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Determination of ketotifen in human plasma by LC–MS

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Abstract

Analytical validation of a new liquid chromatographic–mass spectrometric (LC–MS) method for determination of total amount of ketotifen (unchanged and conjugated) in human plasma is presented. Pizotifen was used as an internal standard. An enzyme hydrolysis of conjugated ketotifen was conducted with a combination of β-glucuronidase and aryl sulfatase. After enzyme hydrolysis a liquid–liquid extraction was performed as a cleaning step. The quantitative determination was obtained using selected ion monitoring (SIM) LC–MS. Chromatographic condition was a combination of reverse phase gradient system and a switching column technique. A satisfactory hydrolysis, acceptable accuracy, improved precision in the linear range from 0.5 to 20.0 ng/ml plasma, absolute recovery of 98.04% for ketotifen and 95.13% for pizotifen and stability for 7 months at −20 °C have been achieved.
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1. Introduction

Ketotifen, 4,9-dihydro-4-(1-methyl-4-piperidinylidene)-10H-benzo[4,5]cyclohepta[1,2-b]thiophen-10-one, is a nonbronochodilator; antialletrgic properties and a specific anti-H1 effect [1]. In human, ketotifen is biotransformed to ketotifen-*N*-glucuronide (primary), *N*-demethyl ketotifen, *N*-oxide ketotifen and reduced ketotifen [2,3] (Fig. 1). The glucuronide conjugate and the *N*-oxide ketotifen readily undergo hydrolysis to parent drug by β-glururonidase [2–9]. Preclinical studies indicated that ketotifen's anti-H1 effect seems to be distinct from its anti-allergic properties. The effectiveness of ketotifen in the chronic management of mild atopic pediatric asthma has been shown in clinical trials. Continued use of ketotifen results in a partial reduction in the frequency, severity and duration of asthma symptoms and attacks.

Ketotifen has been investigated by GC with electron capture detection [5] and by GC–MS methods [1–3,5–11]. Sieradzki et al. [5] and Julien-Larose et al. [6] used alkanization of the plasma samples before the liquid extraction with benzene. Tzvetanov et al. [12] determined ketotifen in human plasma after enzyme hydrolysis using solid-phase extraction technique and GC–MS. The present paper describes the development and validation of the first liquid chromatographic–mass spectrometric (LC–MS)

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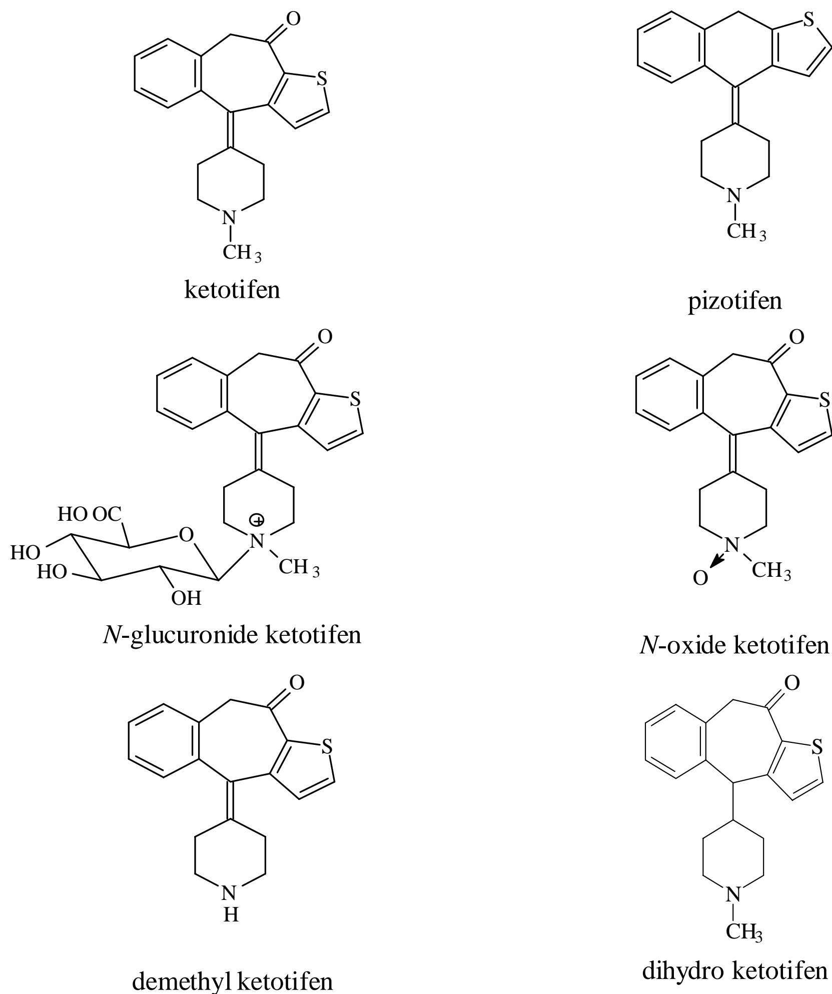

Fig. 1. Chemical structures of pizotifen, ketotifen and its main metabolites.

method for quantification of ketotifen in human plasma after enzyme hydrolysis using switching column technique. For those who adopt LC–MS as a main tool of analysis and find it more practical, this new method will find an application in bioequivalence studies for determination of ketotifen in human plasma.

2. Experimental

2.1. Equipment

Mass spectral analyses were carried out on an AQA single quadruple LC–MS system (ThermoQuest, Finnigan, UK). The LC–MS equipment used was consisted of Symmetry (Waters, USA) C18 (5 μm) (150 mm × 4 mm) column (column 1) and Hypersil (ThermoQuest, Finnigan, UK), BDS C18 (5 μm) (150 mm × 4.6 mm) column (column 2); P2000 pump (TSP, UK) liquid chromatograph, SN4000 system controller (TSP, UK), AS300 autosampler (TSP, UK); PR700-100-01 (switch valve 1) and PR500-101-01 (switch valve 2), Lab PRP (Rheodyne, USA). AQA single quadrupole was equipped with (+)-electrospray ionization (ESI) source, probe temperature set at 340 °C and ionization voltage at 40 V. AQA LC–MS adopts Xcalibur software, Finnigan. Selected ion monitoring (SIM) has been applied on the following

ions: $m/z = 295.9$ for pizotifen and $m/z = 309.9$ for ketotifen.

2.2. Reagents

Ketotifen fumarate was donated kindly from Gulf Pharmaceutical Industries (UAE), and pizotifen malate was donated kindly by Jordan Pharmaceutical Manufacturing (Jordan). Human blood plasma was obtained from the National Center of Blood Transfusion, Amman, Jordan. Methanol HPLC grade and *tert*-butyl methyl ether HPLC grade were obtained from Merck, Germany. Ammonium acetate was obtained from Fisher Scientific, USA. Deionized water was prepared in our laboratories using Easy pure RO and Easy pure UV system, Parnstead Thermolyne, USA. Enzyme stock solution, β-glucoronidase (about 30 U/ml) and arylsulfatase (about 60 U/ml) was obtained from Merck, Germany.

2.3. Analytical procedure

2.3.1. Preparation of stock solutions

Standard stock solution of ketotifen and pizotifen was prepared by dissolving 100 mg of each in 100 ml methanol in volumetric flask to produce a concentration of 1 mg/ml. The standard working solutions were prepared by diluting the standard stock solution of ketotifen 2000-fold and pizotifen 20 000-fold with methanol to afford a concentration of 0.5 μg/ml for ketotifen and 50 ng/ml for pizotifen. The standard enzyme working solution was prepared by diluting 800 μl of enzyme stock solution supplied with 20 ml of 0.1 M tri-sodium citrate, then adjusted to pH 5.20 with 1.0 M citric acid.

2.3.2. Preparation of calibration samples

The calibration plasma samples prepared in 10 ml volumetric flasks as blank, zero standard, 0.5, 1.0, 2.0, 5.0, 10.0, 15.0, and 20.0 ng/ml. Each volumetric flask was vortexed for 5 min and the content of each was split to several 10 ml glass tubes containing 0.5 ml plasma. The quality control samples were prepared in the following concentration: 1.5, 8.0, and 18.0 ng/ml in 25 ml volumetric flask. Each volumetric flask was vortexed and split into 10 ml glass tubes containing 0.5 ml plasma.

2.3.3. Sample preparation

Two hundred and fifty micriliters of the β-glucoronidase/arylsulfatase enzyme working solution were added to 0.5 ml plasma sample (standard sample, control sample or volunteer sample) vortexed for 30 s. The samples were incubated in the oven for 18 h at 37 °C, then 100 μl of internal standard working solution (pizotifen 50 ng/ml) was added to the standard sample, control sample or volunteer sample. The samples were vortexed for 30 s. Seven ml of extraction solvent (*tert*-butyl methyl ether) were added and vortexed for 1 min, then centrifuged for 5 min at 3000 rpm. The supernatant was transferred to another 10 ml glass tube, and evaporated to dryness at 57 °C, then reconstituted with 120 μl of mobile phase and transferred to 250 μl micro glass insert tube, centrifuged for 5 min at 13 000 rpm. Fifty microliters of the aliquot sample was injected and chromatographed using column-switching technique onto a Hypersil BDS C18 (5 μm) (150 mm × 4.6 mm) and a Symmetry C18 (5 μm) (150 mm × 4 mm) column.

2.3.4. Chromatographic conditions

Chromatographic condition was a combination of two switching valves and two C18 columns using the following gradient system and switching time events:

Time (min)	Flow (ml/min)	*A* (%)	*B* (%)
0.00	0.70	100	0
3.50	0.70	100	0
4.50	0.50	0	100
9.00	0.50	0	100
10.00	0.30	0	100
15.00	0.30	0	100
16.00	0.50	0	100
19.10	0.50	0	100
20.00	2.00	100	0
22.00	2.00	100	0
23.00	0.70	100	0

Switching (time events): from 0.00 to 8.00 min (column 1 to waste); from 8.00 to 12.00 min (columns 1–2 to waste); from 12.00 to 19.10 min (columns 1–2 to mass detector); from 19.10 to 23.00 min (column 1 to waste).

The mobile phase used consisted of phase A (40% methanol and 60% 0.01 M ammonium acetate) with

pH adjusted to 3.0 with glacial acetic acid and phase B (80% methanol and 20% 0.01 M ammonium acetate) with pH adjusted to 3.0 with glacial acetic acid. The injection volume was 50 μl.

2.3.5. Standardization and calculation

The standard calibration curve lines were shown to be linear in the range from 0.5 to 20.0 ng/ml for ketotifen in human plasma. Best-fit calibration lines of peak height ratios (peak height analyte/peak height internal standard) versus concentration were determined by single-level calibration curve.

3. Results and discussion

3.1. Method development

The GC–MS method reported by Tzvetanov et al. [12] use solid-phase extraction and had an injection frequency of 22 min. Many sponsored research firms nowadays acquire only LC–MS machines for contract analyses. In our labs, we find it easier and more convenient to develop and validate a new LC–MS method using simple nontoxic liquid–liquid extraction rather than use GC–MS. A high-performance liquid chromatographic/mass spectrometric method for the quantification of ketotifen concentrations in human plasma was developed. To separate ketotifen and pizotifen from interfering endogenous plasma substances, the analysis was performed using column-switching technique. Xcalibur LCQuan Liquid chromatogram of a blank plasma sample is presented in Fig. 2. The analytes were monitored by the measurement, the response of mass detection using (+)-electrospray ionization mode and single ion monitoring at the protonated molecular ions with $m/z = 295.9$ and 309.9 for ketotifen and pizotifen, respectively. These molecular ions were the base peaks and no major adduct ions were observed. The combination of liquid extraction and chromatography provided a rapid assay free from interferences. Xcalibur LCQuan Liquid chromatogram of a standard plasma sample containing ketotifen at a concentration of 8.0 ng/ml (medium QC) and the internal standard pizotifen at a concentration of 10.0 ng/ml is presented in Fig. 3.

3.2. Validation study

The method was evaluated in terms of linearity, accuracy, precision, sensitivity, recovery, specificity and stability [13].

3.2.1. Linearity, accuracy, precision

For the determination of linearity, accuracy, precision and sensitivity two standard calibration curves of seven points (non-zero standards) were prepared on each of three consecutive days: days 1–3. Two sets of calibration standards and five sets of 1.5, 8.0, and 18.0 ng/ml spiked quality control samples were prepared and analyzed. The calibration curves were evaluated individually by linear regression and the concentrations of the calibration standards were back calculated. The concentrations were then normalized,

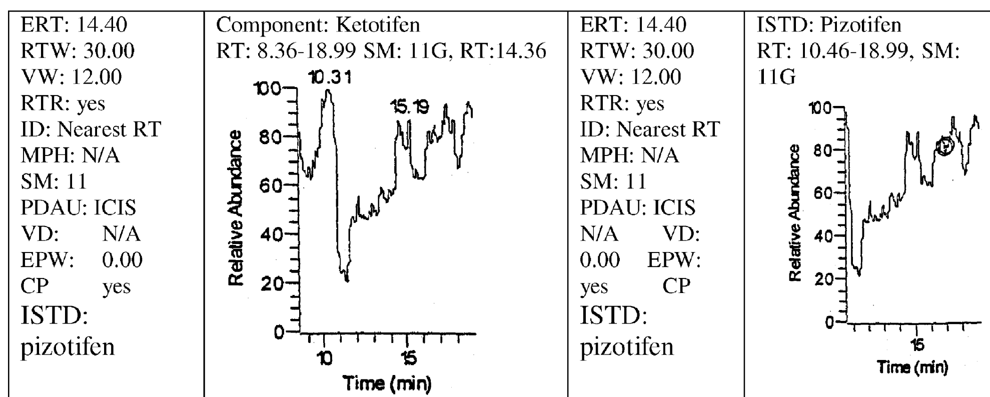

Fig. 2. Xcalibur LCQuan liquid chromatogram of a blank plasma sample.

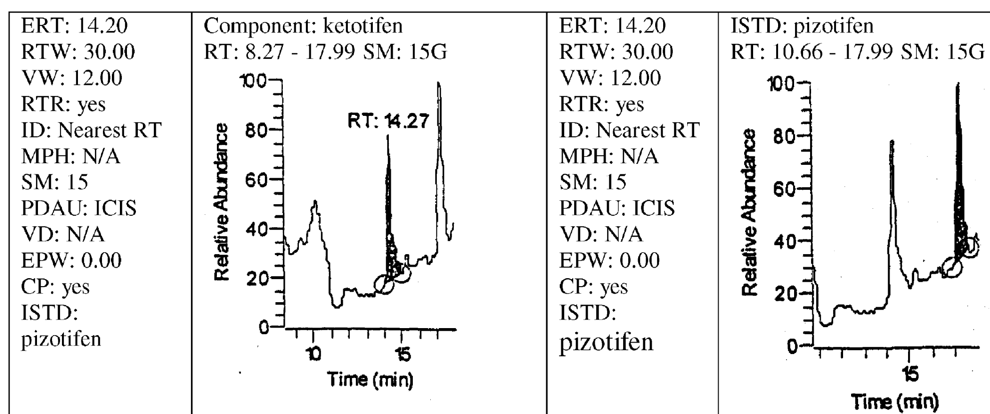

Fig. 3. Xcalibur LCQuan liquid chromatogram of a standard plasma sample containing the drug (ketotifen) at a concentration of 8.0 ng/ml (medium QC) and the internal standard (pizotifen) at a concentration of 10.0 ng/ml.

by dividing by the corresponding theoretical values. The statistical parameters including the means, standard deviations, coefficient of variation, accuracy and relative error (RE (%)) were calculated for the back-calculated normalized concentrations of each calibration curve (Table 1). The coefficient of correlation was consistently greater than 0.9965 during the course of validation.

3.2.2. Intra-day accuracy and precision

The intra-day accuracy and precision of the assay was measured by analyzing five replicates of 1.5, 8.0, and 18.0 ng/ml spiked quality controls samples of ketotifen. Intra-day accuracy of the method for ketotifen ranged from 106.67 to 107.78%, while the intra-day precision ranged from 0.00 to 9.44% at concentration of 1.5, 8.0, and 18.0 ng/ml (Table 2).

Table 1
Statistical analysis of back-calculated normalized ketotifen concentrations of the calibration standards in human plasma

Concentration (ng/ml)	Mean	S.D.	Precision as CV (%)	Accuracy (%)	RE (%)
0.5	1.0100	0.1311	12.98	101.00	1.00
1.0	1.0553	0.0590	5.59	105.53	5.53
2.0	1.0212	0.0942	9.22	102.12	2.12
5.0	1.0460	0.0731	6.99	104.60	4.60
10.0	1.0446	0.0602	5.76	104.46	4.46
15.0	1.0066	0.0441	4.38	100.66	0.66
20.0	0.9824	0.0211	2.15	98.24	−1.76

Table 2
Intra-day accuracy, precision and relative error of ketotifen spiked quality control samples in human plasma

Analyzed on 3 days	Theoretical concentration (ng/ml)	Mean (ng/ml)	S.D. (ng/ml)	Precision as CV (%)	Accuracy (%)	RE (%)
QC 1	1.5	1.60	0.000	0.00	106.67	6.67
QC 2	8.0	8.62	0.814	9.44	107.75	7.75
QC 3	18.0	19.40	1.005	5.18	107.78	7.78

Table 3
Inter-day accuracy, precision and relative error of the ketotifen spiked quality control samples in human plasma

Analyzed on 3 days	Measured ketotifen concentration in human plasma		
	QC 1 (1.5 ng/ml)	QC 2 (8.0 ng/ml)	QC 3 (18.0 ng/ml)
Mean (μg/ml)	1.61	8.22	18.23
S.D. (μg/ml)	0.074	0.895	1.671
Precision CV (%)	4.60	10.89	9.17
Accuracy (%)	107.33	102.75	101.28
RE (%)	7.33	2.75	1.28

Table 4
Absolute analytical recovery of ketotifen and pizotifen

Concentration (ng/ml)	Normalized mean of ketotifen		Recovery (%)
	Peak in human plasma	Peak in direct injection	
1.5	18905	19457	97.16
8.0	21660	21812	99.30
18.0	18160	18594	97.67
Mean			98.04
	Mean peak height of pizotifen		
	86326	90749	95.13

3.2.3. Inter-day accuracy and precision

The inter-day precision of the assay was measured by analyzing 15 replicates of 1.5, 8.0, and 18.0 ng/ml quality controls of ketotifen obtained from days 1 to 3. Inter-day accuracy of the method for ketotifen ranged from 101.28 to 107.33%, while the inter-day precision ranged from 4.60 to 10.89%, at concentration 1.5, 8.0, and 18.0 ng/ml (Table 3).

3.2.4. Sensitivity

The limit of quantitation defined as the concentration with acceptable accuracy and precision (below 15%) was 0.5 ng/ml for ketotifen in human plasma (Fig. 4). It was satisfactory sufficient for bioavailability studies about ketotifen [13].

3.2.5. Analytical recovery

3.2.5.1. Absolute analytical recovery.

The percent absolute recovery was determined by measuring the absolute peak height of ketotifen and pizotifen from a plasma sample prepared according to the method mentioned earlier. The absolute peak height obtained from the injection of the prepared plasma standards were compared to the absolute peak height of an equivalent aqueous standard, which was prepared to contain a concentration of drug and internal standard assuming 100% recovery. The percent absolute recovery of pizotifen and ketotifen is shown in Table 4.

3.2.6. Specificity

The specificity of the method was determined by screening six different batches of controlled human

ERT: 14.25
RTW: 30.00
VW: 12.00
RTR: yes
ID: Nearest RT
MPH: N/A
SM: 11
PDAU: ICIS
VD: N/A
EPW: 0.00
CP: yes
ISTD: pizotifen

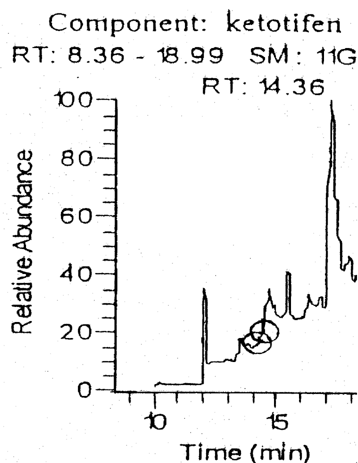

Fig. 4. Xcalibur LCQuan liquid chromatogram of LLOQ for ketotifen.

Table 5
Short-term room temperature stability for the low QC (1.5 ng/ml) for 4 h

QC sample	Initially analyzed concentration (ng/ml)	Analyzed concentration after 4 h	Stability (%)
1.5 ng/ml			
Mean	1.56	1.52	97.57
S.D.	0.114	0.084	3.32
CV (%)	7.31	5.53	3.40
18.0 ng/ml			
Mean	17.92	17.76	99.12
S.D.	0.890	0.847	0.990
CV (%)	4.97	4.77	1.00

Table 6
Sample stability after preparation for (1.5 and 18.0 ng/ml) for 24 h at room temperature

QC sample	Initially analyzed concentration (ng/ml)	Analyzed concentration for 24 h at RT	Stability (%)
1.5 ng/ml			
Mean	1.56	1.50	96.24
S.D.	0.114	0.100	3.44
CV (%)	7.31	6.67	3.57
18.0 ng/ml			
Mean	18.24	18.00	98.72
S.D.	0.590	0.374	1.457
CV (%)	3.23	2.08	1.48

Table 7
Long-term stability (freezer storage stability)

QC sample	Analyzed concentration after the first month (ng/ml)	Analyzed concentration after the seventh month (ng/ml)
1.5 ng/ml		
Mean	1.46	1.54
S.D.	0.055	0.055
CV (%)	3.77	3.57
18.0 ng/ml		
Mean	18.42	19.04
S.D.	0.396	1.232
CV (%)	2.15	6.47

blank plasma, which were free from interfering endogenous plasma components. This was evidenced by the lack of interfering peaks in the chromatograms of plasma samples. Solutions of commonly used drugs including, aspirin, acetaminophen, ascorbic acid, caffeine, nicotine and ibuprofen were prepared in mobile phase and then were injected to check for interference. No interferences were observed.

3.2.7. Stability

3.2.7.1. Short-term room temperature stability (counter stability). Ten replicates of each concentration of two different quality control samples were prepared 1.5 and 18.0 ng/ml. Five of each concentration were analyzed at zero time. The other five samples of each concentration were allowed to stand on the bench top at room temperature without further treatment. The samples were prepared as described in Section 2.3.3 and analyzed after 4 h (Table 5).

3.2.7.2. Sample stability after preparation procedure (auto sampler stability). On a validation day 10, samples of each concentration of two different quality control samples (1.5 and 18.0 ng/ml) were prepared as in Section 2.3.3. The supernatants of samples were pooled. Five samples of each concentration were analyzed immediately after preparation; another five samples of each concentration were stored at room temperature for 24 h. Results are presented in Table 6.

3.2.7.3. Long-term stability (freezer storage stability). Ten replicates of each concentration of the following quality control samples: 1.5 and 18.0 ng/ml were prepared and stored at −20 °C. Five samples of each concentration were prepared as described in Section 2.3.3 and analyzed at the end of the first month. The other five samples of each concentration were prepared and analyzed at the end of the seventh month (Table 7).

4. Conclusion

A new extraction procedure and LC–MS method were validated to ensure reliable determination of ketotifen in human plasma. A combination of liquid–liquid extraction and column-switching technique guaranteed clean analysis. Compared to other liquid chromatography techniques, improved accuracy, precision, sensitivity and high recovery were among the main advantages of this study. Specificity and stability of the method were also conducted. Validation results proved that the method described is suitable for pharmacokinetic studies.

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