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Therapeutic drug monitoring of flecainide in serum using high-performance liquid chromatography and electrospray mass spectrometry

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Abstract

High-performance liquid chromatography with electrospray mass spectrometry (LC–MS) was used for analysis of the drug flecainide in serum. The clean-up was performed by solid-phase extraction, and an aromatic ring positional isomer was used as internal standard. Results from method validation on spiked serum samples showed excellent reproducibility; intra- and inter-assay variations (C.V.% and %Bias) were less than 6% within the therapeutic concentration range of the drug (0.2–1.0 μg/ml). Linearity was demonstrated from 0.05 to 2.0 μg/ml. The limit of detection and quantification was 0.025 and 0.05 μg/ml, respectively. Due to the high selectivity of the mass spectrometric detection, no interferences were observed. Results from clinical samples ($n=18$) from patients in treatment with Tambocor (flecainide acetate) showed excellent correlation with parallel data obtained from a method based on high-performance liquid chromatography (HPLC) with fluorescence detection after liquid/liquid extraction. The chromatographic separation of flecainide and internal standard was improved compared to earlier HPLC methods. The methodology is simple, accurate and requires only 0.25 ml of sample. It is a well suited method for routine therapeutic drug monitoring in a hospital or clinical chemistry laboratory.

Keywords: Flecainide

1. Introduction

Flecainide, 2,5-bis-(2,2,2-trifluoroethoxy)-*N*-(2-piperidylmethyl)benzamide (Fig. 1), marketed under the drug name Tambocor (as the acetate salt), has been used during the last decades for suppression of ventricular arrhythmias in humans. Because of the risk of serious side effects and the narrow therapeutic index, it is a standard clinical practice to monitor serum concentrations for patients treated with this drug [1]. The therapeutic range is 0.2 to 1.0 μg/ml [1,2]. Different analytical techniques have been used

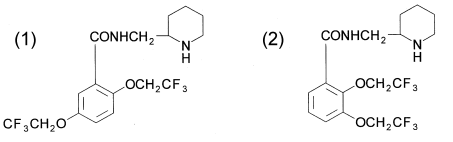

Fig. 1. Structures of flecainide (1) and internal standard (2).

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for initial research and pharmacokinetic studies, including capillary gas chromatography (GC) after derivatization [3] and various high-performance liquid chromatography (HPLC) methods with either fluorescence or ultraviolet detection [4–15]. Some attention has also been devoted to stereoselective pharmacokinetic studies involving analysis of flecainide enantiomers [16–18]. Toxicology cases are usually solved using gas chromatography and mass spectrometry (GC–MS) [19,20]. A practical and widely used technique for quantitative routine measurements of flecainide in serum or plasma has been the fluorescence polarization immunoassay (FPIA) developed by Abbott Laboratories for the TDx system [21]. This immunoassay was characterized by high analytical throughput with low sample volume and no need for sample pretreatments. However, the production of reagents for the FPIA assay has been terminated due to calibration problems and lacking reproducibility for quality control samples [22]. The expiration date for the last reagent batch was May 1999. Laboratories, which have used FPIA for therapeutic drug monitoring of flecainide, are in future forced to implement or develop other methods.

High-performance liquid chromatography with mass spectrometry (LC–MS) is becoming a routine instrumentation for drug analysis at hospital and medical laboratories. At present, no LC–MS applications have been reported for analysis of flecainide in biological samples. The scope of this paper is to describe a new routine method for quantitative determination of flecainide in serum after solid-phase extraction using LC–MS with atmospheric pressure electrospray ionisation.

2. Experimental

2.1. Chemicals

Flecainide acetate (2,5-bis-(2,2,2-trifluoroethoxy)-*N*-(2-pyridinemethyl)benzamide acetate) and internal standard (2,3-bis-(2,2,2-trifluoroethoxy)-*N*-(2-pyridinemethyl)benzamide hydrochloride) with the synonym S-15531, where kindly donated by 3M Pharmaceuticals (St. Paul, MN, USA). All other chemicals were of analytical reagent grade and used without further purification.

2.2. Liquid chromatography–mass spectrometry

The liquid chromatograph was a Hewlett-Packard (Palo Alto, CA, USA) 1100 Series system. Chromatography was performed with a cyanopropyl bonded phase column: Supelcosil LC-CN, 150×4.6 mm I.D, 5 μm, from Supelco (Bellefonte, PA, USA). Mobile phases were (A) 25 m*M* formic acid in water (pH adjusted to 5.2 with concentrated ammonia) and (B) acetonitrile. The mobile phase conditions were isocratic using 50% B. Flow rate was 0.5 ml/min. Column department temperature was 40°C. Injection volume was 2 μl. The quadrupole mass spectrometric detector was a Hewlett-Packard 1100 LC–MSD system equipped with an atmospheric pressure electrospray ionization interface. Selected ion monitoring (SIM) was performed in positive mode on the signal from protonated drug and internal standard $[M+H]^+$: m/z 415. Capillary voltage was 3500 V. Drying gas was 99% pure nitrogen from a gas generator (Whatman, Haverhill, MA, USA) in line with 4000-150 PD3 air compressor from Junair (Nørresundby, Denmark). Drying gas temperature was 320°C and gas flow-rate 7.0 l/min. Nebulizer pressure was 35 p.s.i. Mass calibration (100–1000 amu) was performed using autotune macros and calibrators from the manufacturer. Aqueous standard solutions spiked at four levels (2, 1, 0.5 and 0.1 μg/ml) from a stock solution of flecainide acetate in dimethylformamide (1 mg/ml) was analysed like samples and used for calibrating the assay. The internal standard concentration in the final extract was 0.5 μg/ml. Calculations were based on the peak area ratio of flecainide and internal standard derived from the integrated m/z 415 signals. Least-squares linear regression with equal weighting was used to fit the curves. *Important note*: The above concentrations of calibrators as well as the level of serum samples mentioned below, all corresponds to μg/ml of the acetate salt of flecainide. This procedure is consistent with the scientific literature published on the subject. However, the pharmacological active compound in vivo is flecainide (Fig. 1).

2.3. Sample preparation

Serum, 0.25 ml, was mixed with 0.25 ml phosphate buffer (0.1 *M*, pH 8.0) and 50 μl aqueous internal standard solution (10 μg/ml) and cen-

trifuged at about 1000 *g* for 2 min. A vacuum manifold was used for solid-phase extraction. SPEC-PLUS-3 ML-C_{18} (15 mg) columns (Ansys, Irvine, CA, USA) were conditioned with 0.5 ml methanol and 0.5 ml 0.1 *M* phosphate buffer (pH 8.0). The sample was transferred to and percolated through the column. The column was washed with 500 μl water–methanol (15:85, v/v). Column drying was performed in the manifold for a minimum of 5 min. Finally the analytes were eluted with 1 ml methanol. The eluate was evaporated to dryness in a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA) at a water bath temperature of 45°C using laboratory air at 15 p.s.i. for 10 min. The residue was redissolved in 1 ml 50% acetonitrile in 25 m*M* formic acid (pH 5.2), vortex shaken (5 s) and transferred to autosampler vials.

2.4. Validation

The method was validated for intra-assay variation and accuracy by analysis of human serum samples (from blank donor pool) at six levels (2.29, 1.15, 0.57, 0.29, 0.12 and 0.057 μg/ml) in replicates of ten samples. The serum samples were spiked with an aqueous solution of flecainide acetate (10 mg/ml) and stored at 18°C. Inter-assay variation and accuracy was tested by single determinations of serum samples at five levels (1.15, 0.57, 0.29, 0.12 and 0.057 μg/ml) performed on five different days within one month. External QC-samples, TDM Controls (Level 2, Lot. No. 57080) from Biorad Laboratories (Hercules, CA, USA), made from human serum and provided in lyophilized form, where analysed in single in all sample series. The limit of detection (LOD) for LC–MS analysis was determined at signal-to-noise ratio (S/N) equal to 6. The limit of quantification (LOQ) was determined at the lowest sample concentration that could be analysed using predefined boundaries of ±20% deviation from the nominal values with a coefficient of variation not exceeding 15%.

Randomly selected samples from patients in treatment with Tambocor ($n=18$) were split into two specimens and analysed in parallel using the present LC–MS method and a HPLC method with fluorescence detection after liquid/liquid extraction (performed at the laboratory of 3M Medica (Borken, Germany)). The concentrations from HPLC were, except for a single sample, mean values from two single determinations on two different days. The samples were kept at 18°C.

3. Results and discussion

Results from method validation showed an excellent reproducibility, expressed as intra- and inter-assay precision and accuracy (Table 1). The LOD was 0.025 μg/ml, and LOQ was 0.05 μg/ml. The absolute recoveries of flecainide, estimated by the peak area ratios in extracted and non-extracted aqueous standards were between 93 and 102%. Detector liniarity was demonstrated from 0.05 to 2.0 μg/ml, with correlation coefficients (r^2) of 0.999 or better. The calibration curve equation was typically as follows: $y=0.543x+3.03$ E-3, where y is the area ratio of analyte and internal standard in the final extract and x is analyte concentration in the serum calibrators.

Table 1
Intra- and inter-assay precision and accuracy[a]

Sample concentration (μg/ml)		Intra-assay ($n=10$)		Inter-assay ($n=5$)	
Flecainide	Flecainide acetate	Coefficient of variation (%)	%Bias	Coefficient of variation (%)	%Bias
2.0	2.29	1.2	+1.5	–	–
1.0	1.15	2.5	−0.1	3.1	+0.1
0.5	0.57	1.7	+1.2	3.3	−2.3
0.25	0.29	2.8	+0.9	4.0	−0.3
0.1	0.12	3.0	+7.9	2.8	+0.4
0.05	0.057	2.8	+17.6	5.2	+10.9

[a] Accuracy was calculated as mean deviation from the nominal spiked value, and reported here as %Bias.

The final extract actually ends up being a four-fold dilution of the sample. Thereby the final concentrations are adjusted to fit the sensitive detector and avoid overloading of the electron multiplier (detector) and non-linearity in the therapeutic range. If more sensitivity is needed for other applications than drug monitoring, the method should be easy to adapt to sub-ppb detection limits by adjusting the concentration factor or the autosampler injection volume.

The polarity of flecainide makes the drug well suited for liquid chromatography. However, the separation of flecainide and the structural isomeric internal standard is somewhat difficult to achieve. When mass spectrometric detection is used, the compounds must be separated as they have identical mass to charge ratios (m/z). The final mobile phase parameters chosen thus represents a compromise between optimal chromatography and electrospray detection in positive mode, where acidic conditions are essential. Experiments were carried out with shorter and/or smaller internal diameter cyanopropyl columns of different brands, but no further improvements to chromatography could be made. However, full baseline separation was achieved, which is equal to or better than results from earlier HPLC methods using ordinary column materials (Fig. 2). Obayashi et al. recently reported an excellent HPLC method with the use of a new fluorocarbon-bonded silica gel column for effective separation of flecainide and internal standard [23]. However, the mobile phase contained an ionpair reagent, which seems to be incompatible with electrospray ionization.

A minor disadvantage of the present method is the relative high analysis time (18 min) and broad peak widths (0.3 min). An idea for a more elegant solution would be the use of the research compound 2,5-diethoxy-*N*-(2-piperidylmethyl)benzamide as internal standard (batch synonym S-15277), which was first described by Chang et al. [4] and later by Woollard [12]. This compound has non-fluorinated ethoxy side groups attached to the aromatic ring system, and its molecular ion mass to charge ratio differs from that of flecainide. A fast isocratic run could possibly be performed, because chromatographic separation of drug and internal standard would not be essential.

External QC samples containing flecainide are

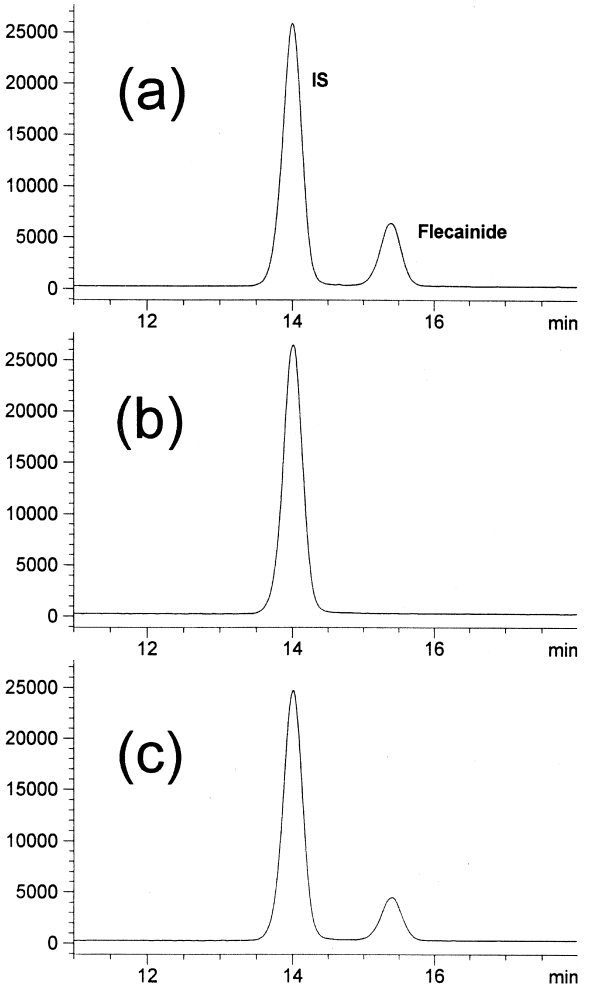

Fig. 2. Ion chromatograms of m/z 415 obtained from analysis of (a) calibration standard 0.5 μg/ml; (b) blank serum sample; (c) patient sample (concentration of flecainide acetate 0.35 μg/ml). The chromatograms are in the same scale. R_t (internal standard): 13.99 min; R_t (flecainide): 15.39 min.

available from Biorad Laboratories as freeze-dried serum for therapeutic drug monitoring. Biorad TDM Controls (Level 2) were included during method validation and showed good stability at room temperature, however, no systematic stability test were performed. Mean value was 0.53 μg/ml, and the inter-day variation over a period of five months was 3.9% ($n=16$). This is lower than the specified value at 0.62 μg/ml (target value: 0.50–0.75 μg/ml). However, the flecainide acetate values for Biorad TDM Controls are based on the FPIA method, which

has been stated to overestimate serum concentrations in the lower range and underestimate them in the higher concentration range, relative to HPLC methods [24].

No signal interferences were observed during the study of patient samples and the Biorad TDM samples, which contain more than forty different drugs, including six important cardiac drugs and the most commonly prescribed antiepileptics and antidepressants. Immunoassays generally cross react with metabolites, which in the present case would be considered unwanted, because flecainide metabolites lack pharmacological activity. Due to the high selectivity of mass spectrometric detection, the LC–MS method will only determine flecainide.

Because of the current replacement of flecainide methods at hospital laboratories worldwide, the author would like to stress the future need for external quality control samples and biological reference materials containing flecainide, and the importance of including this drug in international external quality assessment programs.

With the introduction of new methods, it is a safe approach to demonstrate good correlation with previous techniques. The consistency between methods based on GC, HPLC and FPIA has been well documented in works by others [5,6,12,24–26]. Therefore eighteen patients in treatment with Tambocor was parallel analysed at the laboratories of 3M Medica (Germany) using liquid/liquid extraction and HPLC with fluorescence detection. The results, which are shown in Fig. 3, indicate an excellent correlation in the therapeutic range. The correlation coefficient (r^2) was 0.9960 and the regression line had a slope of 1.019 and an intercept of 0.019. The data from LC–MS are systematically lower (mean deviation=5±2.5%), however, this would have no consequence in the clinical management of quantitative data.

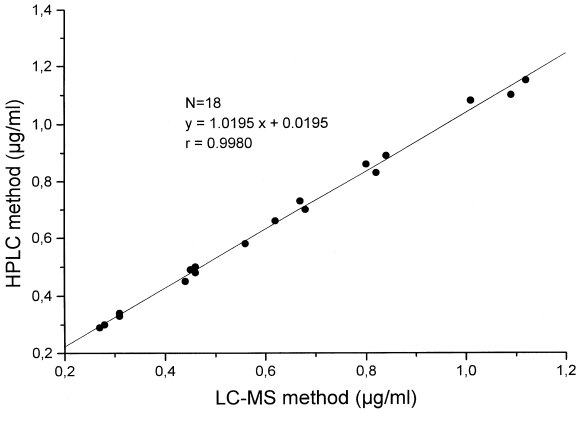

Fig. 3. Comparison of the LC–MS method for the quantification of flecainide acetate in serum with a HPLC method using fluorescence detection after liquid/liquid extraction (n=18). x-axis: LC–MS data (µg/ml). y-axis: HPLC data (µg/ml).

4. Conclusions

Method validation data for a new LC–MS method for quantitative determination of flecainide in serum showed excellent precision, accuracy and selectivity. The method is well suited for therapeutic drug monitoring in a routine laboratory.

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