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## Note

# Use of liquid chromatography–mass spectrometry for the quantitation of dethiobiotin and biotin in biological samples

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During our studies on the biosynthetic pathway dethiobiotin (DTB) → biotin in *Escherichia coli*[1] we needed a method suitable for the analysis of small quantities of DTB and biotin. The existing assays, relying, in most cases, either on microbiological[2] methods or on avidin complexation[3–11], lack the specificity required by our biosynthetic studies*. So, we chose to identify biotin and its analogues by mass spectrometry (MS) which is a very sensitive and selective technique but requires a previous chromatographic purification to estimate compounds in biological mixtures. Gas chromatography–mass spectrometry could not be employed, to solve this problem because of the lack of volatility of biotin and its analogues, and we therefore used the combination of MS and liquid chromatography (LC). In the case of biotin, which does not absorb in the UV region, LC is limited by the availability of a suitable detection system. Until now we performed the conversion DTB → biotin using either a radioactive precursor[13] or a mixture of deuterated and radioactive[1] precursors. All these experiments, involving the isolation and chromatographic purification of amounts of biotin ranging from 1 to 10 μg, have been monitored using radioactivity detection. To avoid the well known difficulties linked with the use of labelled compounds, we have now elaborated a method suitable for the analysis of small quantities of DTB and biotin.

Two techniques were used: the first one is the off-line combination of LC and MS. Biotin and DTB in complex mixtures were derivatized as bromoacetophenone (BAP) esters[14] to enable their UV detection. Then biotin was identified by comparing its chromatographic retention time with a standard and, after fractionation, by MS analysis. However, with this method the high degree of selectivity of LC is lost during the fractionation. So, to avoid this disadvantage, we decided to analyse the biological

* An enzyme-linked immunosorbent assay (ELISA), which uses the development of monoclonal antibody technology, has recently been proposed[12]. This technique may become the method of choice especially in clinical chemistry.

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sample by the second technique: LC–MS of the methyl esters which are more easily prepared than the BAP esters.

## EXPERIMENTAL

Biotin was a gift from Hoffmann-La Roche (France). DTB was obtained from biotin by desulphuration with Raney nickel[15]. The methyl esters were prepared by treatment of the acids with diazomethane[16,17]. The trideuteromethyl esters of DTB and biotin were obtained by refluxing overnight 0.1 g of the corresponding acid in a mixture of 1 ml of $C^2H_3O^2H$ and 0.1 ml of sulphuric acid.

*Conversion of DTB into biotin*

This was performed by using *Bacillus sphaericus* as described by Izumi *et al.*[18]. After incubation, the cells were harvested and the supernatant evaporated to dryness under reduced pressure. The DTB and biotin were extracted with 99% ethanol and purified by column chromatography on a Dowex AG 1-X2 formate column as previously described[19–21]. The methyl esters were obtained from the acid using freshly prepared diazomethane and chromatographed on a silica gel 60 column (70–200 mesh) using ethyl acetate–methanol (9:1) as eluent. The fractions corresponding to the DTB and biotin methyl esters (compared to standard column) were collected, concentrated *in vacuo* and used for LC–MS studies.

*Equipment*

A Hewlett-Packard 1084B liquid chromatograph equipped with a variable volume automatic injector was used. The mass spectrometer was a Hewlett-Packard 5985B quadrupole driven by a PH 1000 data system. The sample was introduced into the mass spectrometer by means of the Hewlett-Packard direct liquid introduction probe with a 5-$\mu$m stainless-steel diaphragm allowing 20 $\mu$l/min of effluent into the mass spectrometer source.

*Experimental conditions*

The LC studies were carried out on a Hewlett-Packard RP 8 column (10 × 0.32 cm, particle diameter 5 $\mu$m), with methanol–water (40:60) as mobile phase. The flow-rate was 0.6 ml/min and 20 $\mu$l of solution were injected each time.

For sample introduction, water cooling of the probe allowed the diaphragm to operate around 40°C. The effective splitting ratio at introduction was 600/20 = 30. The mass spectrometer was operated in the positive chemical ionization mode using the LC solvent mixture as a reagent gas under the following conditions: source temperature, 200°C; electron energy; 150 eV; electron emission current; 300 $\mu$A. The quadrupole was adjusted for unit resolution over the mass range used for the analysis.

## RESULTS AND DISCUSSION

In preliminary work, reversed-phase separation of biotin and DTB methyl esters was carried out using an isocratic system, methanol–water (40:60), as mobile phase and refractometric detection[1]. In this study we used the same chromatographic conditions with MS detection in positive ion chemical ionization mode. Fig. 1 shows

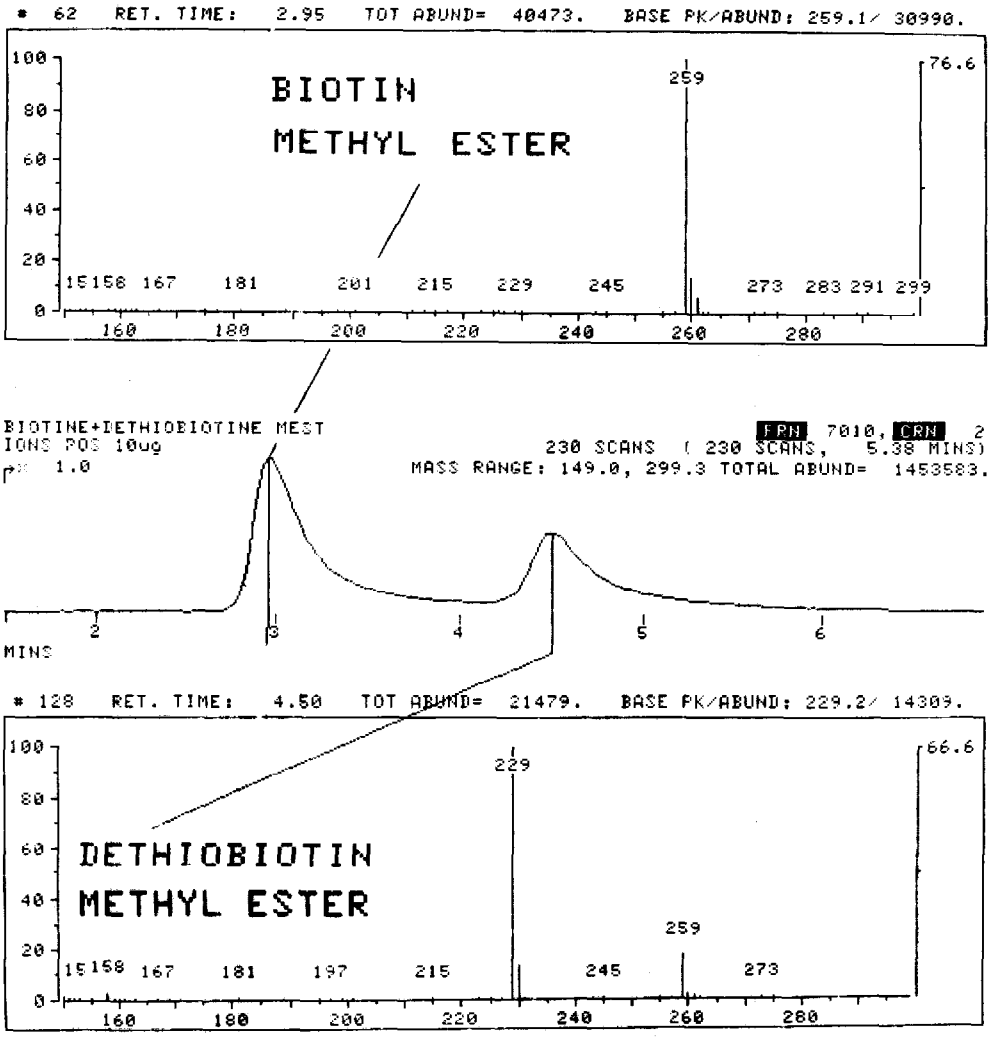


Fig. 1. LC–MS chromatogram of a mixture of 10 μg of DTB methyl ester and 10 μg of biotin methyl ester. Column: RP 8, 5 μm, 10 × 0.32 cm. Mobile phase: methanol–water (40:60). Flow-rate: 0.6 ml/min. Injection volume: 20 μl.

the LC–MS chromatogram obtained by injection of a mixture of 10μg of biotin and DTB methyl esters in a volume of 20 μl. In the corresponding spectra, only the quasimolecular peaks, $m/z$ = 259 and 229, respectively, were observed. Based on this result, in the subsequent measurements, mass fragmentography was employed which allows more sensitive analysis.

In order to test the usefulness of the method in quantitative analysis, we injected standard samples of DTB or biotin methyl esters ranging from 10 ng to 1 μg in a volume of 20 μl and compared the resulting peak areas. The limit of detection was found to be lower than 10 ng (50 pmol). The regression between the detector response and the injected sample amount is linear only from 0 to 300 ng. For quan-

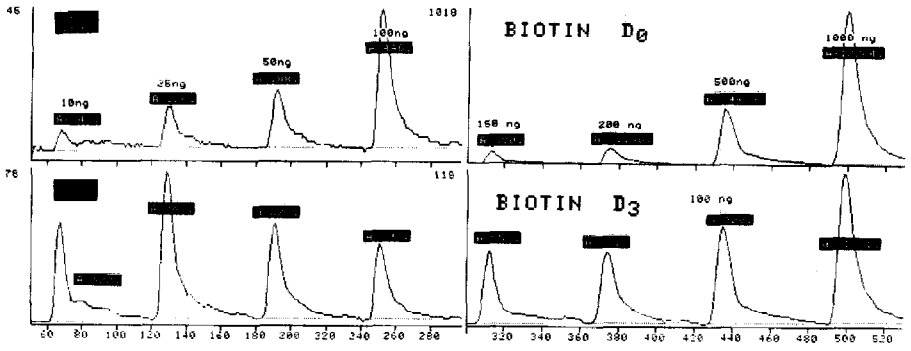


Fig. 2. LC–MS chromatograms of increasing quantities of biotin methyl ester (Biotin $D_0$) (from 10 ng to 1 μg) mixed with 100 ng of biotin [$^2H_3$]methyl ester (Biotin $D_3$) as internal standard. Ion monitoring at $m/z$ = 259 (biotin) and 262 (internal standard).

tities above 300 ng the response increases more rapidly. This phenomenon is known in chemical ionization.

So, to ameliorate the linearity of the analysis, the determinations were then performed using an internal standard. For this purpose we used the trideuteromethyl esters of biotin and DTB which are easily obtained from $C^2H_3O^2H$ with an excellent isotopic purity (>99%). Each standard sample of biotin methyl ester was injected together with 100 ng of biotin [$^2H_3$]methyl ester and MS fragmentography employed

TABLE I

CHARACTERISTICS OF THE CALIBRATION LINE ($y = ax + b$) FOR THE QUANTITATION OF BIOTIN AND DTB METHYL ESTERS

| *Methyl ester* | *Correlation coefficient* | $a \times 10^{-2}$ | $b$ | *Confidence limit (%)* |
|---|---|---|---|---|
| Biotin | 0.988 | 0.94 ± 0.06 | −0.047 ± 0.089 | 98 |
| DTB | 0.981 | 1.18 ± 0.04 | −0.060 ± 0.069 | 98 |

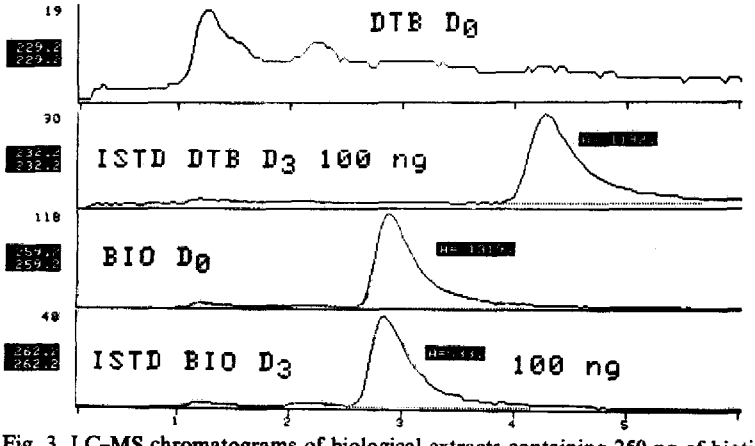


Fig. 3. LC–MS chromatograms of biological extracts containing 250 ng of biotin methyl ester (Bio $D_0$) plus 100 ng of biotin [$^2H_3$]methyl ester (Bio $D_3$) and DTB[$^2H_3$]methyl ester (DTB $D_3$) as internal standards (ISTD). Ion monitoring at $m/z$ = 229 (DTB), 232, 259 (biotin), 262.

for ions at $m/z$ = 259 and 262 corresponding to the quasimolecular peaks of the unlabelled and labelled biotin esters (Fig. 2). These measurements were repeated three times. In each case the relationship between the quantity of biotin injected and the peak area ratio for the ions at $m/z$ = 259 and 262 was determined: the regression graphs were linear over the range 0.5–50 μg/ml (10 ng–1 μg of biotin ester injected) (Table I).

A linear correlation was also observed for the methyl ester of DTB over the range 10 ng–1 μg using 100 ng of the DTB [$^2H_3$]methyl ester as internal standard (Table I).

This method has been applied to the quantitation of the conversion DTB → biotin by *Bacillus sphaericus*. The reaction was carried out as described by Izumi *et al.*[18] and the biotin was extracted and purified as its methyl ester[19–21]. To test the technique, known amounts of biotin methyl ester, 50 ng (a) and 250 ng (b), were added to biological extracts obtained after incubation of *Bacillus sphaericus* in a medium without DTB. The mean values obtained by this method were 59 ± 19 ng (a) and 270 ± 38 ng (b) (Fig. 3).

In conclusion, LC–MS appears to be a specific and convenient technique to identify and quantitate biotin and DTB as their methyl esters in complex matrices. The detection is five times more sensitive than the UV detection of BAP esters[14] and nearly as sensitive as fluorimetric detection of methyl methoxycoumarin esters[14]. Furthermore, methyl esters are easily obtained in quantitative yields after treatment with diazomethane.

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