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Short communication

Determination of the terfenadine metabolite azacyclonol in human serum using gas chromatography–mass spectrometry

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Abstract

In this work, a method for the determination of azacyclonol, a primary metabolite of terfenadine, in human serum is described. Sample preparation is carried out by liquid–liquid extraction under basic conditions. For an efficient clean up, the analytes are back-extracted into diluted hydrochloric acid and, after alkalinization, are once again extracted into the organic phase. No derivatisation step is performed. The samples are measured by gas chromatography–mass spectrometry with good selectivity. The limit of detection is 2 ng/ml. The coefficients of variation are 12.6% at 10 ng/ml and 6.44% at 200 ng/ml in the day-to-day control measurements.

Keywords: Terfenadine; Azacyclonol

1. Introduction

Terfenadine is a widely used antihistaminic drug without central nervous depressant activities. Its serum levels, due to the extensive first pass metabolization, are very low. Peak levels reach only about 4 ng/ml after oral administration of 180 mg to healthy volunteers [1]. The two major metabolic pathways are the oxidation of the *tert.*-butyl group of terfenadine to the corresponding acid and the cleavage of the N-alkyl chain to yield the product azacyclonol (α,α-diphenyl-4-piperidinemethanol, AZA). Both metabolites are formed in constant ratios by the enzyme cytochrome P450 3A4 [2]. To monitor the low serum concentrations of terfenadine, RIA is the method of choice [1]. For the acid metabolite, an HPLC procedure with fluorescence detection is capable of determining serum levels in therapeutic ranges [3]. Both metabolites can be detected in human urine by thermospray liquid chromatography–mass spectrometry [4]. The determination of AZA in human serum and urine using GC–MS is described in [5]. This method uses a derivatisation step of the secondary amine moiety with heptafluorobutyric anhydride prior to GC analysis.

In the method presented here, the derivatisation step, which is quite expensive and can be a source of errors, is avoided. The serum extract, containing AZA and the I.S., is injected directly onto the GC column and the eluent is monitored by mass spectrometry with good selectivity and sensitivity.

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2. Experimental

2.1. Apparatus

A gas chromatograph (HP 5890 Series II plus), with an electronic pressure programmer and cool-on-column-injector is used. The analytes are separated on a HP-5 capillary column (30 m × 0.320 mm I.D., 0.33 μm film thickness). The detection is carried out in a HP 5972 MSD mass-selective ion detector (all from Hewlett-Packard, Waldbronn, Germany). Data are collected and analyzed by a Hewlett-Packard DOS ChemStation (software version C.01.05).

2.2. Chemicals

Azacyclonol (>98%, batch A0876) is obtained from Applichem (Heidelberg, Germany), the I.S., 4-diphenylmethoxy-1-methylpiperidine hydrochloride (>97%, batch 132-18-3), is from Aldrich (Steinheim, Germany). n-Hexane is purchased from Baker (Gross-Gerau, Germany; quality grade: Baker-analyzed), methanol, isoamyl alcohol, HCl, NaOH and NaCl in quality grade p.A. are from Merck (Darmstadt, Germany). Helium, quality 5.6, is purchased from Messer-Griessheim (Magdeburg, Germany).

2.3. Sample collection

The blood samples (about 10 ml) are collected into glass tubes. To separate serum from blood cells, the samples are centrifuged for 10 min at 2400 *g*. After centrifugation, the serum samples are frozen at −20°C until analysis.

2.4. Sample preparation

To 1 ml of serum, 0.1 ml of a solution of the I.S., 4-diphenylmethoxy-1-methyl-piperidine hydrochloride (2 μg/ml in water), and 0.5 ml of 1 *M* NaOH in 6% aqueous NaCl are added. This mixture is extracted with 4 ml of 1.5% isoamyl alcohol in *n*-hexane by shaking for 30 min. After phase separation, the organic phase is transferred into a tube containing 1 ml 0.1 *M* hydrochloric acid and is re-extracted by shaking for 15 min. The remaining organic phase is discarded and the aqueous phase is alkalinized by adding 0.15 ml 1 *M* NaOH in 6% aqueous NaCl. This mixture is extracted again by shaking with 0.1 ml of 1.5% isoamyl alcohol in *n*-hexane. Finally, the organic phase is evaporated, the residue reconstituted in 10 μl of *n*-hexane and 1 μl is injected manually onto the GC–MS system.

2.5. Calibration and quality control samples

An initial solution of AZA is made by dissolving 10 mg in 100 ml of methanol. This solution is diluted by a factor of ten with methanol and drug free serum is spiked with this solution to yield calibration samples in the range of 10 to 160 ng/ml.

A stock of quality control samples are prepared at concentrations of 10 and 200 ng/ml and frozen until usage. Both levels are measured before and after each sample block to assure the reproducibility of the assay.

2.6. Chromatographic conditions

Carrier gas for the chromatographic separation is helium at a flow-rate of 1.2 ml/min. This flow-rate is held constant over the run time of the temperature program. The temperature program starts at 65°C, is held constant for 1 min, then the temperature is raised by 15°C/min up to 290°C and held constant for 1 min. The total run time is 17 min. The injector is tracking the oven temperature and the MS interface is heated to 300°C. A 1-μl volume is injected manually direct onto the column. Under these conditions, the retention time of the I.S. is 13.0 min and that of AZA is 14.0 min. For the I.S., the ions 114.1 *m/z*, 152.1 *m/z* and 167.1 *m/z* and for AZA the ions 105.0 *m/z* and 183.1 *m/z* are observed.

3. Results and discussion

3.1. Chromatography and mass spectrometry

Fig. 1 shows some typical chromatograms obtained by the method described in Section 2. Since AZA, because of its polar secondary amine moiety, cannot be injected on standard split injectors with satisfying results, the usefulness of the on-column

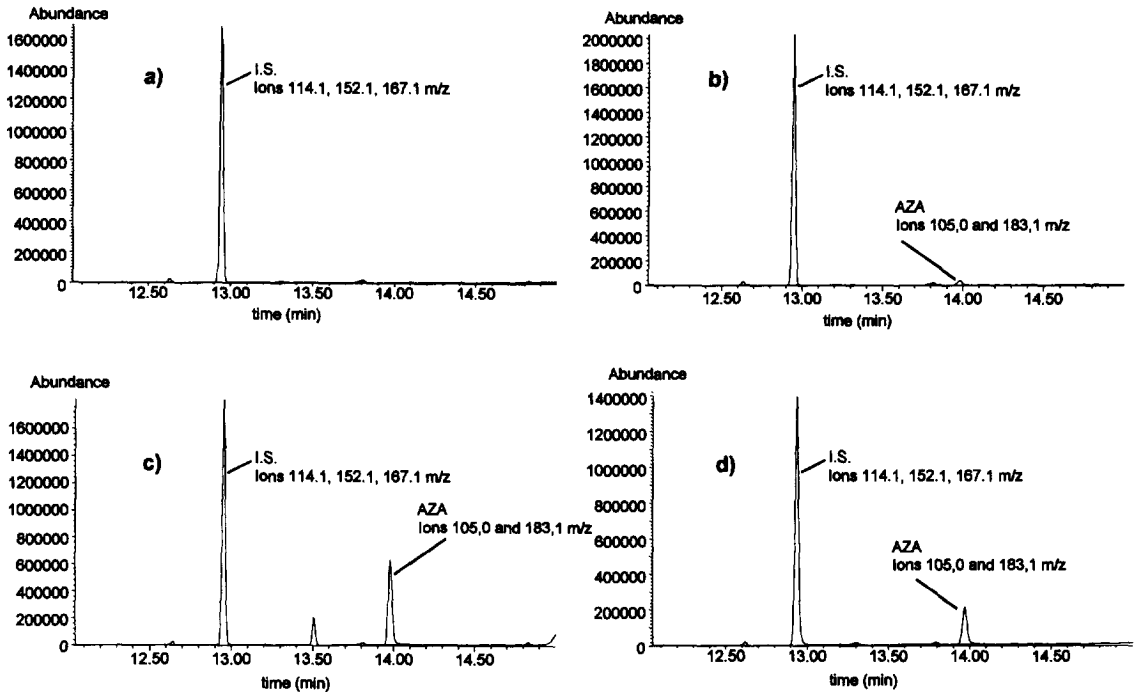

Fig. 1. Typical chromatograms obtained using the method described in Section 2 (the sum of the monitored ions is shown). (a) Blank serum with 200 ng/ml of I.S. (b) Serum spiked with 10 ng/ml of AZA and 200 ng/ml of I.S. (c) Serum spiked with 200 ng/ml of AZA and 200 ng/ml of I.S. (d) Sample from a patient containing 83 ng/ml of AZA.

injection here is illustrated by the sharp and symmetrical peak-shape for both AZA and the I.S. As can be seen in Fig. 1, no endogenous substances are interfering with the assay.

Mass spectra obtained for AZA and for the I.S., by electron impact ionization at 70 eV, are depicted in Fig. 2. The main fragmentation pattern of the molecules are pointed out by the hatched lines in the formula schemes. The mass spectra correspond to those published in [5] for AZA and in [6] for the I.S. The detection is carried out in the selected-ion monitoring mode of the mass spectrometer and the ions 105 m/z and 183 m/z are observed in the case of AZA and 114 m/z, 152 m/z and 167 m/z for the I.S. These fragment ions exhibit high intensities in the mass spectra at low background noise levels. The quantitation is carried out with the fragment ions 105 m/z for AZA and 167 m/z for the I.S. The other fragment ions serve as qualifiers for peak identification.

3.2. Extraction efficiency

The recovery of AZA in the extraction procedure is determined by comparing peak areas of extracted samples with pure solutions of AZA at concentrations corresponding to 100% recovery. A trial with five sample pairs results in a 67% extraction yield. The sophisticated extraction procedure produces very clean extracts, thus the on-column injector and the analytical column do not need maintenance due to non-volatile residues over a period of more the 200 samples.

3.3. Quantitation

The calibration function for AZA is linear in the range of 10 to 160 ng/ml and has the parameters (nine concentration levels) slope = 5.15×1.0^{-3} (S.D. = 1.7×10^{-4}), intercept = −0.017 (S.D. = 0.015) and r = 0.9962.

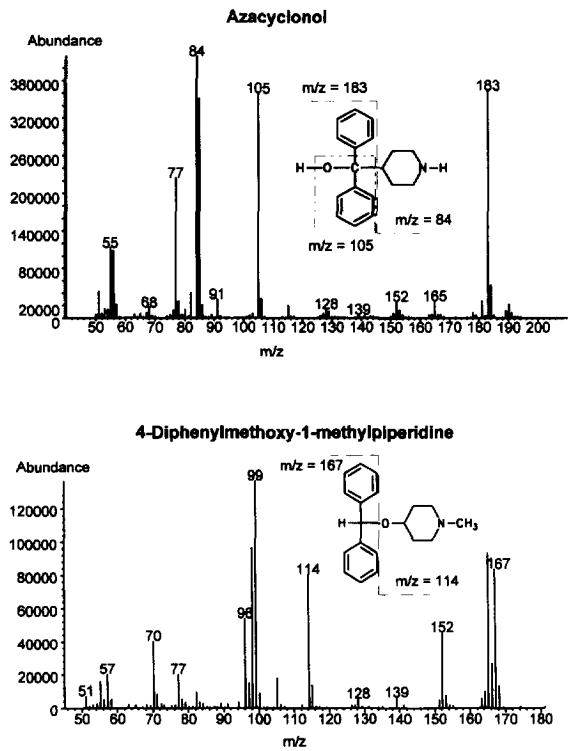

Fig. 2. Mass spectra of AZA and the I.S. obtained by electron impact ionization at 70 eV. The main fragmentation pattern of the molecules are pointed out by the hatched lines in the formula schemes.

The limit of detection is defined as three times the noise level of a blank chromatogram and results in a concentration of 2 ng/ml. The limit of quantitation is defined as three times the limit of detection, which is 6 ng/ml.

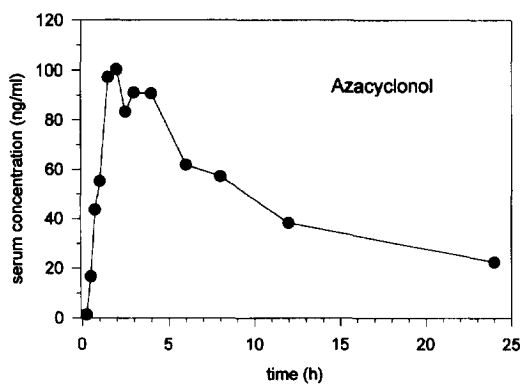

Fig. 3. Concentration–time curve of AZA after oral intake of 240 mg of terfenadine, together with 300 ml of milk, by a healthy volunteer.

3.4. Precision and accuracy

The intra-day R.S.D. ($n=10$) of the 10 ng/ml spike level is 9.51% with an average result of 11.0 ng/ml and for the 160 ng/ml spike level is 10.6%, with an average result of 157.4 ng/ml. On ten different days of analysis, the quality control samples show an average of 11.5 ng/ml with an R.S.D. of 12.6%, at the 10 ng/ml level, and an average of 203.9 ng/ml with an R.S.D. of 6.44%, at the 200 ng/ml level. None of the quantitative results differ by more than 1.5% from the spike level and none of the R.S.D.s exceed the limit of 15%, which is the highest acceptable value in our laboratory.

4. Application of the assay in clinical trials

The described method has been used in a clinical trial investigating the resorption properties of terfenadine under different feeding conditions. In the course of this trial, we have measured about 500 serum samples with this procedure. A typical serum concentration–time curve of AZA, after a single oral dose of 240 mg terfenadine, is depicted in Fig. 3.

5. Conclusions

Due to the very low serum levels of terfenadine caused by its first pass metabolization, the drug can only be determined in serum with great difficulty. Its metabolite, AZA, is a possible substitute for monitoring absorption properties of terfenadine. The described procedure for the determination of AZA in human serum is selective and sensitive enough to be used in clinical trials as well as in compliance investigations in patients. Its lack of complicated and expensive derivatisation steps in the sample preparation eliminates a probable source of errors. A further facilitation of the assay can be the use of an autosampler for more sample throughput and a further improvement in precision and accuracy.

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