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Short communication

Gas chromatographic–mass spectrometric method for quantitative determination of ketotifen in human plasma after enzyme hydrolysis of conjugated ketotifen

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Abstract

A validated method for determination of total amount of ketotifen (unchanged and conjugated) in human plasma has been presented. An enzyme hydrolysis of conjugated ketotifen was conducted with combination of β-glucuronidase and arylsulfatase. After the enzyme hydrolysis a solid-phase extraction was applied as a cleaning step. The quantitative determination by gas chromatography with mass-spectrometry detection (GC–MS) was performed. Pizotifen has been used as an internal standard. A reliable hydrolysis as well as a satisfactory accuracy, improved precision in the linear region from 0.500 to 10.0 ng/ml plasma, limit of detection of 0.010 ng/ml and prolonged capillary column life have been achieved.

Keywords: Ketotifen

1. Introduction

Ketotifen, [4-(1-methyl-4-piperilidine)-4H-benzo-(4,5)-cyclohepta-(1,2-*b*)-thiophen-10(9H)-one] (Fig. 1a) belongs to the group of cycloheptathiophenones. The drug is H_1-receptor blocking agent with antiasthmatic effect and mechanism of action associated with inhibition of histamine release from mastocytes. In humans ketotifen is biotransformed to ketotifen-*N*-glucuronide (primary), *N*-demethyl ketotifen, *N*-oxide ketotifen and reduced ketotifen [1,2]. The glucuronide conjugate [1] and the *N*-oxide ketotifen [2] readily undergo hydrolysis to the parent drug. The enzyme hydrolysis of glucuroconjugated ketotifen have been conducted by β-glucuronidase [1–8].

The investigations of ketotifen have been conducted by GC with electron capture detection [4] and by GC–MS methods [1–3,5–10]. Sieradzki at al. [4] and Julien-Larose at al. [5] used alkalization of the plasma samples before the liquid extraction with benzene. We found out that these extracts were not pure enough for GC–MS analysis. To establish a better procedure for sample clean-up, also avoiding toxic benzene, and to obtain a more reliable determination of ketotifen in human plasma we used a solid-phase extraction technique with RP-CN-E cartridge.

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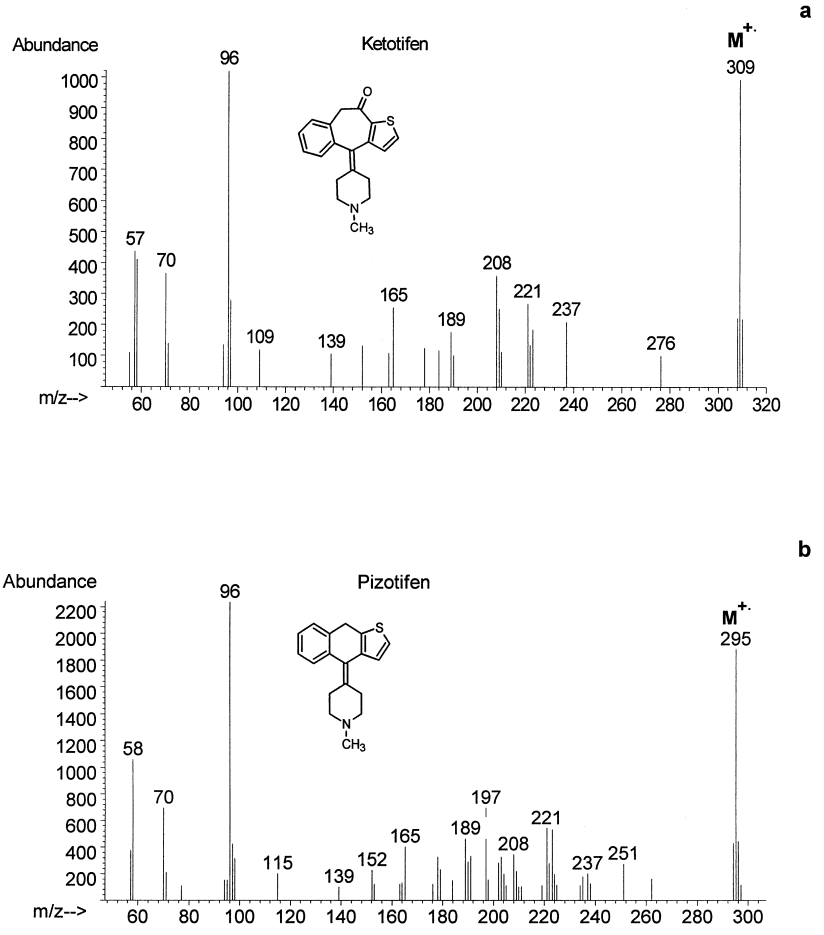

Fig. 1. Mass spectra and structures of ketotifen (a) and pizotifen (b).

2. Experimental

2.1. Equipment

The GC–MS equipment consisted of Hewlett-Packard 5890 SERIES II Plus gas chromatograph and 5972 mass-selective detector (Hewlett-Packard, Palo Alto, CA, USA) and an autosampler HP 7673 with a controller. The column was a HP-5MS fused-silica (15 m×0.25 mm I.D.) with cross-linked 5% PH ME siloxane stationary phase with a film thickness 0.25 μm, fixed through the transfer line about 2 mm from the ion source block. The transfer line temperature was 280°C; ionization voltage was 70 eV; vacuum 1.5×10^{-3} Pa. A split–splitless injector was used at 270°C with split opening time 1.0 min. after the injection. The carrier gas was helium at a flow-rate of 0.900 ml/min. An HP G 1034C MS ChemStation Software has been used. The column temperature program began at 100°C for 3.0 min, increased with 20°C/min to the final temperature of 250°C, which was held for 2.0 min. A purification step at 300°C for 3.0 min was included. The injection frequency in the automatic process was approximately 22 min.

Pizotifen was used as an internal standard (I.S.). The selected-ion monitoring (SIM) mode for identification and quantitation has been applied on the following conditions: group 1 for I.S.: m/z 295.10

and 295.20, (Fig. 1b); group two for ketotifen: *m/z* 309.10 and 309.20, (Fig. 1a) both at dwell time 100 ms, EMV delta 500 V. Autotune at masses: 219, 264 and 414 was used for daily calibration of the mass-selective detector.

2.2. Chemicals and reagents

Ketotifen and pizotifen were synthesized in NIHFI (Sofia, Bulgaria), human drug free plasma was obtained from the National Center for Blood Transfusion. The solvents used were: methanol for chromatography, chloroform for chromatography, 2-propanol for chromatography and *n*-hexane-p.a. obtained from Merck (Darmstadt, Germany). The enzyme hydrolysis was made with β-glucuronidase (~30 U/ml)–arylsulfatase (~60 U/ml) (from Helix promatia), (Merck). The chemicals used were: trisodium citrate dihydrate-p.a., citric acid monohydrate-p.a., and sodium hydroxide-p.a., (Merck). The solid-phase extraction was carried out with ''Varian Bond Elut®'' cartridges, 3 ml, 500 mg reversed-phase CN-E from Varian Associates (Harbor City, CA, USA).

2.3. Analytical procedure

2.3.1. Preparation of the calibration samples

Both stock solutions of ketotifen and I.S. were made at a concentration of 50 μg/ml in methanol, stored at 4°C and protected from direct light. The I.S. working solution was prepared by dilution of the stock solution to gave a final concentration of 140 ng/ml. Analytical solutions for the preparation of plasma calibration samples were made at the following concentrations of ketotifen: 50, 100, 200, 400, 700 and 1000 ng/ml in methanol. A 1.0 ml volume of each analytical solution was diluted with blank plasma to 100 ml. The final concentrations of the plasma calibration samples were: 0.500, 1.00, 2.00, 4.00, 7.00 and 10.0 ng/ml plasma. All plasma samples were stored at −20°C in plastic tubes.

2.3.2. Sample preparation

2.3.2.1. Solution for the enzyme hydrolysis. To 40 ml citric buffer (pH=5.20) were added 1.6 ml β-glucuronidase (~30 U/ml)–arylsulfatase (~60 U/ml) solution (solution A). The final concentrations of β-glucuronidase and arylsulfatase were approximately 1.15 and 2.31 U/ml, respectively. This solution A was daily prepared for the enzyme hydrolysis of plasma samples.

2.3.2.2. Enzyme hydrolysis. Plasma samples (1 ml) were incubated at 37°C for 18 h [3–5] with 0.5 ml of the fresh solution A.

2.3.2.3. Extraction procedure. After the enzyme hydrolysis each plasma sample was diluted with 4 ml bidistilled water and made alkaline with 0.5 ml of 0.1 *M* NaOH. A 50 μl volume of working solution of I.S. (7.00 ng/ml plasma) was added and mixed at 100 rpm in a test-tube for 30 s. The SPE-CN-E cartridge was conditioned with 3 ml methanol and 3 ml bidistilled water. The sample was aspirated through the SPE-CN-E cartridge. The cartridge was washed with four cartridge volumes bidistilled water and 1 ml mixture of bidistilled water–2-propanol (2:1, v/v). After vacuum drying of the cartridge ketotifen and I.S. were eluted with five portions of 0.5 ml methanol and the total eluate was evaporated to dryness at 37°C under N_2. The dry residue was dissolved in 300 μl chloroform, transferred into a microvial and evaporated to dryness at room temperature under N_2. The residue was dissolved in 50 μl methanol and 1 μl of it was injected into the GC–MS.

2.3.3. Washing of SPE-RP cartridges and glassware

After the elution the cartridges were regenerated by rinsing with chloroform, methanol and bidistilled water. The glassware were washed with a detergent solution, soaked in a bichromic mixture, rinsed with bidistilled water, soaked in 0.02 *M* NaOH and finally rinsed with bidistilled water.

3. Results

The method was validated in terms of selectivity, sensitivity, linearity, accuracy, precision, analytical recovery and stability.

3.1. Selectivity

No interfering peaks have been observed in blank plasma. The SIM chromatograms for blank plasma and plasma calibration samples with 0.500 and 7.00 ng/ml ketotifen and 7.00 ng/ml I.S. were presented in Fig. 2.

3.2. Sensitivity

The detection limit, defined as three times the baseline noise for the plasma sample examined was 0.010 ng/ml human plasma. The limit of quantitation chosen, defined as the concentration assessed with acceptable accuracy and precision (below 15%), was

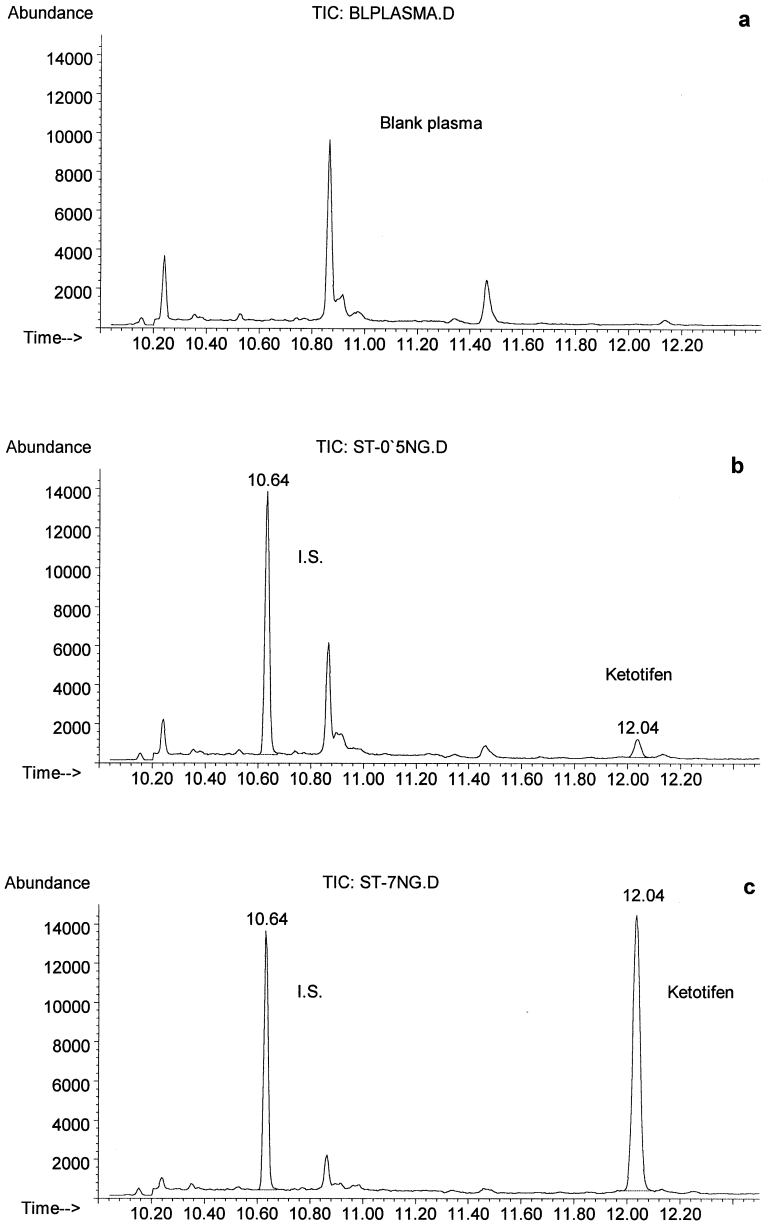

Fig. 2. SIM chromatograms of blank plasma (a) and plasma calibration samples contained 0.500 (b) and 7.00 (c) ng/ml ketotifen and 7.00 ng/ml I.S.

Table 1
Intra-day variation ($n=7$)

Concentration of ketotifen added (ng/ml)	Mean concentration of ketotifen found (ng/ml)	Accuracy (%)	SD	Coefficient of variation (%)
0.500	0.505	+ 1.00	0.0164	3.25
1.00	0.983	−1.70	0.0482	4.90
2.00	1.95	−2.50	0.0517	2.65
4.00	4.15	+ 3.75	0.1195	2.88
7.00	6.97	−0.43	0.1519	2.18
10.0	10.1	+ 1.00	0.3373	3.34

0.500 ng/ml plasma. It was completely sufficient for bioavailability studies of ketotifen.

3.3. Precision and accuracy

Six concentration levels, each replicated seven times have been measured for the evaluation of intra-day precision and accuracy (Table 1).

Replicates at each level for the calibration range from 0.500 to 10.0 ng/ml plasma have been measured daily. Fifteen calibration curves were constructed on fifteen consecutive days. Table 2 presented inter-day precision and accuracy ($n=30$).

3.4. Linearity and curve fitting

The calibration curve was studied over the concentration range from 0.500 to 10.0 ng/ml plasma and shown to be linear. For a better fitting a linear regression with weighting factor inverse of concentration has been chosen: $y = a_0 + a_1 \times x$, where $y = (\textit{peak area of ketotifen})/(\textit{peak area of I.S.})$ was the response ratio and $x = (\textit{concentration of ketotifen})/(\textit{concentration of I.S.})$ was the concentration ratio. In general, the r^2 values obtained were at a range of 0.999. The equation obtained from six calibration levels ($n=7$) was: $\textit{response ratio} = -1.38 \times 10^{-2} + 2.26 \times \textit{concentration ratio}$.

3.5. Analytical recovery

The analytical recovery of extraction procedure was determined at all concentration levels (each with seven repetitions) as the ratio of the peak area of ketotifen obtained from the calibration plasma sample to the peak area of ketotifen obtained from the analytical solution at the same concentration. The recovery for the internal standard was calculated in the same way. The values of the mean analytical recovery of extraction procedure were 81% and 60% for ketotifen and I.S., respectively.

3.6. Stability

Under storage conditions for more than three months the plasma calibration samples showed no degradation.

Table 2
Inter-day variation ($n=30$)

Concentration of ketotifen added (ng/ml)	Mean concentration of ketotifen found (ng/ml)	Accuracy (%)	SD	Coefficient of variation (%)
0.500	0.503	+0.60	0.0172	3.41
1.00	0.987	−1.30	0.0373	3.78
2.00	1.99	−0.50	0.0667	3.35
4.00	4.08	+2.00	0.1281	3.14
7.00	6.96	−0.57	0.1162	1.67
10.0	9.96	−0.40	0.1484	1.49

4. Conclusion

A suitable extraction procedure and GC–MS method were set up to guarantee a reliable determination of ketotifen in human plasma. The extracts obtained by our extraction procedure have been considerably cleaner compared to the ones described in the literature [4,5]. The most important advantages are: (1) limit of detection 0.010 ng/ml plasma; (2) improvement in the method precision and accuracy; (3) sparing of the capillary column and MS detector; (4) clean SP-extracts provided working with large volumes of plasma samples (1 ml and more); (5) using of toxic benzene was avoided.

Time consuming purification procedures have been avoided because of the high degree of purification of the plasma samples using SP-RP extraction. The results from the validation proved that the method described was suitable for pharmacokinetic studies.

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