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APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE ANALYSIS OF PROPIONYL-L-CARNITINE BY A STEREOSPECIFIC ENZYME ASSAY

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SUMMARY

Propionyl-L-carnitine was converted by chemical hydrolysis (0.3 *M* potassium hydroxide, pH 12.8, room temperature) into L-carnitine, which competes with crotonoylbetaine formation and can be quantitatively evaluated by an enzyme assay. Under the conditions selected, hydrolysis of propionyl-L-carnitine to L-carnitine was completed in a few minutes with less than 0.5% of crotonoylbetaine being formed. The method described is enantioselective and possesses the analytical requirements for assaying propionyl-L-carnitine during chemical synthesis procedures and in pharmaceutical formulations.

INTRODUCTION

Propionyl-L-carnitine (PLC) is an odd-carbon-number short-chain L-carnitine ester present in humans and animals as a minor component compared with L-carnitine and acetyl-L-carnitine (ALC)[1,2]. The interest in PLC results from the inotropic and fat-clearing activity that this substance has proved to possess in isolated perfused rat heart and in *in vivo* investigations of rats, dogs and humans. Hence a quantitative stereospecific assay is required in order to monitor the chemical synthesis of PLC and its presence in pharmaceutical formulations.

As the enzyme assay routinely used for L-carnitine and ALC[3–5] is not applicable to PLC, a method that involves alkaline hydrolysis of PLC to L-carnitine, with quantitative stereospecific determination of L-carnitine and high-performance liquid chromatographic (HPLC) monitoring to ascertain the complete hydrolysis of PLC and the net crotonoylbetaine (CB) formation, was developed and validated.

EXPERIMENTAL

Materials

Solvents and chemicals, all of analytical-reagent or HPLC grade, were supplied by Merck (Bracco, Milan, Italy), BDH (Milan, Italy) and Boehringer (Milan, Italy).

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L-Carnitine, PLC and CB were supplied by Sigma Tau (Rome, Italy).

A Varian Model DMS 70 spectrophotometer (Segrate, Milan, Italy) was used for the enzyme assay. A Hitachi Model 655A-11 liquid chromatograph, equipped with a Hitachi Model 655A variable-wavelength UV detector (Bracco, Milan, Italy), was used to monitor the hydrolysis. The statistical computations were performed on a Macintosh Plus personal computer (Apple, Reggio Emilia, Italy).

PLC hydrolysis

Hydrolysis, which allows the complete conversion of PLC into L-carnitine and the net formation of CB to be restricted to $\leq 0.5\%$, as monitored by HPLC, is achieved with 0.3 *M* potassium hydroxide solution (pH 12.8) at room temperature (20–25°C) in 15 min.

A number of hydrolysing agents at various concentrations and temperatures and with different times of assay were tested in order to obtain optimal conditions for PLC hydrolysis [0.5 *M* phosphate buffer at pH 8 and 10, room temperature, no hydrolysis; 0.2 *M* ammonia solution, pH 10 at 80°C, 55% hydrolysis after 2 h and 2% CB formation; 0.5 *M* ammonia solution, pH 11 at room temperature, 15% hydrolysis after 1 h and 1.5% CB formation; 0.1 *M* sodium hydrogencarbonate, pH 7.7 at 80°C, 15% hydrolysis after 2 h; 0.5 *M* potassium hydroxide solution, pH 13 at room temperature, 100% hydrolysis after 15 min but very large CB formation; 0.5 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer, pH 10 at room temperature, no hydrolysis].

HPLC

A Supelcosil NH_2 (5 μm) column (250 × 4.6 mm I.D.) supplied by Supelco (Supelchem, Milan, Italy) was used. The mobile phase was acetonitrile–0.02 *M* phosphate buffer (pH 3) (75:25) at a flow-rate of 1 ml/min. Absorbance was monitored at 205 nm. The retention times were 2.15 min for PC, 4.80 min for PLC, 8.65 min for L-carnitine and 10.80 min for CB. Fig. 1 shows typical chromatograms of PLC before and after chemical hydrolysis.

Enzyme assay

L-Carnitine formed by alkaline hydrolysis of PLC was determined using an enzyme method based on a colorimetric reaction[6]. The addition of L-carnitine to a system containing carnitine acetyltransferase (CAT) and acetyl coenzyme A leads to the formation of CoASH and ALC. CoASH reduces dithionitrobenzoic acid, breaking the S–S bond and leading to 5-thio-2-nitrobenzoic acid with an absorption peak at 412 nm ($\varepsilon = 13\,600$ l mol^{-1} cm^{-1}).

RESULTS

Linearity

The assay proved to be linear in the range 185–1110 nmoles/ml of L-carnitine, with a r^2 value of 0.9998 (Fig. 2). Linearity was also obtained for the overall procedure described, starting from PLC, over the range 250–1495 nmoles/ml.

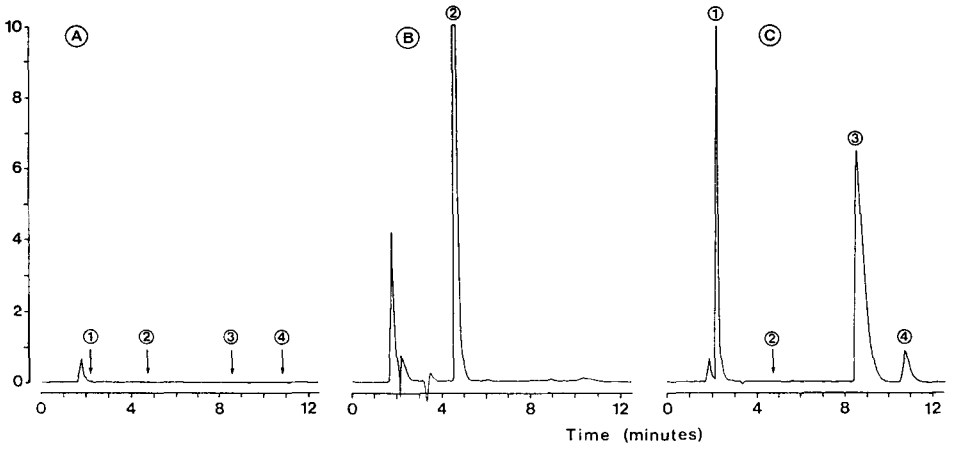

Fig. 1. Typical chromatograms obtained in monitoring PLC hydrolysis. (A) Solvent injection; (B) PLC before chemical hydrolysis; (C) PLC after chemical hydrolysis. Peaks: 1 = propionic acid; 2 = PLC; 3 = L-carnitine; 4 = CB.

Reproducibility

Reproducibility was investigated as the coefficient of variation (C.V.) over a series of 24 evaluations of PLC after hydrolysis to L-carnitine. The inter-assay C.V. was 1.04% (Table I).

Specificity

The method proved to be specific for the L-enantiomers of both carnitine and propionylcarnitine. When applied to L–D-enantiomer mixtures (1:0, 3:1, 1:1), the same results were obtained, indicating that the D-enantiomer does not interfere. Its specificity was tested by determining small amounts of L-carnitine in the presence of large amounts of PLC (Table II), in order to validate the method for the determination

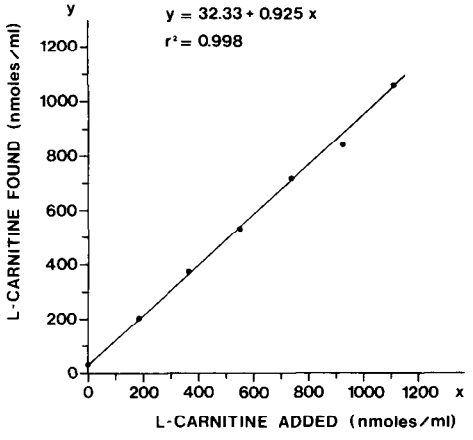

Fig. 2. Linearity of L-carnitine enzyme assay.

TABLE I

ANALYTICAL REPRODUCIBILITY OF LC ENZYME ASSAY

Six individual assays were carried out, each in quadruplicate.

Absorbance values	*Mean absorbance*	*S.D.*	*C.V. (%)*
0.512 0.507 0.505 0.513	0.509	0.0039	0.76
0.492 0.499 0.495 0.494	0.495	0.0029	0.59
0.502 0.495 0.505 0.494	0.499	0.0054	1.07
0.494 0.498 0.492 0.502	0.496	0.0044	0.89
0.499 0.496 0.491 0.500	0.496	0.0040	0.81
0.501 0.495 0.503 0.495	0.498	0.0041	0.83
Mean values	0.499	0.0052	1.04

TABLE II

SPECIFICITY OF THE ENZYME ASSAY FOR THE DETERMINATION OF SMALL AMOUNTS OF L-CARNITINE IN THE PRESENCE OF LARGE AMOUNTS OF PLC

Values in μmoles/ml.

L-Carnitine	*Propionyl-L-carnitine*	*Absorbance (mean of two determinations)*
0.19	3.0	0.134
0.19	5.9	0.135
0.19	11.8	0.132
0.19	23.7	0.131
		Mean: 0.133 S.D.: 0.0018 C.V. (%): 1.37

of L-carnitine when present as an impurity in PLC. In addition, the determination is not affected by the presence of propionic acid or potassium chloride in amounts equimolar to L-carnitine formed after PLC hydrolysis. In this respect, the method was validated for both PLC inner salt and hydrochloride.

DISCUSSION

We investigated the optimal hydrolysis conditions that convert PLC into L-carnitine by an enantiospecific enzyme assay. PLC hydrolysis proved to compete with CB formation, which in most instances was restricted to 0.2% and was never greater than 0.5%. The method also determines the L-carnitine before the hydrolysis as a likely impurity in PLC with high specificity. The percentage of PLC can be evaluated as follows:

$$[\text{PLC}] = A + B - \text{C}$$

where

$$A = \frac{A_c \cdot p_s \cdot 100 \cdot t}{A_s \cdot p_c};$$

A_c = absorbance of sample at 412 nm;
A_s = absorbance of standard at 412 nm;
p_c = weight of sample (mg);
p_s = weight of standard (mg);
t = fraction of reference standard title;
B = net percentage formation of CB;
C = percentage of L-carnitine in PLC raw material.

This method is therefore specific, selective, reproducible, sensitive and enantiospecific, allowing PLC to be determined in synthetic procedures and pharmaceutical formulations. It is also inexpensive, requiring only chemicals and enzymes that are readily available.

The method was not standardized for analyses of biological fluids, as the radioenzyme procedure proposed by Kerner and Bieber[7] seems to be more suitable for this purpose.

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