in that of carcinoma [1]. As all the methods for the determination of esterolytic activity currently in use are lacking either sensitivity or specificity [2—8], there is need for a new sensitive and specific method with good accuracy and precision for measuring slight differences in serum levels of esterolytic activity for purposes of detecting early stages of pathological disorders. The technique reported here, which is based on gas—solid chromatography (GSC) with a special synthetic support and detection by a flame ionization detector appears to meet such need.

EXPERIMENTAL

Reagents

Ethyl butyrate of analytical grade (Koch-Light Labs., (Colnbrook, Great

Britain) was further purified as follows. Traces of fatty acids were removed by treatment with 5% aqueous sodium hydrogen carbonate followed by washing five times with distilled water. The ester was then distilled over a 40-plate column, and the fraction with boiling-point range 120.5—121° was collected and stored in a glass-stoppered dark bottle.

Chromatographically purified *n*-octane (purity 99%) was obtained as a gift from the Technological Institute, Prague, Czechoslovakia.

Analytical reagent grade butyric acid (Lachema, Brno, Czechoslovakia) was further purified by preparative gas—liquid chromatography (GLC) to achieve purity $\geqslant$ 99%, which was checked by GLC of the methyl ester.

Diethyl ether of analytical reagent grade was purchased from Lachema.

Apparatus, instrumental and chromatographic conditions

Gas chromatographic analyses were performed on a Perkin-Elmer Model F 33 gas chromatograph equipped with an all-glass system, flame ionization detector and a glass column, 100 cm × 2 mm I.D., packed with adsorbent Spheron-BD, 75—120 mesh (synthetic material developed by the Research and Development Chemical Department of The Laboratory Instruments Works, Prague, Czechoslovakia). Further information about this support will be reported elsewhere.

The column was conditioned at 170° for 12 h with a nitrogen flow-rate of 39.2 ml/min. The operating conditions were: oven temperature, 170°; detector temperature, 225°; nitrogen flow-rate, 39.2 ml/min; air flow-rate, 400 ml/min; and hydrogen flow-rate, 52 ml/min.

A Perkin-Elmer Model 56 recorder was used with a chart speed of 5 mm/min and sensitivity set at 2 mV.

Calibration

The standard solutions of butyric acid were prepared by dissolving known amounts of butyric acid in diethyl ether containing 0.02% (v/v) of *n*-octane as internal standard. The concentration range of the butyric acid standards was 0.31 to 2.76 μmoles per μl. A 5.0-μl aliquot of each standard was injected into the gas chromatograph and the ratio of the butyric acid and *n*-octane peak areas was determined and plotted against the amount of butyric acid injected. A typical standard curve is shown in Fig. 1, curve A.

In a similar manner, standard aqueous solutions of butyric acid were prepared in 0.5 *M* phosphate buffer (pH 8.0); 1 ml of each solution was mixed with 1 ml of 1 *M* orthophosphoric acid and extracted into diethyl ether containing internal standard as described in the next section. A 5-μl aliquot of each diethyl ether extract was injected into the gas chromatograph and the ratio of the butyric acid and *n*-octane peak areas was determined and plotted against the amount of butyric acid injected. A typical standard curve is presented in Fig. 1, curve B.

Assay of biological sample for esterolytic activity

An assay procedure similar to that reported by Skořepa *et al.* [2] was used. Serum and buffered substrate were equilibrated for 5 min at 37°. Serum (0.5 ml) was added to 3.78×10^{-2} *M* buffered substrate (4.5 ml) in a glass-

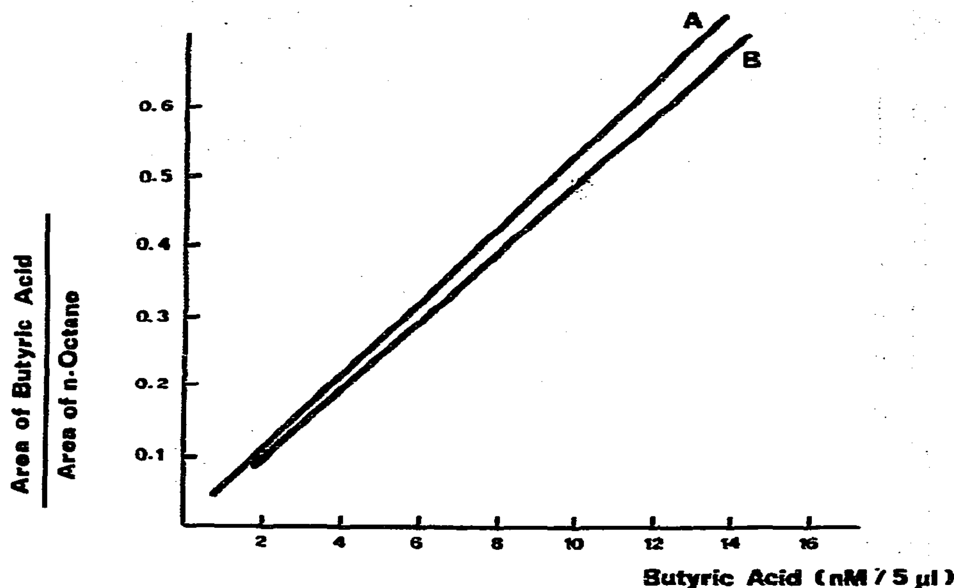

Fig.1. Calibration and extraction curves for quantitative analysis of butyric acid. Curve A: diethyl ether solution of butyric acid with internal standard was chromatographed directly. Curve B: butyric acid was extracted from buffered serum solution into diethyl ether solution of internal standard.

stoppered test-tube (180X14 mm), and the mixture was incubated in a water-bath at 37°. The enzymic reaction was stopped after a definite period by pipetting 1 ml of the incubated mixture into another glass-stoppered test-tube (150X12 mm) which contained 1 ml of 1 *M* orthophosphoric acid. The mixture was shaken, then stored in a refrigerator at 4° for 5 min before it was extracted into 2 ml of cooled diethyl ether solution which contained 0.02% (v/v) of *n*-octane as internal standard. The mixture was well shaken and stored in the refrigerator for at least 5 min before the injection of 5-μl aliquots of the upper diethyl ether layer into the gas chromatograph.

In the control test-tube, heat-inactivated serum or physiological saline was used instead of serum. Esterolytic activity assays were carried out in duplicate.

The pH optimum was determined by measuring the extent of hydrolysis of substrate dissolved in buffer solutions of different pH values (6.5—9.5). The period of incubation was fixed at 2 h. Similarly, esterolytic activity was determined in buffer solutions of different ionic strengths (0.006—1.6 *M*). Determination of the effect of dilution was carried out by diluting the serum with heat-inactivated sera or physiological saline.

RESULTS AND DISCUSSION

A typical chromatogram is shown in Fig. 2. Butyric acid is conveniently separated from the solvent (diethyl ether), and the substrate (ethyl butyrate).

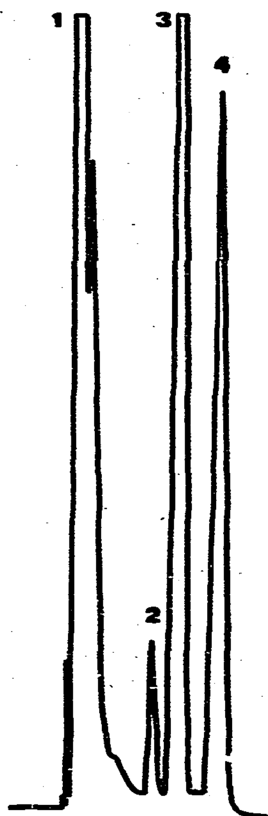

Fig.2. Typical pattern of separation of a mixture of butyric acid, ethyl butyrate, diethyl ether (solvent) and *n*-octane (internal standard). GSC conditions are as described under Experimental. 1=diethyl ether; 2=butyric acid; 3=ethyl butyrate; 4=*n*-octane.

Calibration and extraction efficiency

Standard samples of butyric acid dissolved in diethyl ether in concentrations ranging from 0.31 to 2.76 nmoles per μl were assayed. The calibration curve shown in Fig. 1, curve A, was linear. To determine the efficiency of the extraction of butyric acid from buffered aqueous solutions into diethyl ether, buffered serum solution supplemented with butyric acid concentrations ranging from 0.246 to 2.46 nmoles per μl were assayed after a one-step extraction into diethyl ether according to the procedure described. The calibration curve shown in Fig. 1, curve B, was linear over this range, which covers the range of concentrations of butyric acid produced on hydrolysis of ethyl butyrate by serum for 1—4 h. The single diethyl ether extraction proved to be effective enough, the recovery of butyric acid being 92.50± 0.81%. Since the calibration curve shown in Fig. 1, curve B, was constructed under experimental conditions similar to those used in the assay of butyric acid from hydrolysis of ethyl butyrate by biological samples, this calibration curve rather than Fig. 1, curve A, was used as the standard calibration curve in the determination of esterolytic activity in biological samples.

Reproducibility and stability

The reproducibility of chromatographic analysis was determined by injection of ten samples of 5 µl of the same solution. Three solutions with different concentrations of butyric acid were analysed and evaluated statistically. For each concentration the coefficient of variation (C.V.) did not exceed 1.8%.

The stability of the serum hydrolysate after extraction into diethyl ether was checked by injecting 5-µl samples of three mixtures that were incubated for 1, 2 and 3 h, respectively, after the following periods of storage in the refrigerator at 4°: 5 min, 30 min, 1 h, 3 h, 6, 24 h, 48 h, and 72 h. The mixtures were stable at 4°, as is evident from the results in Table I.

The high reproducibility of the method deserves comment. Heptane [9] and toluene [10] have previously been used for the extraction of free fatty acids. Under the conditions of the GSC procedure, these solvents were not suitable on elution, their peaks interfering with the elution peaks of either butyric acid or ethyl butyrate. Diethyl ether as an extraction solvent gave quantitative yields in a one-step procedure. After storage of the solution in the refrigerator at 4°, the reproducibility of results was better than ±3% Table I).

Effect of enzyme concentration on the rate of hydrolysis

The rate of esterolytic activity measured by hydrolysis of ethyl butyrate was directly proportional to the volume of serum over a 15-fold range up to 1.5 ml.

Results presented in Fig. 3 show a linear relationship between the amount of hydrolysis product, butyric acid and time up to 4 h with undiluted and diluted sera. There was no significant difference in the rate of hydrolysis when heat-inactivated serum or physiological saline was used for diluting the serum, which indicates that the blood serum probably does not contain any inhibitor of esterolytic activity.

TABLE I

EFFECT OF STORAGE ON THE SERUM HYDROLYSATE IN DIETHYL ETHER SOLUTION

Serum hydrolysate in ether	Extent of hydrolysis (nmoles per 5-µl aliquot)								Remarks
	Period of storage								
	5 min	30 min	1 h	3 h	6 h	24 h	48 h	72 h	
1-h hydrolysate	2.80	2.90	2.88	2.80	2.92	2.88	2.90	2.90	Average: 2.87 C.V.: 1.62
2-h hydrolysate	5.70	5.72	5.68	5.72	5.90	5.70	5.70	5.72	Average: 5.73 C.V.: 1.22
3-h hydrolysate	8.50	8.64	8.84	8.50	8.90	8.84	8.70	8.70	Average: 8.70 C.V.: 1.75

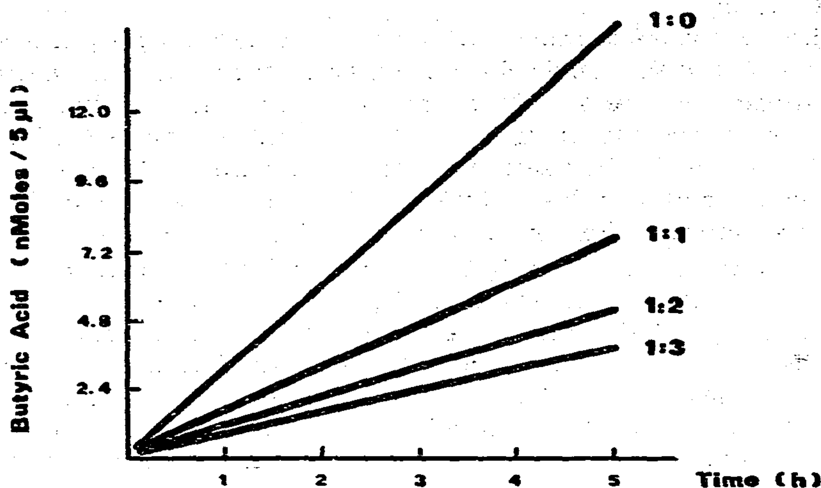

Fig.3. Time course of hydrolysis of ethyl butyrate by undiluted serum and serum diluted with heat-inactivated serum in the ratio indicated.

Effect of pH and ionic strength

The pH—activity curve for serum hydrolysis of ethyl butyrate is shown in Fig. 4. Optimal activity occurred near pH 8.0. The curve is rather broad near its optimum and in this respect resembles the pH—activity curve for tryptic hydrolysis of benzoylarginine methyl ester [11]. At pH 8.0, non-enzymic hydrolysis was almost negligible, being 0.005% of the substrate in 1 h at 37°. The stability of ethyl butyrate to non-enzymic hydrolysis makes it a suitable choice as a substrate for the determination of esterolytic activity, unlike methyl butyrate which was more volatile and far more readily split by non-enzymic hydrolysis. Other workers [12] have also reported appreciable rates of non-enzymic hydrolysis of methyl butyrate even at pH 6.8 and 37°.

Table II shows that the optimal ionic strength for the esterolytic activity of blood serum on ethyl butyrate was 0.5 *M*.

Accuracy and sensitivity of the GSC method

The accuracy of the GSC method was determined from duplicates of esterolytic assays performed on twenty different samples of blood sera. The coefficient of variation was 0.43%.

The detection limit of butyric acid was about 1 µg/ml

CONCLUSION

The GSC method proposed has several advantages in comparison with methods in current use which include: titration [2], colorimetry [3—5], fluorimetry [6], and manometry [7, 8]. The restrictions in these methods have been reported by Ikezawa *et al.* [12]. These authors proposed a GLC method which itself has a few limitations. First, the methyl butyrate used

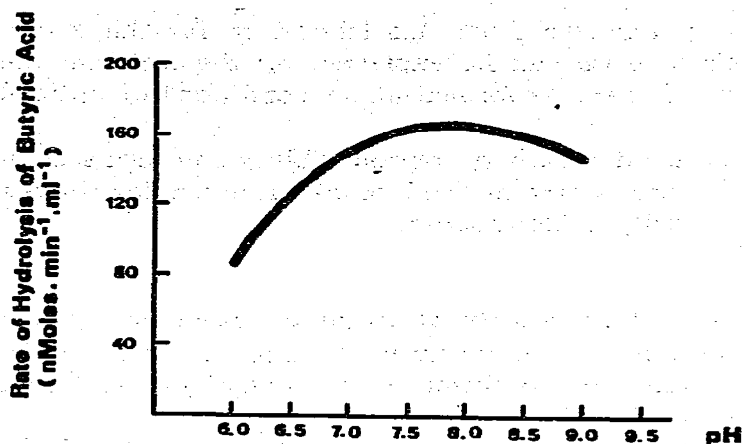

Fig.4. The pH—activity curve for the hydrolysis of ethyl butyrate by serum.

TABLE II

RATE OF SERUM HYDROLYSIS OF ETHYL BUTYRATE AS A FUNCTION OF IONIC STRENGTH

Ionic strength of phosphate buffer (pH 8.0) (*M*)	Rate of hydrolysis (nmoles of butyric acid produced per minute per ml of serum)
1.0	195.0
0.75	233.3
0.50	240.0
0.20	201.6
0.10	188.3
0.05	175.0
0.025	155.0
0.0125	141.7
0.006	135.0

in their GLC method is not a suitable substrate because of its high rate of non-enzymic hydrolysis as previously discussed [12]. Secondly, the method proposed included determination of esterolytic activity by assay of the substrate concentration. An enzymic reaction shows the desirable characteristic linear relationship between the substrate transformed and time only if it is zero order with respect to the substrate concentration. This implies only slight changes in substrate concentration and such changes cannot be determined with as high a precision as the change in the concentration of the product.

The accuracy of the method proposed is about ten times as high as that of the micro-titration method since the coefficient of variation of the method described was estimated to be 0.43% and that of the micro-titration method was 4.5%. The activities measured with the optimized GSC method were

higher than those previously reported from this laboratory for the micro-titration method [2]. This increase can be explained by the fact that the assays using the GSC method were performed under conditions of optimal pH and ionic strength.

The proposed method has good sensitivity, reproducibility and high accuracy. The speed of analysis permits this method to be proposed for routine clinical assay of esterolytic activity in blood serum.

Normal values

The average value for esterolytic activity of serum as determined by the hydrolysis of ethyl butyrate in the micro-titrimetric method was 82.98± 9.51 nmoles·min^{-1}·ml^{-1} serum and the normal values statistically evaluated were 59.18—106.78 nmoles·min^{-1}·ml^{-1}.

Preliminary results with this method gave an average value of 235.30± 61.70 nmoles·min^{-1}·ml^{-1} and a statistically evaluated normal range of 81—389 nmoles·min^{-1}·ml^{-1} serum. The differences in these values can be accounted for by reasons already discussed.

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