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Note

Determination of progesterone by liquid chromatography—mass spectrometry using a moving-belt interface and isotope dilution

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In the past decade several new interfaces for liquid chromatography—mass spectrometry (LC—MS) have been developed [1, 2] and successfully applied in the analysis of complex mixtures. Of these, the moving-belt interface has the advantage that removal of the mobile phase enables the use of the ionization method of choice. The combination of electron impact (EI) and chemical ionization (CI) is favourable with respect to identification problems, because both structural and molecular weight information is obtained. Furthermore, a library search with the EI spectra obtained is possible with the standard libraries. In addition, self-interpretative retrieval systems can provide substructural information [3—7].

For thermally labile or very polar compounds, "soft ionization" methods as fast atom bombardment (FAB), secondary ion MS (SI-MS) and laser desorption (LD) [8—10] can be used in combination with the moving belt. For the latter class of compounds, LC—MS with thermospray ionization seems to be very promising [11, 12].

The construction of a moving-belt interface strongly influences its performance, as has been shown in a comparative study [13]. It was found that desorption characteristics comparable with those of "direct" CI can be obtained if flash evaporation is accomplished inside the ionization source.

Several gas chromatographic—mass spectrometric (GC—MS) methods have

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been reported for the determination of progesterone by isotope dilution [14, 15]. For GC—MS analysis, however, derivatization is necessary, which is not the case when LC—MS [16] is used.

In the study described here, we applied isotope-dilution LC–MS for the determination of lyophilized progesterone in serum kits — designed as standards for measurement of progesterone with radioimmunoassays — and in human serum.

EXPERIMENTAL

Instrumentation

A Finnigan MAT "moving-belt" interface was coupled to a Finnigan MAT 8230 double-focusing mass spectrometer equipped with an EI-CI ion source. A Waters M6000 pump, a Waters U6K injector and a Nucleosil C_{18} column (300 × 4.0 mm I.D.; particle size 5 μm) were used, with methanol as mobile phase at flow-rates of 0.6—1.2 ml/min. Other typical conditions were: source temperature, 200°C; CI (ammonia) source pressure, 0.5—1.0 Torr; vaporizer temperature, 150°C.

Samples

Isotope dilution was performed with [3,4-$^{13}C_2$] progesterone (CEA, France). For the determination of lyophilized progesterone in a standard kit (Eurodiagnostics, The Netherlands), 4.8 μg of the labelled compound were added to 1 ml of serum. After equilibration, 1 ml of water was added followed by extraction with 2 ml of hexane. After washing with 1 ml of dilute sodium chloride solution, the solution was evaporated to dryness under a nitrogen stream. For the LC—MS analysis the residue was dissolved in 250 μl of methanol. GC—MS analysis was performed after derivatization with 50 μl of methoxylamine hydrochloride (2% in pyridine) which results in formation of dimethoxime-progesterone. Calibration was performed, using standard solutions containing a fixed amount of the isotopically labelled substance (4.8 ng/μl) and with various concentrations of unlabelled progesterone (0, 2.15, 4.3 and 8.6 ng/μl). The standards were directly used for the LC—MS analysis as well as for GC—MS analysis after derivatization as described above.

Samples of human serum (0.75 ml) were analysed after addition of 24 ng of labelled progesterone by the same method. After evaporation to dryness, however, the residue was dissolved in 50 μl of methanol.

RESULTS

Injection of a standard solution containing 4.3 ng of unlabelled progesterone (mol. wt. 314) and 4.3 ng of [3,4-$^{13}C_2$] progesterone resulted in the mass fragmentogram shown in Fig. 1A. Despite maximal clean-up heating and the application of a washing procedure, severe memory effects due to the moving belt are apparent. In order to reduce this effect the moving belt was treated with a solution containing 50 ppm of Carbowax 20M in chloroform. This solution was deposited on the moving belt during one cycle. Although an increase of the background was observed, the memory effect almost disappeared and the

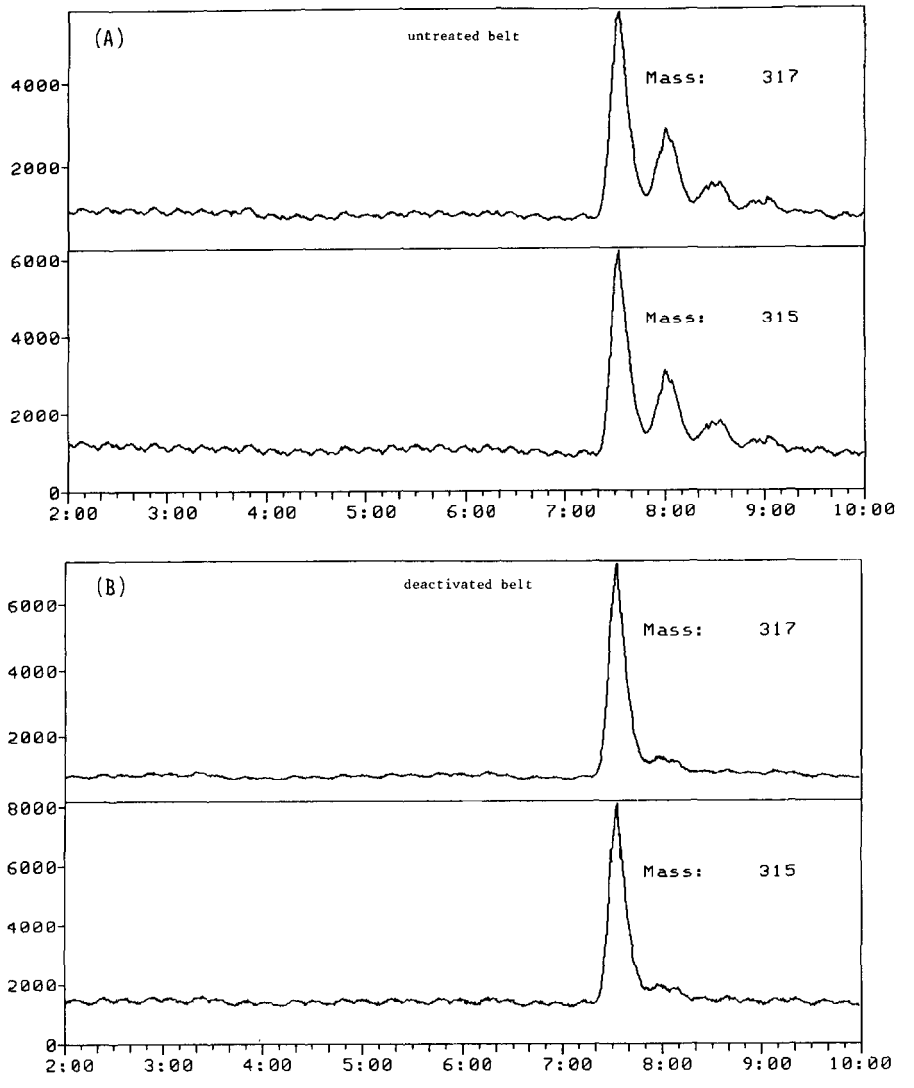

Fig. 1. LC—MS analysis of a standard solution of progesterone with isotope dilution before (A) and after (B) deactivation of the belt.

sensitivity improved slightly (Fig. 1B). Two deactivation treatments completely removed the memory effects, but a day later a small memory effect was detectable again. Therefore, the belt was deactivated at the start of each day. The belt memory was found to be strongly compound-dependent whereas the stability of the deactivation depends on the mobile phase used (shorter for high concentrations of water and acetonitrile). The desorption characteristics of the moving belt are positively influenced by the deactivation procedure [17].

The detection limit of the moving-belt LC—CI-MS system for progesterone is ca. 4 ng/ml (25 μl injected) as illustrated in Fig. 2, which shows the detection of 200 pg of both labelled and unlabelled progesterone. The baseline peak

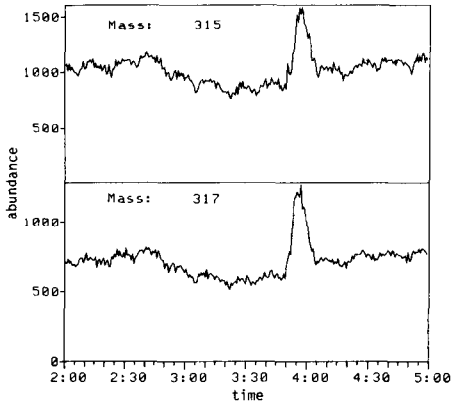

Fig. 2. LC—MS isotope dilution determination of 200 pg of progesterone and [3,4-$^{13}C_2$]-progesterone.

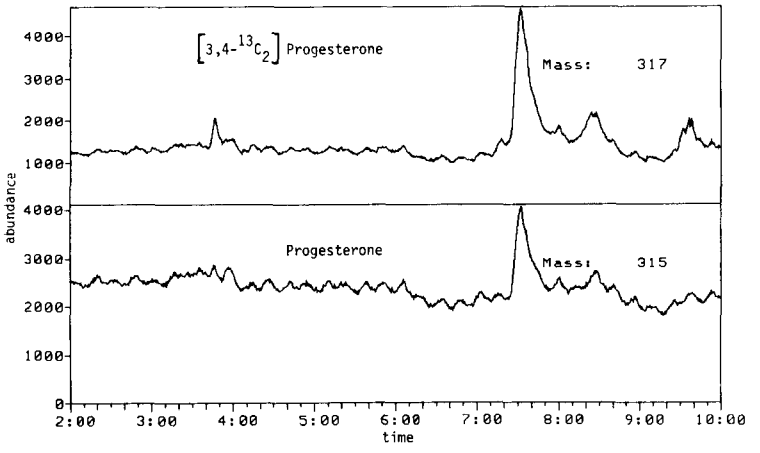

Fig. 3. Determination of progesterone in a human serum sample with isotope dilution.

width in this experiment, with methanol as mobile phase at a flow-rate of 1.2 ml/min, is 13 sec.

The progesterone calibration was performed by measurement of four standard solutions containing a fixed amount of the labelled substance (4.3 ng/μl) and various concentrations of the unlablled progesterone (0, 2.15, 4.3 and 8.6 ng/μl). A calibration line via linear regression was determined as $y = 1.11 - 0.081$, with $r^2 = 0.997$.

Samples were worked up in duplicate and measured in triplicate by LC—MS. In this way a value of 4.7 ± 0.3 ng/ml was measured for a standard serum kit. The accuracy of the result was mainly determined by the spread in the duplicates, indicating that possibly the equilibration between labelled and unlabelled material was not complete in both cases. GC—MS analysis of the same samples after derivatization confirmed this. The results were identical with those obtained by LC—MS analysis. However, the LC—MS method is quicker than the GC—MS method, because no derivatization is needed and measurement of the standard solutions can be performed much faster. The latter

measurements can be made by injecting every 30 sec without having to wait for elution of the previous sample. With GC—MS this is not possible owing to solvent effects occurring during the injections.

Human serum samples were also analysed by the LC—MS method and a typical example of a mass chromatogram is shown in Fig. 3. No interference was observed at the retention time of progesterone while quantitation was performed as usual. For the serum sample a progesterone level of 20.0 ± 0.5 ng/ml was found, using the same baseline definition for both masses in the isotope dilution experiment, which is in good agreement with a radioimmunoassay value of 17.7 ng/ml.

CONCLUSIONS

Progesterone can be determined rapidly and accurately with the method presented, using isotope dilution LC—MS with a moving-belt interface. Deactivation is very useful in that it minimizes belt memory effects and optimizes belt desorption characteristics. Since the moving-belt properties were found to change during time with respect to the parameters mentioned, isotope dilution is of importance in order to obtain accurate results.

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